

NTP TECHNICAL REPORT
ON THE
TOXICOLOGY AND CARCINOGENESIS
STUDIES OF 2,4-HEXADIENAL
(89% *trans,trans* isomer, CAS No. 142-83-6;
11% *cis,trans* isomer)
IN F344/N RATS AND B6C3F₁ MICE
(GAVAGE STUDIES)

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

October 2003

NTP TR 509

NIH Publication No. 04-4443

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 NIEHS 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> (866-541-3841 or 919-653-2590). 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 back cover.

NTP TECHNICAL REPORT
ON THE
TOXICOLOGY AND CARCINOGENESIS
STUDIES OF 2,4-HEXADIENAL
(89% *trans,trans* isomer, CAS No. 142-83-6;
11% *cis,trans* isomer)
IN F344/N RATS AND B6C3F₁ MICE
(GAVAGE STUDIES)

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

October 2003

NTP TR 509

NIH Publication No. 04-4443

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

CONTRIBUTORS

National Toxicology Program

Evaluated and interpreted results and reported findings

P.C. Chan, Ph.D., Study Scientist
 D.W. Bristol, Ph.D.
 J.R. Bucher, Ph.D.
 J.R. Hailey, D.V.M.
 J.K. Haseman, Ph.D.
 R.A. Herbert, D.V.M., Ph.D.
 J. Mahler, D.V.M.
 R.R. Maronpot, D.V.M.
 S.D. Peddada, Ph.D.
 G.N. Rao, D.V.M., Ph.D.
 J.H. Roycroft, Ph.D.
 C.S. Smith, Ph.D.
 G.S. Travlos, D.V.M.
 K.L. Witt, M.S., ILS, Inc.

Microbiological Associates, Inc.

Conducted 16-day studies and evaluated pathology findings

M.L. Wenk, Ph.D., Principal Investigator
 L.L. Lanning, D.V.M.

Southern Research Institute

Conducted 14-week and 2-year studies and evaluated pathology findings

J.D. Prejean, Ph.D., Principal Investigator
 W.R. Richter, D.V.M., M.S., Principal Investigator
 D.R. Farnell, D.V.M., Ph.D.
 J.E. Heath, D.V.M.
 R.B. Thompson, D.V.M., Ph.D.

Experimental Pathology Laboratories, Inc.

Provided pathology quality assurance

J.F. Hardisty, D.V.M., Principal Investigator
 A.E. Brix, D.V.M., Ph.D.
 C.V. Okerberg, D.V.M., Ph.D.

Dynamac Corporation

Prepared quality assurance audits

S. Brecher, Ph.D., Principal Investigator

NTP Pathology Working Group

*Evaluated slides and prepared pathology report on rats
 (January 16, 2001)*

C. Picut, V.M.D., J.D., Chairperson
 ILS, Inc.
 G.P. Flake, M.D.
 National Toxicology Program
 J.R. Hailey, D.V.M.
 National Toxicology Program
 R.A. Herbert, D.V.M., Ph.D.
 National Toxicology Program
 J. Mahler, D.V.M.
 National Toxicology Program
 A. Nyska, D.V.M.
 National Toxicology Program
 C.V. Okerberg, D.V.M., Ph.D.
 Experimental Pathology Laboratories, Inc.
 G. Pearse, B.V.M. & S.
 National Toxicology Program
 R.C. Sills, D.V.M., Ph.D.
 National Toxicology Program
 D. Wolf, D.V.M., Ph.D.
 Environmental Protection Agency

*Evaluated slides and prepared pathology report on mice
 (November 16, 2000)*

C. Picut, V.M.D., J.D., Chairperson
 ILS, Inc.
 A.E. Brix, D.V.M., Ph.D.
 Experimental Pathology Laboratories
 J.R. Hailey, D.V.M.
 National Toxicology Program
 R.A. Herbert, D.V.M., Ph.D.
 National Toxicology Program
 J. Mahler, D.V.M.
 National Toxicology Program
 G. Pearse, B.V.M. & S., Observer
 National Toxicology Program
 R.C. Sills, D.V.M., Ph.D.
 National Toxicology Program
 V. Turusov, M.D.
 Carcinogenesis Institute of Moscow
 D. Wolf, D.V.M., Ph.D.
 Environmental Protection Agency

Analytical Sciences, Inc.

Provided statistical analyses

P.W. Crockett, Ph.D., Principal Investigator

L.J. Betz, M.S.

K.P. McGowan, M.B.A.

J.T. Scott, M.S.

Biotechnical Services, Inc.

Prepared Technical Report

S.R. Gunnels, M.A., Principal Investigator

M.P. Barker, B.A.

P.A. Gideon, B.A.

L.M. Harper, B.S.

D.C. Serbus, Ph.D.

P.A. Yount, B.S.

CONTENTS

ABSTRACT	7
EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY	11
TECHNICAL REPORTS REVIEW SUBCOMMITTEE	12
SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS	13
INTRODUCTION	15
MATERIALS AND METHODS	23
RESULTS	35
DISCUSSION AND CONCLUSIONS	59
REFERENCES	63
APPENDIX A Summary of Lesions in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal	71
APPENDIX B Summary of Lesions in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal	111
APPENDIX C Summary of Lesions in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal	143
APPENDIX D Summary of Lesions in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal	185
APPENDIX E Genetic Toxicology	225
APPENDIX F Clinical Pathology Results	233
APPENDIX G Organ Weights and Organ-Weight-to-Body-Weight Ratios	241
APPENDIX H Reproductive Tissue Evaluations and Estrous Cycle Characterization	249
APPENDIX I Chemical Characterization and Dose Formulation Studies	253
APPENDIX J Ingredients, Nutrient Composition, and Contaminant Levels in NTP-2000 Rat and Mouse Ration	267
APPENDIX K Sentinel Animal Program	273

APPENDIX L	Measures of 2,4-Hexadienal-Induced Oxidative Stress in the Forestomach of F344/N Rats	277
APPENDIX M	DNA Adduct Characterization Studies	283

SUMMARY

Background

2,4-Hexadienal occurs naturally as an oxidation product of fatty acids, especially in heating and cooking of oils and fats. It is also used as a flavoring agent and as the starting material for making sorbic acid, a preservative.

Methods

We deposited solutions of 2,4-hexadienal dissolved in corn oil through a tube directly into the forestomachs of male and female rats and mice daily, five times a week, for 2 years. Rats received doses of 22.5, 45, or 90 milligrams of 2,4-hexadienal per kilogram of body weight; mice received doses of 30, 60, or 120 milligrams of 2,4-hexadienal per kilogram of body weight. Control groups received corn oil with no 2,4-hexadienal added. At the end of the study, tissue samples from over 40 different organs were examined for each animal.

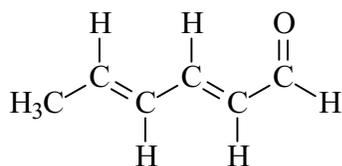
Results

In all four study sets (male and female rats and mice), animals receiving 2,4-hexadienal had significantly greater occurrences of neoplasms of the forestomach. The forestomach in rodents is similar in tissue type to the esophagus in humans. These tumors included papillomas and malignant carcinomas. Normally such tumors of the forestomach are rare in rodents.

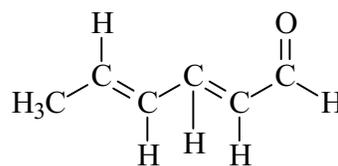
Conclusion

We conclude that 2,4-hexadienal caused neoplasms of the forestomach in male and female rats and mice.

ABSTRACT



(89% *trans,trans*)



(11% *cis,trans*)

2,4-HEXADIENAL

(89% *trans,trans* isomer, CAS No. 142-83-6)

Chemical Formula: C₆H₈O Molecular Weight: 96.13

Synonyms: Hexa-2,4-dienal; 2,4-hexadienal; 2,4-hexadien-1-al; 2,4-Hx; 1,3-pentadiene-1-carboxaldehyde; 2-propylene acrolein; sorbaldehyde; sorbic aldehyde

2,4-Hexadienal, a colorless to yellow liquid with a pungent "green" or citrus odor, is used as a food additive for flavor enhancement, as a fragrance agent, as a starting material or intermediate in synthetic reactions in the chemical and pharmaceutical industries, as a fumigant, and as a corrosion inhibitor for steel. 2,4-Hexadienal was nominated for study by the National Cancer Institute because of the potential for carcinogenicity based on its α,β -unsaturated aldehyde structure and the potential link between exposure to lipid peroxidation products in the diet and human malignancies. The commercial product is a mixture containing chiefly *trans,trans*-2,4-hexadienal in equilibrium with *cis,trans*-2,4-hexadienal. Male and female F344/N rats and B6C3F₁ mice received 2,4-hexadienal (89% *trans,trans*; 11% *cis,trans*) in corn oil by gavage for 16 days, 14 weeks, or 2 years. Tissues and plasma from dosed rats were examined for malondialdehyde and glutathione concentrations, and DNA adducts were characterized in liver and forestomach samples from dosed rats and mice. Genetic toxicology studies were conducted in *Salmonella typhimurium*, rat and mouse bone marrow cells, and mouse peripheral blood erythrocytes.

16-DAY STUDY IN RATS

Groups of five male and five female rats were administered 0, 3, 9, 27, 80, or 240 mg 2,4-hexadienal/kg body weight in corn oil by gavage, 5 days per week, for 16 days. Three male and three female 240 mg/kg rats died before the end of the study. Mean body weight gains of 240 mg/kg rats were significantly less than those of the vehicle controls. Clinical findings included diarrhea, ataxia, lethargy, and nasal/eye discharge in males, and lethargy, paleness, and abnormal breathing in females in the 240 mg/kg groups. Liver weights of 240 mg/kg females were significantly greater than those of the vehicle controls. Gross and microscopic lesions indicative of forestomach necrosis and ulceration were present in most 240 mg/kg rats, and forestomach epithelial hyperplasia was microscopically evident in most 80 mg/kg rats.

16-DAY STUDY IN MICE

Groups of five male and five female mice were administered 2,4-hexadienal in corn oil by gavage at doses of 0,

3, 9, 27, 80, or 240 mg/kg, 5 days per week, for 16 days. Chemical-related deaths occurred in one male and one female in the 240 mg/kg groups. Female mice in the 240 mg/kg group lost weight during the study. Gross and microscopic lesions indicative of forestomach necrosis and ulceration were present in all 240 mg/kg mice, and forestomach epithelial hyperplasia and hyperkeratosis were microscopically evident in 80 mg/kg mice.

14-WEEK STUDY IN RATS

Groups of 10 male and 10 female rats were administered 2,4-hexadienal in corn oil by gavage at doses of 0, 7.5, 15, 30, 60, or 120 mg/kg, 5 days per week, for 14 weeks. All rats survived to the end of the study. Mean body weights of 30, 60, and 120 mg/kg males were significantly less than those of the vehicle controls. The only clinical finding attributed to 2,4-hexadienal administration was hypersalivation in 30 and 120 mg/kg males and females. The incidences of forestomach hyperplasia and nasal olfactory atrophy or necrosis were significantly increased in 120 mg/kg rats. Nasal lesions occurred in most 120 mg/kg male rats.

14-WEEK STUDY IN MICE

Groups of 10 male and 10 female mice were administered 2,4-hexadienal in corn oil by gavage at doses of 0, 7.5, 15, 30, 60, or 120 mg/kg, 5 days per week, for 14 weeks. No deaths were attributed to administration of 2,4-hexadienal. Mean body weights of males and females were similar to those of the vehicle controls throughout the study. Clinical findings included salivation and anal wetness in males and females. Kidney weights of 60 and 120 mg/kg males and liver weights of 60 mg/kg males and females were significantly greater than those of the vehicle controls. The incidences of forestomach hyperplasia and/or nasal olfactory atrophy or necrosis were significantly increased in 120 mg/kg mice.

2-YEAR STUDY IN RATS

Groups of 50 male and 50 female rats were administered 2,4-hexadienal in corn oil by gavage at doses of 0, 22.5, 45, or 90 mg/kg, 5 days per week, for up to 105 weeks.

Survival of all dosed groups of rats was similar to that of the vehicle control groups. The mean body weights of 90 mg/kg males were generally less than those of the vehicle controls throughout the study.

The incidences of squamous cell papilloma of the forestomach occurred with positive trends in male and female rats. This neoplasm was found in 58% of males and 34% of females in the 90 mg/kg groups. In the forestomach of male rats, papilloma multiplicity was increased in the 90 mg/kg group, and squamous cell carcinomas were found in one 45 mg/kg male and two 90 mg/kg males. Epithelial hyperplasia of the forestomach occurred in most 45 and 90 mg/kg rats.

2-YEAR STUDY IN MICE

Groups of 50 male and 50 female mice were administered 2,4-hexadienal in corn oil by gavage at doses of 0, 30, 60, or 120 mg/kg, 5 days per week, for up to 105 weeks. Survival of dosed mice was similar to that of the vehicle controls. The mean body weights of all dosed groups were generally similar to those of the vehicle controls throughout the study. The incidences of squamous cell papilloma of the forestomach occurred with positive trends in male and female mice; squamous cell carcinomas were present in 120 mg/kg males and females. Epithelial hyperplasia of the forestomach occurred in many 120 mg/kg mice. Two 120 mg/kg males had uncommon squamous cell carcinoma of the oral cavity (tongue).

GENETIC TOXICOLOGY

2,4-Hexadienal was mutagenic in *S. typhimurium* strain TA100 with and without induced hamster or rat liver enzymes; no mutagenic activity was detected with strains TA1535 or TA98, with or without S9. Results of bone marrow tests in male rats and male mice given intraperitoneal injections of 2,4-hexadienal showed a small increase in the induction of micronucleated erythrocytes. However, neither test was repeated, and the test results were judged to be inconclusive. Results of peripheral blood micronucleus tests in male and female mice treated with 2,4-hexadienal by gavage for 14 weeks were negative.

CONCLUSIONS

Under the conditions of these 2-year gavage studies, there was *clear evidence of carcinogenic activity** of 2,4-hexadienal in male and female F344/N rats and male and female B6C3F₁ mice based on increased incidences of squamous cell neoplasms of the forestomach. The occurrence of squamous cell carcinoma of the oral cavity

(tongue) in male B6C3F₁ mice may have been related to the administration of 2,4-hexadienal.

Hyperplasia of the forestomach in male and female rats and mice was associated with administration of 2,4-hexadienal.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 11. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 13.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of 2,4-Hexadienal

	Male F344/N Rats	Female F344/N Rats	Male B6C3F ₁ Mice	Female B6C3F ₁ Mice
Doses in corn oil by gavage	0, 22.5, 45, or 90 mg/kg	0, 22.5, 45, or 90 mg/kg	0, 30, 60, or 120 mg/kg	0, 30, 60, or 120 mg/kg
Body weights	90 mg/kg group less than the vehicle control group	Dosed groups similar to the vehicle control group	Dosed groups similar to the vehicle control group	Dosed groups similar to the vehicle control group
Survival rates	37/50, 35/50, 33/50, 30/50	37/50, 39/50, 41/50, 31/50	44/50, 39/50, 44/50, 39/50	42/50, 37/49, 37/50, 39/50
Nonneoplastic effects	<u>Forestomach</u> : epithelium, hyperplasia, (3/50, 19/50, 42/50, 50/50)	<u>Forestomach</u> : epithelium, hyperplasia (2/50, 16/50, 37/50, 41/50)	<u>Forestomach</u> : epithelium, hyperplasia, squamous (14/50, 7/50, 9/50, 26/50)	<u>Forestomach</u> : epithelium, hyperplasia, squamous (4/50, 8/49, 12/50, 31/50)
Neoplastic effects	<u>Forestomach</u> : squamous cell papilloma (0/50, 3/50, 10/50, 29/50); squamous cell carcinoma (0/50, 0/50, 1/50, 2/50); squamous cell papilloma or carcinoma (0/50, 3/50, 11/50, 29/50)	<u>Forestomach</u> : squamous cell papilloma (0/50, 1/50, 5/50, 17/50)	<u>Forestomach</u> : squamous cell papilloma (2/50, 4/50, 5/50, 8/50); squamous cell carcinoma (0/50, 1/50, 0/50, 2/50); squamous cell papilloma or carcinoma (2/50, 4/50, 5/50, 10/50)	<u>Forestomach</u> : squamous cell papilloma (2/50, 2/49, 11/50, 13/50); squamous cell carcinoma (0/50, 0/49, 0/50, 7/50); squamous cell papilloma or carcinoma (2/50, 2/49, 11/50, 18/50)
Equivocal findings	None	None	<u>Oral cavity (tongue)</u> : squamous cell carcinoma (0/50, 0/50, 0/50, 2/50)	None
Level of evidence of carcinogenic activity	Clear evidence	Clear evidence	Clear evidence	Clear evidence
Genetic toxicology				
<i>Salmonella typhimurium</i> gene mutations:		Positive in strain TA100; negative in strains TA1535 and TA98 with and without S9		
Micronucleated erythrocytes				
Rat bone marrow <i>in vivo</i> :		Inconclusive		
Mouse bone marrow <i>in vivo</i> :		Inconclusive		
Mouse peripheral blood <i>in vivo</i> :		Negative		

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 2,4-hexadienal on October 18, 2001, 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.

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

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

Harold Davis, D.V.M., Ph.D.*
Preclinical Safety Assessment
Amgen, Inc.
Thousand Oaks, CA

Yvonne P. Dragan, Ph.D.*
School of Public Health
Ohio State University
Columbus, OH

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

James E. Klaunig, Ph.D., Principal Reviewer*
Division of Toxicology
Department of Pharmacology and Toxicology
Indiana University/Purdue University at Indianapolis
Indianapolis, IN

David E. Malarkey, D.V.M., Ph.D., Principal Reviewer
Department of Microbiology, Pathology, and Parasitology
College of Veterinary Medicine
North Carolina State University
Raleigh, NC

Michele Medinsky, Ph.D.
Durham, NC

Walter W. Piegorsch, Ph.D.
Department of Statistics
University of South Carolina
Columbia, SC

Mary Anna Thrall, D.V.M.
Department of Pathology
College of Veterinary Medicine and Biomedical Sciences
Colorado State University
Fort Collins, CO

* Did not attend

SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On October 18, 2001, the draft Technical Report on the toxicology and carcinogenesis studies of 2,4-hexadienal 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. P.C. Chan, NIEHS, introduced the toxicology and carcinogenesis studies of 2,4-hexadienal by describing 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 and nonneoplastic lesions in rats and mice. The proposed conclusions were *clear evidence of carcinogenic activity* of 2,4-hexadienal in male and female F344/N rats and male and female B6C3F₁ mice.

Dr. Klaunig, a principal reviewer, was unable to attend the meeting, and Dr. M.S. Wolfe, NIEHS, read his comments for the record. Dr. Klaunig agreed with the proposed conclusions regarding forestomach neoplasms but did not feel the oral cavity carcinomas in two male mice constituted evidence of carcinogenic activity. He also noted that mutagenicity findings from different laboratories were inconsistent.

Dr. Drinkwater, the second principal reviewer, felt the statement that oral cavity carcinomas may have been

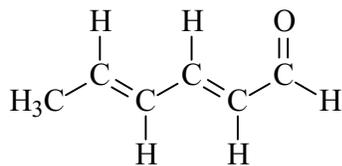
treatment related could be included in the conclusions. He asked for clarification of the description of the isomeric mixture at the start of the report.

Dr. Malarkey, the third principal reviewer, agreed with the proposed conclusions and felt the oxidative stress and DNA adduct studies were worthwhile additions.

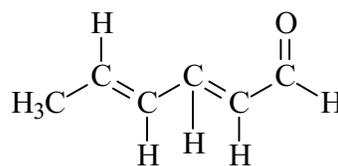
Dr. Chan explained that different concentrations of S9 metabolic activation enzymes were used in different mutagenicity assays and that a lack of response in tests at one laboratory does not negate positive responses at another. He noted that carcinomas of the tongue are rare in NTP studies, and the intent was to note their presence without implying statistical significance. Dr. J.R. Bucher, NIEHS, explained that the term "may have been related" to chemical exposure proposed for the oral cavity neoplasms was meant to distinguish these lesions from those constituting the "clear evidence" of carcinogenic activity in the same sex/species group. By themselves, the tongue neoplasms would be considered only an equivocal finding.

Dr. Drinkwater moved, and Dr. Thrall seconded, that the second sentence of the conclusion statement regarding oral cavity carcinomas be deleted. The motion was defeated by a vote of three to two. Dr. Malarkey then moved, and Dr. Drinkwater seconded, that the conclusions be accepted as originally written. The motion was carried unanimously with five votes.

INTRODUCTION



(89% *trans,trans*)



(11% *cis,trans*)

2,4-HEXADIENAL

(89% *trans,trans* isomer, CAS No. 142-83-6)

Chemical Formula: C₆H₈O Molecular Weight: 96.13

Synonyms: Hexa-2,4-dienal; 2,4-hexadienal; 2,4-hexadien-1-al; 2,4-Hx; 1,3-pentadiene-1-carboxaldehyde; 2-propylene acrolein; sorbaldehyde; sorbic aldehyde

CHEMICAL AND PHYSICAL PROPERTIES

2,4-Hexadienal is a colorless or yellowish liquid with a pungent sweet, citrusy odor (Ford *et al.*, 1988). It is insoluble in water, soluble in alcohol, and reacts with strong oxidizing and reducing agents (Bedoukian, 1985). 2,4-Hexadienal has a boiling point of 147° F at 15 mm Hg (Lancaster Synthesis, Inc., 1991), a specific gravity of 0.871, a vapor density of greater than 1, and a vapor pressure of 1.6 mm Hg at 20° C. The refractive index of 2,4-hexadienal is 1.540 and its flash point is 154° F (Aldrich, 1991; MSDS, 1992). The commercial-grade 2,4-hexadienal occurs as an isomeric mixture of 89% *trans,trans*-2,4-hexadienal and 11% *cis,trans*-hexadienal.

PRODUCTION, USE, AND HUMAN EXPOSURE

2,4-Hexadienal is prepared by condensation of acetaldehyde (Keller *et al.*, 1983). Current production levels are not available.

2,4-Hexadienal is used as a flavoring agent in the manufacture of the aromatic chemical 3,5,7-nonatrien-2-one, as a chemical intermediate in various organic synthetic reactions, and as the starting material for the manufacture of sorbic acid, a widely used food preservative (Keller *et al.*, 1983). It is also used as a chemical intermediate in the manufacture of polymethine dyes (Sturmer and Diehl, 1982), a pharmaceutical intermediate for the manufacture of mitomycins and antihypercholesteremics (STN, 1992), a corrosion inhibitor for steel used in oil field operations (Growcock *et al.*, 1989), a monomer for reaction with silane comonomers in polyalkenyloxysilane polymer manufacture, and a fumigant against larvae of the Caribbean fruit fly (STN, 1992).

2,4-Hexadienal occurs naturally as an auto-oxidation product of polyunsaturated fatty acids of plant and animal origin. During auto-oxidation of polyunsaturated fatty acids, the radical that initiates the process, usually following exposure to light or metal ions, reacts with the α -methylene group adjacent to the carbon-carbon double

bonds by abstraction of a hydrogen adjacent to a double bond leading to the formation of a lipid radical L•. The lipid radical L• combines with ground state oxygen to give the peroxyradical LOO•, which in turn attacks another α -methylene group yielding a lipid hydroperoxide (LOOH) and a new lipid radical, propagating the chain reaction. The monohydroperoxides LOOH, which are the first products of peroxidation, are unstable and easily decompose into aldehydes and other products termed secondary auto-oxidation products that include saturated and unsaturated aldehydes, di- and epoxyaldehydes, lactones, furans, ketones, oxo- and hydroxyacids, and saturated and unsaturated hydrocarbons (Esterbauer, 1982). 2,4-Hexadienal is one of the unsaturated aldehydes produced by this decomposition.

It has been shown that the amount of secondary auto-oxidation products (carbonyl compounds) in soybean oil can increase during storage in the dark at room temperature (White and Hammond, 1983). The increase was accelerated at higher temperature or under fluorescent light. During cooking, the auto-oxidation process in oil and fat is enhanced. The generated concentration of these polyunsaturated fatty acid-derived auto-oxidation products depends on the polyunsaturated fatty acid content of the oil, the nature and capacity of the heating vessel used (surface area), and the durations and conditions of heating and storage (Haywood *et al.*, 1995). This is seen in samples of repeatedly used frying oils obtained from fast-food/take-out establishments and in cooked beef fat, butter, lard, and ovine fat (Suzuki and Bailey, 1985; Claxon *et al.*, 1994).

2,4-Hexadienal has been identified in numerous oxidized glyceridic oils, including canola (low erucic acid rapeseed) oil, soybean oil, cottonseed oil, sunflower oil, sesame oil, and palm oil; it has also been detected in the essential oils of lovage, thyme leaf, and dill, and in solid alfalfa extract. It has been detected in fish, including farm-raised catfish, Gulf of Mexico menhaden, and Upper Wisconsin River walleye, and northern pike (Heil and Lindsay, 1988). It occurs naturally in a variety of plant products, including cotton, tomatoes, mango, kiwi, and Chinese quince, and is a component of tobacco leaf and tobacco-smoke volatiles (Wright and Harris, 1985; Takeoka *et al.*, 1986; Weeks *et al.*, 1989; Zeringue and McCormick, 1989). It has been detected as a volatile of piled (rather than picked) Toyama Kurocha tea processed in Japan. 2,4-Hexadienal was not found in fresh, steamed, or fermented tea leaves but was reported at a concentration of 0.4 mg/100 mg in the solar-dried

product and 0.2 mg/100 mg in the product stored for 1 year (Kawakami and Shibamoto, 1991).

2,4-Hexadienal was identified in polluted urban air (Dumdei *et al.*, 1988), and it was cited in a Russian review of aldehydic environmental pollutants. 2,4-Hexadienal has been identified as a low-level carbonyl impurity in commercial-grade ethanol as well as in distilled premium grades (Sherman and Kavasmaneck, 1980).

The presence of 2,4-hexadienal in oysters at 35 $\mu\text{g}/\text{kg}$ (35 ppb) and clams at 7.5 $\mu\text{g}/\text{kg}$ (7.5 ppb) from Lake Pontchartrain in Louisiana has been attributed to water pollution by volatile organic chemicals (Ferrario *et al.*, 1985).

Humans are continually exposed to 2,4-hexadienal in oxidized oils and fats in the diet. 2,4-Hexadienal has been detected in tobacco and tobacco smoke (Florin *et al.*, 1980; Pettersson *et al.*, 1980) and is present in seafood (Ferrario *et al.*, 1985), oxidized edible fats and oils, heated oils for food frying and cooking, and fish oils (Selke and Rohwedder, 1983; White and Hammond, 1983; Suzuki and Bailey, 1985; Przybylski and Hougen, 1989; Claxon *et al.*, 1994). Other food products in which it has been detected include meat fat, cow's milk fat, potato chips, bread crust, dried and stored piled tea, herbs and spices, and tropical fruits. Based on the large number of foods and food products that contain 2,4-hexadienal either naturally or as an additive, low-level human exposure to this compound is widespread.

According to the Flavor and Extract Manufacturers' Association (personal communication, 1994), the total amount of 2,4-hexadienal used as a flavor ingredient was 0.9 kg. This amounted to a per capita exposure of 9.9 ng/person per day via foods and flavor ingredients. Ford *et al.* (1988) reported that the maximum concentration of this chemical as an ingredient in consumer products could reach as high as 0.1%; the types of food products were not indicated.

REGULATORY STATUS

2,4-Hexadienal is listed in the U.S. Environmental Protection Agency's Toxic Substances Control Act Chemical Substance Inventory (USEPA, 2000). No standards or guidelines have been set for allowable occupational exposures or environmental concentrations

of 2,4-hexadienal. The American Conference of Governmental Industrial Hygienists has not adopted a time-weighted average threshold limit value for this compound.

2,4-Hexadienal was given Generally Recognized as Safe status after a review of flavoring ingredients and food additives by the Flavoring Extract Manufacturers' Association and was listed in 1981 by the Council of Europe as a flavoring substance that may be added to food (Ford *et al.*, 1988).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

No absorption, distribution, metabolism, or excretion studies of 2,4-hexadienal or other dienals were found in a search of the available literature. Absorption, metabolism, and urinary excretion of aldehydes such as malondialdehyde (McGirr *et al.*, 1985) and acrolein (Kaye, 1973) have been reported. Grootveld *et al.* (1998) reported that *trans*-2-alkenals (*trans*-2-nonenal and *trans*-2-pentenal) are readily absorbed from the gut into the systemic circulation *in vivo*, metabolized (primarily via the addition of glutathione across their electrophilic C-C double bonds), and excreted in the urine as C3-mercapturate conjugates.

Aldehydes are principally metabolized in the liver. Alcohol dehydrogenase is capable of catalyzing the metabolic reduction of aldehydes to primary alcohols in the reversal of the metabolic reaction involving the oxidation of primary alcohols to aldehydes. In mammals, however, this does not necessarily occur, because aldehydes are preferentially oxidized to the corresponding acids. The acids formed as major metabolites of aldehydes may then be excreted or form conjugates that are excreted. Aldehyde dehydrogenase, with NADH as cofactor, has been shown to dehydrogenate short chain aliphatic aldehydes as well as aromatic aldehydes (McMahon, 1982).

Humans

No information on the absorption, distribution, metabolism, or excretion of 2,4-hexadienal in humans was found in a review of the available literature.

BIOLOGIC EFFECTS

While free radicals and lipoperoxides produce direct damage at the cell structures where they are produced, aldehydes are more diffusible and long-lived and may induce damage at distant sites. The secondary peroxidation products react *in vivo* with biomolecules such as glutathione, free amino acids, proteins, and DNA (Esterbauer, 1985).

α,β -Unsaturated aldehydes ingested or produced endogenously as a result of lipid peroxidation during normal metabolic processes or induced by exogenous chemicals such as CCl₄, CHCl₃, DDT, and PCB are strong electrophilic reagents and react readily with nucleophilic groups. Thus, 2,4-hexadienal is expected to interact with DNA. Eder *et al.* (1993) demonstrated that β -alkyl-substituted acrolein congeners (pentenal, hexenal, 3,3-dimethylacrolein) form 1,N²-cyclic adducts and 7,8-cyclic adducts with deoxyguanosine in a cell-free system *in vitro* similar to those observed with crotonaldehyde. Their data showed that 2,4-hexadienal formed similar adducts with deoxyguanosine in a cell-free system, although they could not isolate the adducts in sufficient quantity for exact characterization. They postulated that because these adducts are premutagenic DNA lesions and crotonaldehyde is carcinogenic, the β -alkyl-substituted acrolein congeners are to be considered procarcinogenic. A fluorescence associated with singlet oxygen is generated when DNA interacts with a lipid degradation product. Using this technique, Frankel *et al.* (1987) demonstrated that 2,4-alkadienals readily interact with calf thymus DNA *in vitro* in the presence of ferric chloride and ascorbic acid.

Glutathione *S*-transferases catalyze intracellular detoxification of a wide range of xenobiotics and chemotherapeutic agents, including the α,β -unsaturated aldehydes, by catalyzing the conjugation of chemically reactive electrophiles and glutathione. Oral administration of acrolein causes a dose-dependent depletion of glutathione in the liver, and conjugation of reduced glutathione and acrolein mediated by glutathione transferases is considered a detoxification mechanism (Witz, 1989). But evidence indicates that such conjugates may also transport the chemical to be activated at a new site. Thus, the biological fate of thiol conjugates of α,β -unsaturated aldehydes remains to be explored. Thiol reactivity could play a role in the induction of

DNA-protein cross-links because depletion of cellular glutathione could lead to elevated levels of reactive oxygen species and lipid peroxidation products that have been implicated in DNA-protein cross-link formation *in vitro* and *in vivo*.

Aldehydes have been shown to inhibit cell proliferation. Tumor cells are more sensitive to aldehydes than are normal cells, due to reduced aldehyde dehydrogenase activity. The mechanism may involve interaction with tubulin or inhibition of polyamine metabolism, adenylylase, or lysosomes (Dianzani, 1982). In addition, the sulfhydryl reactivity of α,β -unsaturated aldehydes may play a role in their carcinostatic action. For example, the reactivity of the double bond with sulfhydryls could produce adducts such as 1,2-crotonaldehyde-cysteine and 1,1-*trans*-4-hydroxypentenal-cysteine; the essential sulfhydryl groups might be located in enzymes.

2,4-Hexadienal was investigated for use as a food preservative but was found inactive in retarding the growth of food molds (fungi) (Troller and Olsen, 1967). However, Gueldner *et al.* (1985) reported that naturally occurring 2,4-hexadienal appeared to act as an endogenous mycostatic insecticide in corn ears, inhibiting growth of *Aspergillus flavus*.

The effect of α,β -unsaturated aldehydes on rat liver microsomal glucose-6-phosphatase has been studied. Depending on the chain length, the Michaelis constant, K_m , and the maximal rate of reaction, V_{max} , were affected. However, 2,4-hexadienal did not alter the kinetic constant and K_m of the enzyme. These results may be attributed to the rather rigid planar structure around the two conjugated double bonds in 2,4-hexadienal, which give rise to a severe steric hindrance at the α - and β -carbon atoms (Jorgensen *et al.*, 1992). Other enzymes known to be inhibited include cytochrome P450, aminopyrine demethylase, adenylylase, and O6-methylguanine DNA methyltransferase.

2,4-Hexadienal is cytotoxic, and the cytotoxicity may be related to a decrease in membrane lipid fluidity (Witz, 1989). For example, in a study of the effects of tobacco smoke components, Thelestam *et al.* (1980) found that 2,4-hexadienal at a concentration of 25 mM caused an increase of 20% in the membrane permeability of human lung fibroblasts incubated for 30 minutes. Growth of murine Ascites sarcoma BP8 cells were inhibited 44% by 0.01 mM 2,4-hexadienal in ethanol while 0.1 and 1.0 mM concentrations were 100% cytotoxic (Pilotti *et al.*, 1975). Noradrenaline-induced oxidative

metabolism in isolated hamster brown fat cells was inhibited by 0.1 mM 2,4-hexadienal; inhibition increased to 100% at 1 mM (Pettersson *et al.*, 1980). Complete cessation of ciliary activity of chicken embryo tracheal organ cultures was induced for 6 minutes by 5 mM 2,4-hexadienal.

α,β -Unsaturated aldehydes, including 2,4-hexadienal, react with thiobarbituric acid to form a reddish pigment which is the basis of lipid peroxidation analyses; Kosugi *et al.* (1988) observed synergism between 2,4-alkadienals and other aldehydes and hydroperoxides as evidenced by the intensity of this red pigment.

No DNA-protein crosslinks were observed 4 hours after treatment of HL60 cells with the *trans,trans*-muconaldehyde (MUC) metabolites 6-hydroxy-*trans,trans*-2,4-hexadienal, 6-oxo-*trans,trans*-2,4-hexadienoic acid, or *trans,trans* muconic acid each at 100 μ M (Schoenfeld and Witz, 2000). However, increases in DNA-protein crosslink formation at higher concentrations cannot be ruled out. The MUC metabolites did not decrease cell viability at 100 μ M.

STRUCTURE/ACTIVITY RELATIONSHIPS

2,4-Hexadienal is a representative of the family of α,β -unsaturated aldehydes. The benzene metabolite, MUC, an α,β -unsaturated six-carbon diene dialdehyde, is hematotoxic, mutagenic, and clastogenic (Witz *et al.*, 1996). It is a reactive multifunctional alkylating agent, capable of cross-linking cellular components such as proteins (Schoenfeld and Witz, 2000), and forming adducts with deoxyguanosine 5'-phosphate and DNA (Latriano *et al.*, 1989; Schatz-Kornbrust *et al.*, 1991). The MUC metabolite 6-hydroxy-*trans,trans*-2,4-hexadienal is also hematotoxic and mutagenic (Witz *et al.*, 1996).

α,β -Unsaturated aldehydes react with sulfhydryl groups (Witz *et al.*, 1987; Kline *et al.*, 1993) and with amino groups of proteins and DNA (Latriano *et al.*, 1989; Udupi *et al.*, 1994). A number of carcinogenic aldehydes such as formaldehyde (Casanova-Schmitz and Heck, 1983) and acrolein (Crook *et al.*, 1986) induced DNA-protein cross-links.

TOXICITY

Little is known about the *in vivo* effects of 2,4-hexadienal, its mechanisms of toxicity, or which tissues it may target. However, α,β -unsaturated aldehydes are

direct-acting alkylating agents capable of covalent binding without prior metabolism to cellular nucleophilic groups (Eder *et al.*, 1993). Accordingly, 2,4-hexadienal is potentially toxic and/or capable of modifying cellular processes.

Experimental Animals

The irritating effect of 2,4-hexadienal may cause cellular injury and cell proliferation in esophageal tissue and other parts of the alimentary tract following oral administration. 2,4-Hexadienal is listed in the Registry of Toxic Effects of Chemical Substances database as a severe irritant in rabbits following dermal and ocular administration and a severe irritant and sensitizer in guinea pigs following dermal administration (RTECS, 1992). Acute toxicity values for 2,4-hexadienal are given in Table 1.

The feeding of lipid oxidation products and oxidized fats has been reported to cause adverse biologic effects in laboratory animals, including growth retardation, teratogenicity, tissue damage, and increased liver and kidney weights (Izaki *et al.*, 1984; Kanazawa *et al.*, 1985, 1986; Alexander *et al.*, 1987), as well as cellular damage to the testes and epididymides, increased peroxidation of membrane and tissue lipids, and induction of cytochrome P450 activities in the liver and colon (Crawford and Wheeler, 1983; Haywood *et al.*, 1995).

Tanaka (1979) applied peroxidized linoleic acid on the shaved skin of guinea pigs in a patch test experiment and found that it produced necrosis and bleeding. When the

abdominal skin of a guinea pig was patched for 8 days with a cream sample containing 25 nmol (in terms of malondialdehyde) of lipid peroxides per gram, a thickening of the epidermis was found (Tanaka and Hayakawa, 1986).

Humans

Health hazard advisory information in the Aldrich Material Safety Data Sheet for 2,4-hexadienal includes severe irritant and toxic effects following inhalation or dermal absorption, with tissue destruction of mucous membranes of the upper respiratory tract, eyes, and skin (MSDS, 1992). Ford *et al.* (1988) cited the results of dermal 48-hour closed patch tests using 1% 2,4-hexadienal (in petroleum) on the backs of 59 volunteers; one case of sensitization and no irritation was reported.

CARCINOGENICITY

Experimental Animals

No information on long-term studies of the carcinogenicity of 2,4-hexadienal in experimental animals was found in the literature.

Humans

No epidemiology studies or case reports examining the relationships between exposure to 2,4-hexadienal and cancer in humans were found in the literature. However, free radicals, singlet oxygen, and other reactive species formed in the peroxidation of lipids are considered biologically harmful and are implicated in cellular

TABLE 1
Acute Toxicity Data for 2,4-Hexadienal^a

Species	Route	LD ₅₀
Rat	Oral	300 mg/kg
Rabbit	Dermal	270 mg/kg ^b
Guinea pig	Dermal	2,500 mg/kg
Guinea pig	Dermal	5,000 mg/kg
Rat	Inhalation	2,000 ppm/4 hours ^c

^a RTECS, 1992

^b LD₁₀

^c LC₁₀

damage and cancer (Frankel *et al.*, 1987). It has been postulated that high colon cancer incidences may be linked to a high-fat diet or one low in fruits and vegetables and low in vitamin A (Urbany, 1992). Marnett *et al.* (1985) have postulated that, "since carbonyl compounds are widely distributed in foods, are generated during cellular metabolism, and are present in body fluids, they make a significant contribution to the risk of human cancer." It is difficult to assess the cancer risk from multiple low level exposures to a wide variety α,β -unsaturated aldehydes.

GENETIC TOXICOLOGY

2,4-Hexadienal has been shown to be mutagenic in bacterial mutation assays; no reports of *in vivo* mutagenicity testing were identified. Although Florin *et al.* (1980) reported negative results with 2,4-hexadienal (3 μmol per plate) in a spot test using *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537, with and without S9, Marnett *et al.* (1985) reported a clear dose response over a concentration range of 0.1 to 1.0 μmol 2,4-hexadienal in *S. typhimurium* strain TA104 with a liquid preincubation assay in the absence of liver S9 activation enzymes. Results of studies reported by Eder *et al.* (1992) showed that several members of a series of substituted acrolein congeners including 2,4-hexadienal were mutagenic in a modified preincubation test using a 90-minute preincubation period with *S. typhimurium* strain TA100, with and without S9 from Aroclor-induced rats. 2,4-Hexadienal (99.5% pure) was tested over a concentration range of 0.02 to 0.4 μL per plate. In these studies, mutagenic activity for the class of compounds was shown to be inversely related to toxicity and chain length. However, the presence of the second double bond within the 2,4-hexadienal molecule overrode this generalization and conferred decreased toxicity and increased mutagenicity over what chain length alone might have predicted. Further mutagenicity studies with 2,4-hexadienal by Eder *et al.* (1992) using the SOS chromotest yielded negative results. The lack of demonstrated genotoxicity in this assay was attributed to the toxicity of 2,4-hexadienal to the *Escherichia coli* tester strains PQ37 and PQ243. However, it was noted that the negative response in the SOS chromotest appeared to be solvent specific; changing the solvent from dimethylsulfoxide to ethanol provoked a weakly positive response (Eder *et al.*, 1993). Additional studies by Eder *et al.* (1993) showed significant mutagenic activity for 2,4-hexadienal (0.01 to 0.75 μL per plate) in *S. typhimurium* strain TA100 using a standard 30-minute

preincubation period (Eder *et al.*, 1992). In addition, increased levels of DNA strand breakage were measured by the alkaline elution technique in 1,210 mouse leukemia cells treated with relatively high doses of 2,4-hexadienal that also produced evidence of cytotoxicity (Eder *et al.*, 1993). In further investigations, 7,8-cyclic guanine and 1,2-cyclic deoxyguanosine adducts were isolated by chromatography from a cell-free reaction mixture containing 2,4-hexadienal and various nucleosides (Eder *et al.*, 1993).

STUDY RATIONALE

2,4-Hexadienal is a natural constituent of meat, vegetable, and fish oils. 2,4-Hexadienal is also used as a food additive or flavoring agent. It is one of the lipid peroxidation products of polyunsaturated oils that undergo auto-oxidation especially during storage (Snyder *et al.*, 1985) and has been implicated in the development of off or tainted flavor. Lipid hydroperoxides have been shown to give rise to low intracellular levels of α,β -unsaturated aldehydes, including 2,4-hexadienal and 2,4-decadienal. Some of the α,β -unsaturated aldehydes have been shown to be reactive with DNA (Frankel *et al.*, 1987). Ingested lipid oxidation products and oxidized fats have been reported to cause increased excretion of mutagens, cellular injury to the liver and kidney, increased cell proliferation in the gastrointestinal tract, nonspecific tissue injury, and irritation resulting from induced oxidative stress.

2,4-Hexadienal was nominated by the National Cancer Institute for study. The Interagency Testing Committee has classified the α,β -unsaturated aldehydes as a group of closely-related chemicals likely to be associated with adverse health and ecological effects. The Committee's concern for potential health effects resulting from exposures to this group of chemicals included potential oncogenicity, mutagenicity, and membrane irritation.

The NTP decided to evaluate the metabolism, distribution, mutagenicity, and carcinogenicity and perform mechanistic studies of 2,4-hexadienal with 2,4-decadienal as a matched pair, because there is an overall lack of data generated from testing dienals for carcinogenicity and a lack of studies on 2,4-hexadienal exposure related to cancer in humans. The role of consumed oxidized oils in gastrointestinal carcinogenesis including the effects of oral intake of different doses of various biologically active compounds present in heated oils, effects of oxidative stress induced by chronic consumption of

repeatedly heated oils, as well as interactions with other modulating dietary factors, including both macro- and micronutrients has not been investigated (Hageman *et al.*, 1991). Gavage in a corn oil medium was selected as the route of administration because the chemical is insoluble in water and is unstable when mixed in feed preparations.

Because 2,4-decadienal is less toxic (oral LD₅₀ in rats is greater than 5 g/kg) than 2,4-hexadienal, 2-year studies were not conducted. The NTP 90-day study results at doses up to 800 mg/kg showed that the lesions induced by 2,4-decadienal were similar to those induced by

2,4-hexadienal, specifically forestomach hyperplasia accompanied by inflammation and olfactory epithelial necrosis and atrophy (NTP, unpublished data). 2,4-Hexadienal was selected for 2-year studies because the two chemicals have similar chemical and biological properties. Chemical disposition studies were recommended for 2,4-hexadienal; however, these studies were not conducted because of the poor stability of the radio-labeled chemical. Toxicokinetic studies were also considered but not attempted because 2,4-hexadienal was expected to quickly react with blood components making it unlikely that a toxicokinetic study would be successful.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF 2,4-HEXADIENAL

2,4-Hexadienal was obtained from Lancaster Synthesis, Inc. (Windham, NH), in two lots (90000345 and P09653). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Research Triangle Institute (Research Triangle Park, NC) (Appendix I). Reports on analyses performed in support of the 2,4-hexadienal studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a pale to dark yellow liquid, was identified as 2,4-hexadienal by the analytical chemistry laboratory and the study laboratory using infrared and nuclear magnetic resonance spectroscopy. The purity of lot 90000345 was determined by the study laboratory using gas chromatography, which indicated a purity of 95.2% with one major impurity peak and four minor impurity peaks. The purity of lot P09653 was determined by the analytical chemistry laboratory using high-performance liquid chromatography (HPLC) and by the study laboratory using gas chromatography. Discrepancies in the results with these two methods were large but were ultimately traced, during the 2-year study, to incomplete resolution of an impurity peak and overestimation of the purity of the bulk chemical with gas chromatographic methods. HPLC indicated a purity of approximately 89% with one impurity peak representing approximately 11% of the total integrated area. Gas chromatography indicated a purity of 98.4%. The impurity in both lots was identified at the analytical laboratory as *cis,trans*-2,4-hexadienal using infrared spectroscopy and gas chromatography/mass spectrometry. Inconsistencies in the purity of the test articles were resolved during the chronic study. Optimized HPLC method was used, and both lots of 2,4-hexadienal contained approximately 89% *trans,trans*-2,4-hexadienal and approximately 11% *cis,trans*-2,4-hexadienal.

To ensure stability, the bulk chemical was stored refrigerated and protected from light in sealed containers under a nitrogen headspace. Stability was monitored

relative to a frozen reference sample by the study laboratories using gas chromatography. Gas chromatography was used to allow comparison of data to previous bulk chemical analysis. No degradation of the bulk chemical was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared once for the 16-day studies and every 4 weeks for the 14-week and 2-year studies by mixing 2,4-hexadienal with corn oil to give the required concentrations (Table I3). The dose formulations were stored refrigerated and protected from light under nitrogen in amber glass containers for up to 35 days.

Stability studies of 0.290, 0.292, and 0.298 mg/kg dose formulations were performed by the analytical chemistry laboratory using HPLC. Homogeneity studies of the 0.75 and 24 mg/mL dose formulations for the 14-week studies and stability studies of a 0.77 mg/mL dose formulation were performed by the study laboratory using HPLC. Homogeneity was confirmed; stability was confirmed for dose formulations stored under a nitrogen headspace protected from air at room temperature for at least 35 days and for dose formulations, open to air for up to 3 hours.

Periodic analyses of the dose formulations of 2,4-hexadienal were conducted by the analytical chemistry laboratory (16-day studies) and by the study laboratory (14-week and 2-year studies) using HPLC. During the 16-day studies, the dose formulations were analyzed once; four of five dose formulations for rats and mice were within 10% of the target concentrations (Table I4). One formulation was used at 84% of target. During the 14-week studies, the dose formulations were analyzed at the beginning, midpoint, and end of the studies, and all were within 10% of the target concentrations (Table I5). During the 2-year studies, the dose formulations were analyzed every 8 to 12 weeks (Table I6). All dose formulations analyzed for rats and 32 of 33 for mice were

within 10% of the target concentrations; the dose formulation for mice that was not within the acceptable range was remixed and was found to be within 10% of the target concentration. Periodic analyses of the corn oil vehicle by the study laboratories demonstrated that peroxide concentrations were within the acceptable limit of 3.0 mEq/kg.

16-DAY STUDIES

Male and female F344/N rats and B6C3F₁ mice were obtained from Taconic Farms (Germantown, NY). On receipt, the rats and mice were 4 weeks old. Animals were quarantined for 11 (rats) or 12 (mice) days and were 6 weeks old on the first day of the studies. Groups of five male and five female rats and mice were administered 2,4-hexadienal in corn oil by gavage at doses of 0, 3, 9, 27, 80, or 240 mg 2,4-hexadienal/kg body weight, 5 days per week, for 16 days. Feed and water were available *ad libitum*. Rats and female mice were housed five per cage; male mice were housed individually. Clinical findings were recorded and animals were weighed initially, on day 8, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 2.

Necropsies were performed on all rats and mice. The liver and right kidney from each animal were weighed. Histopathologic examinations were performed on rats and mice in the vehicle control, 27, 80, and 240 mg/kg groups. Table 2 lists the tissues and organs examined.

14-WEEK STUDIES

The 14-week studies were conducted to evaluate the cumulative toxic effects of repeated exposure to 2,4-hexadienal and to determine the appropriate doses to be used in the 2-year studies.

Male and female F344/N rats and B6C3F₁ mice were obtained from Taconic Laboratory Animals and Services (Germantown, NY). On receipt, the rats and mice were 4 weeks old. Animals were quarantined for 11 or 12 days (rats) and 13 or 14 days (mice) and were 6 weeks old on the first day of the studies. Before the studies began, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. At the end of the studies, serologic analyses were performed on five male and five female vehicle control rats and mice using the

protocols of the NTP Sentinel Animal Program (Appendix K).

Groups of 10 male and 10 female core study rats and mice and groups of 10 male and 10 female clinical pathology study rats were administered 2,4-hexadienal in corn oil by gavage at doses of 0, 7.5, 15, 30, 60, or 120 mg/kg, 5 days per week, for 14 weeks. Feed and water were available *ad libitum*. Rats and female mice were housed five per cage while male mice were housed individually. Clinical findings were recorded once a week for the duration of the studies beginning on day 1 (mice) or day 4 (rats) and at the end of the study. The animals were weighed initially, on day 4 (rats), weekly, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 2.

Blood was collected from the retroorbital sinus of clinical pathology study rats under carbon dioxide anesthesia on days 4 and 19. Using the same method, blood was collected from core study rats and mice surviving to the end of the studies for hematology and clinical chemistry (rats) analyses. For hematology analyses, blood from each animal was collected into a tube containing EDTA. Erythrocyte, platelet, and leukocyte counts, hematocrit values, hemoglobin concentration, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration analyses were determined using the Technicon H-1™ hematology analyzer (Technicon Corporation, Tarrytown, NY). Reagents were manufactured or supplied by Technicon Corporation, R&D Systems (Minneapolis, MN), or Fisher Scientific, Inc. (Hampton, NH). Reticulocyte counts were conducted using a Coulter Model Elite Flow Cytometer (Coulter Corporation, Miami, FL). Blood smears were prepared to evaluate platelet and erythrocyte morphologies by light microscopy; these smears were also used to manually verify reticulocyte and leukocyte differential counts, as necessary. For clinical chemistry analyses, blood was collected into a tube containing no anticoagulant. Clinical chemistry analyses were performed using the Roche Cobas Fara™ automated analyzer (Roche Diagnostic Systems, Inc., Montclair, NJ). Reagents were manufactured or supplied by Roche, Sigma Chemical Company (St. Louis, MO), or Ciba Corning Diagnostics Corporation (Norwood, MA), as applicable. The parameters measured are listed in Table 2.

At the end of the 14-week studies, samples were collected for sperm motility and vaginal cytology evaluations on vehicle control, 30, 60, and 120 mg/kg core

study rats and mice. The parameters evaluated are listed in Table 2. Methods used were those described in the NTP's Sperm Morphology and Vaginal Cytology Evaluations protocol (NTP, 1992). For 12 consecutive days prior to scheduled terminal sacrifice, the vaginal vaults of the females were moistened with 0.9% saline, if necessary, and samples of vaginal fluid and cells were stained. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus, estrus, and metestrus). Male animals were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk (rats) or modified Tyrode's buffer (mice) was applied to slides and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then heat fixed at 65° C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline centering 10% dimethylsulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

Necropsies were performed on all core study animals. The heart, right kidney, liver, lungs, spleen, right testis, and thymus of core study rats and mice were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed on all vehicle control and 120 mg/kg core study rats and mice. Table 2 lists the tissues and organs examined.

2-YEAR STUDIES

Study Design

Groups of 50 male and 50 female rats were administered 2,4-hexadienal in corn oil by gavage at doses of 0, 22.5, 45, or 90 mg/kg, and groups of 50 male and 50 female

mice received 2,4-hexadienal in corn oil by gavage at doses of 0, 30, 60, or 120 mg/kg, 5 days per week, for 104 to 105 weeks. Additional groups of 10 male and 10 female rats were administered 0, 90, or 120 mg/kg 2,4-hexadienal for oxidative stress studies. Additional groups of five male rats were administered 0 or 90 mg/kg and five or 10 male mice were administered 0 or 120 mg/kg 2,4-hexadienal for DNA adduct characterization studies.

Source and Specification of Animals

Male and female F344/N rats and B6C3F₁ mice were obtained from Taconic Laboratory Animals and Services (Germantown, NY) for use in the 2-year studies. Rats and mice were quarantined for 10 days before the beginning of the studies. Five male and five female rats and mice were randomly selected for parasite evaluation and gross observation of disease. Rats and mice were approximately 5 weeks old at the beginning of the studies. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix K).

Animal Maintenance

Male rats were housed two or three per cage, and female rats and mice were housed five per cage. Male mice were housed individually. Feed and water were available *ad libitum*. Cages and racks were rotated every 2 weeks. Further details of animal maintenance are given in Table 2. Information on feed composition and contaminants is provided in Appendix J.

Clinical Examinations and Pathology

All animals were observed twice daily for mortality and moribundity. Body weights were recorded initially, and clinical findings and body weights were recorded every 4 weeks beginning with week 3 or week 4 (female rats).

Liver (rats) and forestomach (rats and mice) tissue was taken from DNA adduct characterization study animals at 118 (0 mg/kg rats) or 90 days (Appendix M). Additional details for these studies are presented in Table 2. Complete necropsies and microscopic examinations were performed on all surviving rats and mice. 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 paraffin, sectioned to a thickness of 5 µm, and stained with hematoxylin and eosin for microscopic examination. For all paired organs

(e.g., adrenal gland, kidney, ovary), samples from each organ were examined. Tissues examined microscopically are listed in Table 2.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The slides, paraffin blocks, and residual wet tissues were sent to the NTP 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 laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a quality assessment pathologist evaluated slides from all tumors and all potential target organs, which included the forestomach of rats and mice; spleen of rats; thyroid gland, heart, and prostate gland of male rats; lung and kidney of female rats; tongue, testis, liver, nose, and adrenal cortex of male mice; and kidney of female mice.

The quality assessment report and the reviewed slides were submitted to the NTP Pathology Working Group

(PWG) chairperson, who reviewed the selected tissues and addressed any inconsistencies in 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 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 2
Experimental Design and Materials and Methods in the Gavage Studies of 2,4-Hexadienal

16-Day Studies	14-Week Studies	2-Year Studies
Study Laboratory Microbiological Associates, Inc. (Bethesda, MD)	Southern Research Institute (Birmingham, AL)	Southern Research Institute (Birmingham, AL)
Strain and Species F344/N rats B6C3F ₁ mice	F344/N rats B6C3F ₁ mice	F344/N rats B6C3F ₁ mice
Animal Source Taconic Farms (Germantown, NY)	Taconic Laboratory Animals and Services (Germantown, NY)	Taconic Laboratory Animals and Services (Germantown, NY)
Time Held Before Studies Rats: 11 days Mice: 12 days	Rats: 11 (males) or 12 (females) days Mice: 13 (males) or 14 (females) days	10 days
Average Age When Studies Began 6 weeks	6 weeks	5 weeks
Date of First Dose Rats: November 20, 1995 Mice: November 21, 1995	Rats: August 5 (males) or 6 (females), 1996 Mice: August 7 (males) or 8 (females), 1996	Rats: July 11, 1997 Mice: July 25, 1997
Duration of Dosing 5 days/week for 16 days	5 days/week for 14 weeks	5 days/week for 104 to 105 weeks
Date of Last Dose Rats: December 5, 1995 Mice: December 6, 1995	Rats: November 6-7, 1996 Mice: November 8-9, 1996	Rats: July 8-11 (males) or 11-14 (females), 1999 Mice: July 22-25 (males) or 25-28 (females), 1999
Necropsy Dates Rats: December 6, 1995 Mice: December 7, 1995	Rats: November 6-7, 1996 Mice: November 8-9, 1996	Rats: July 9-12 (males) or 12-15 (females), 1999 Mice: July 23-26 (males) or 26-29 (females), 1999
Average Age at Necropsy 8 weeks	Rats: 19 weeks Mice: 19 to 20 weeks	109 to 110 weeks
Size of Study Groups 5 males and 5 females	10 males and 10 females	50 males and 50 females
Method of Distribution Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 16-day studies	Same as 16-day studies
Animals per Cage Rats: 5 Mice: 1 (males) or 5 (females)	Rats: 5 Mice: 1 (males) or 5 (females)	Rats: 2 or 3 (males) or 5 (females) Mice: 1 (males) or 5 (females)

TABLE 2
Experimental Design and Materials and Methods in the Gavage Studies of 2,4-Hexadienal

16-Day Studies	14-Week Studies	2-Year Studies
Method of Animal Identification		
Tail tattoo	Tail tattoo	Tail tattoo
Diet		
NTP-2000 open formula pelleted diet (Zeigler Brothers, Inc., Gardners, PA), available <i>ad libitum</i> , changed weekly	Same as 16-day studies except diet was irradiated	Same as 16-day studies except diet was irradiated
Water		
Tap water (Washington Suburban Sanitary Commission Potomac Plant) via automatic watering system, available <i>ad libitum</i>	Tap water (City of Birmingham municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available <i>ad libitum</i>	Same as 14-week studies
Cages		
Polycarbonate	Polycarbonate (Lab Products, Maywood, NJ), changed twice weekly	Polycarbonate (Lab Products, Maywood, NJ), changed twice weekly or once weekly (male mice)
Bedding		
Sani-Chips [®] hardwood (P.J. Murphy Forest Products, Montville, NJ), changed twice weekly	Heat-treated hardwood chips (P.J. Murphy Forest Products, Inc., Montville, NJ), changed twice weekly or once weekly (male mice)	Same as 14-week studies
Cage Filters		
Reemay spun-bonded polyester (Andico, Birmingham, AL), changed once every 2 weeks	Same as 16-day studies	Same as 14-week studies
Racks		
Stainless steel, changed every 2 weeks	Stainless steel (Lab Products, Inc.), rotated every 2 weeks	Same as 14-week studies
Animal Room Environment		
Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: 10/hour	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: 10/hour	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: 10/hour
Doses		
0, 3, 9, 27, 80, or 240 mg/kg in corn oil by gavage (dosing volume 2.5 mL/kg)	0, 7.5, 15, 30, 60, or 120 mg/kg in corn oil by gavage [dosing volume 5 mL/kg (rats) or 10 mL/kg (mice)]	Rats: 0, 22.5, 45, or 90 mg/kg in corn oil by gavage (dosing volume 5 mL/kg) Mice: 0, 30, 60, or 120 mg/kg in corn oil by gavage (dosing volume 10 mL/kg)
Type and Frequency of Observation		
Observed twice daily; animals were weighed and clinical findings were recorded initially, on day 8, and at the end of the studies.	Observed twice daily; core study animals were weighed initially, on day 4 (rats), weekly, and at the end of the studies; clinical findings were recorded weekly.	Observed twice daily; animals were weighed initially and body weights and clinical findings were recorded every 4 weeks.
Method of Sacrifice		
Carbon dioxide asphyxiation	Carbon dioxide asphyxiation	Carbon dioxide asphyxiation

TABLE 2
Experimental Design and Materials and Methods in the Gavage Studies of 2,4-Hexadienal

16-Day Studies	14-Week Studies	2-Year Studies
<p>Necropsy Necropsies were performed on all animals. Organs weighed were the liver and right kidney.</p>	<p>Necropsies were performed on all core study animals. Organs weighed were the heart, right kidney, liver, lung, spleen, right testis, and thymus.</p>	<p>Necropsies were performed on all animals.</p>
<p>Clinical Pathology None</p>	<p>Blood was collected from the retroorbital sinus of clinical pathology study rats on days 4 and 19 and from core study animals at the end of the studies for hematology and clinical chemistry (rats). Hematology: hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; erythrocyte and platelet morphology; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials Clinical chemistry: urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and bile acids</p>	<p>None</p>
<p>Histopathology In addition to gross lesions, the forestomach of vehicle control and 27, 80, and 240 mg/kg rats and mice; and the liver and kidney of vehicle control and 80 and 240 mg/kg rats and mice were examined.</p>	<p>Complete histopathology was performed on vehicle control and 120 mg/kg core study rats and mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, gallbladder (mice only), heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland (except male mice), nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, and uterus. The forestomach and nose were also examined in all remaining groups of core study rats and mice.</p>	<p>Complete histopathology was performed on all rats and mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, gallbladder (mice only), heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland (except male mice), nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, and uterus.</p>

TABLE 2
Experimental Design and Materials and Methods in the Gavage Studies of 2,4-Hexadienal

16-Day Studies	14-Week Studies	2-Year Studies
Sperm Motility and Vaginal Cytology None	<p>At the end of the studies, sperm samples were collected from core study male animals in the vehicle control and 30, 60, and 120 mg/kg groups for sperm motility evaluations. The following parameters were evaluated: spermatid heads per testis and per gram testis, sperm heads per cauda and per gram cauda, and epididymal sperm motility. The left cauda, left epididymis, and left testis were weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies from females in the vehicle control and 30, 60, and 120 mg/kg groups for vaginal cytology evaluations. The percentage of time spent in the various estrous cycle stages and estrous cycle length were evaluated.</p>	None
Oxidative Stress Study None	None	<p>Forestomach samples were collected from groups of 10 male and 10 female rats administered 0, 90, or 120 mg/kg 2,4-hexadienal for 28 days.</p>
DNA Adduct Characterization None	None	<p>Samples were taken from DNA adduct characterization study male rats and mice for determinations of DNA adducts in the liver of rats and forestomach of rats and mice. The forestomach and liver were collected from five 90 mg/kg rats and 10 120 mg/kg mice at 90 days and from five vehicle control rats at 118 days and five vehicle control mice at 90 days.</p>

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 two sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions are presented in Tables A1, A5, B1, B5, C1, C5, D1, and D5 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 A3, B3, C3, and D3) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., harderian gland, intestine, mammary gland, and skin) before microscopic evaluation, or when neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed. Tables A3, B3, C3, and D3 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 and nonneoplastic lesion 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 B6C3F₁ 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 lesion incidence, and reported P values are one sided. The significance of lower incidences or decreasing trends in lesions is represented as $1-P$ with the letter N added (e.g., $P=0.99$ is presented as $P=0.01N$).

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology, clinical chemistry, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for

pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1951) were examined by NTP personnel, and implausible values were eliminated from the analysis. Average severity values were analyzed for significance with the Mann-Whitney U test (Hollander and Wolfe, 1973). Because vaginal cytology data are proportions (the proportion of the observation period that an animal was in a given estrous stage), an arcsine transformation was used to bring the data into closer conformance with a normality assumption. Treatment effects were investigated by applying a multivariate analysis of variance (Morrison, 1976) to the transformed data to test for simultaneous equality of measurements across doses.

Historical Control Data

The concurrent control group represents the most valid comparison to the treated groups and is the only control group analyzed statistically in NTP bioassays. However, historical control data are often helpful in interpreting potential treatment-related effects, particularly for uncommon or rare neoplasm types. For meaningful comparisons, the conditions for studies in the historical database must be generally similar. One significant factor affecting the background incidence of neoplasms at a variety of sites is diet. In 1995, the NTP incorporated a new diet (NTP-2000) that contains less protein and more fiber than the NIH-07 diet used previously in toxicity and carcinogenicity studies (Rao, 1996, 1997). The current NTP historical database contains all 21 studies that use the NTP-2000 diet with histopathology findings completed up to the present. A second potential source of variability is route of administration. In general the historical database for a given study will include studies using the same route of administration, and the overall incidences of neoplasms for all routes of administration are included for comparison.

Currently, the database includes 11 (10 for male rats) studies by various routes in which the NTP-2000 diet was used. Based on the extensive NTP historical database using the NIH-07 diet, incidences of the vast majority of spontaneous neoplasms are similar among control groups regardless of the route of administration. There is no reason to expect this to be different with the NTP-2000 diet. For example, control animals from dosed feed and dosed water studies are treated no differently and no differences in incidence of neoplasms are

expected. Exceptions exist for some neoplasms/routes, and if comparisons are necessary for these neoplasm types, only studies with similar routes of administration will be used.

QUALITY ASSURANCE METHODS

The 14-week and 2-year studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 2-year studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assurance contractor. 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 NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Technical Report.

GENETIC TOXICOLOGY

The genetic toxicity of 2,4-hexadienal was assessed by testing the ability of the chemical to induce mutations in various strains of *Salmonella typhimurium*, micronucleated erythrocytes in male rat and male mouse bone marrow, and increases in the frequency of micronucleated erythrocytes in mouse peripheral blood. The protocols for these studies and the results are given in Appendix E.

The genetic toxicity studies have evolved from an earlier effort by the NTP to develop a comprehensive database permitting a critical anticipation of a chemical's carcinogenicity in experimental animals based on numerous considerations, including the molecular structure of the chemical and its observed effects in short-term *in vitro* and *in vivo* genetic toxicity tests (structure-activity relationships). The short-term tests were originally developed to clarify proposed mechanisms of chemical-induced DNA damage based on the relationship between electrophilicity and mutagenicity (Miller and Miller, 1977) and the somatic mutation theory of cancer (Straus, 1981; Crawford, 1985). However, it should be noted that not all cancers arise through genotoxic mechanisms.

DNA reactivity combined with *Salmonella* mutagenicity is highly correlated with induction of carcinogenicity in multiple species/sexes of rodents and at multiple tissue sites (Ashby and Tennant, 1991). A positive response in the *Salmonella* test was shown to be the most predictive *in vitro* indicator for rodent carcinogenicity (89% of the *Salmonella* mutagens are rodent carcinogens) (Tennant *et al.*, 1987; Zeiger *et al.*, 1990). Additionally, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. However, these other tests can provide useful information on the types of DNA and chromosomal damage induced by the chemical under investigation.

The predictivity for carcinogenicity of a positive response in acute *in vivo* bone marrow chromosome aberration or micronucleus tests appears to be less than

that in the *Salmonella* test (Shelby *et al.*, 1993; Shelby and Witt, 1995). However, clearly positive results in long-term peripheral blood micronucleus tests have high predictivity for rodent carcinogenicity (Witt *et al.*, 2000); negative results in this assay do not correlate well with either negative or positive results in rodent carcinogenicity studies. Because of the theoretical and observed associations between induced genetic damage and adverse effects in somatic and germ cells, the determination of *in vivo* genetic effects is important to the overall understanding of the risks associated with exposure to a particular chemical. Most organic chemicals that are identified by the International Agency for Research on Cancer as human carcinogens, other than hormones, are genotoxic. The vast majority of these are detected by both the *Salmonella* assay and rodent bone marrow cytogenetics tests (Shelby, 1988; Shelby and Zeiger, 1990).

RESULTS

RATS

16-DAY STUDY

Three male rats administered 240 mg/kg 2,4-hexadienal died on day 2, 3, or 5 of the study; three 240 mg/kg females died on day 3, 5, or 10 of the study (Table 3). Mean body weight gains of 240 mg/kg rats were significantly less than those of the vehicle controls. Clinical findings included diarrhea, ataxia, lethargy, and nasal/eye discharge in males, and lethargy, paleness, and abnormal breathing in females in the 240 mg/kg groups. Liver weights of 240 mg/kg females were significantly greater than those of the vehicle controls (Table G1). Marked ulceration and/or necrosis of the forestomach

were present in most 240 mg/kg rats, in some cases associated with grossly visible adhesions between the stomach, liver, and spleen (data not shown). Lesser incidences of focal forestomach ulceration occurred in 80 mg/kg rats in addition to more diffuse mild to moderate epithelial hyperplasia. No forestomach effect was seen microscopically at 27 mg/kg.

Dose Selection Rationale: Based on the early deaths, increased severity of forestomach lesions, and decreased body weight gains in 240 mg/kg males and females, the highest dose concentration selected for the 14-week study in rats was 120 mg/kg.

TABLE 3
Survival and Body Weights of Rats in the 16-Day Gavage Study of 2,4-Hexadienal

Dose (mg/kg)	Survival ^a	Mean Body Weight ^b (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	93 ± 3	175 ± 5	81 ± 3	
3	5/5	95 ± 3	181 ± 4	86 ± 3	103
9	5/5	91 ± 3	178 ± 7	87 ± 4	102
27	5/5	93 ± 5	177 ± 6	84 ± 4	101
80	5/5	93 ± 2	176 ± 1	83 ± 2	101
240	2/5 ^c	92 ± 5	145 ± 12*	45 ± 5**	83
Female					
0	5/5	85 ± 5	131 ± 5	46 ± 1	
3	5/5	89 ± 4	134 ± 4	46 ± 2	103
9	5/5	88 ± 5	133 ± 6	46 ± 1	102
27	5/5	87 ± 5	130 ± 2	43 ± 3	99
80	5/5	87 ± 4	131 ± 2	44 ± 2	100
240	2/5 ^d	87 ± 5	120 ± 15	28 ± 3**	92

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunnett's test

** $P \leq 0.01$

^a Number of animals surviving at 16 days/number initially in group

^b Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.

^c Day of death: 2, 3, 5

^d Day of death: 3, 5, 10

14-WEEK STUDY

All rats survived to the end of the study (Table 4). Final mean body weights and body weight gains of 30, 60, and 120 mg/kg males were significantly less than those of the vehicle controls. The only clinical finding attributed to 2,4-hexadienal administration was hypersalivation in 30 and 120 mg/kg males and females during week 4; the 120 mg/kg rats continued to have hypersalivation after week 4.

Hematology and clinical chemistry data for rats are listed in Table F1. Numerous alterations in hematology or clinical chemistry variables were identified statistically. Most changes were minor, sporadic, and did not demonstrate a treatment relationship; therefore, they were not considered toxicologically relevant. At all time points, there were minimal to mild decreases in leukocyte and lymphocyte counts and increases in neutrophil counts in 120 mg/kg males and females. These leukocyte count changes, however, were not considered to be toxicity-related but were consistent with a stress leuko-

gram and a secondary treatment-associated stress effect. On day 4, there were minimal decreases in total protein and albumin concentrations (an approximately 8% decrease) in the 120 mg/kg males and females. Additionally, the 120 mg/kg rats demonstrated minimal to mild decreases in alkaline phosphatase activity on days 4 and 19. The changes in protein concentrations and alkaline phosphatase activity were transient and, by day 94, values had returned to control levels. Albumin concentration is sensitive to nutritional influences (Kaneko, 1989). Additionally, in rats, circulating alkaline phosphatase is primarily of intestinal and bone origin (Righetti and Kaplan, 1971), and fasting or food restriction causes decreases in serum alkaline phosphatase activity (Jenkins and Robinson, 1975; Imai *et al.*, 1991). If rats decreased their food intake due to treatment-related toxicity or poor food palatability, decreases in albumin (and consequently total protein) concentrations and alkaline phosphatase activity might be related to altered protein metabolism and loss of the normally circulating intestinal fraction of alkaline phosphatase. Thus, the transient decreases in protein

TABLE 4
Survival and Body Weights of Rats in the 14-Week Gavage Study of 2,4-Hexadienal

Dose (mg/kg)	Survival ^a	Mean Body Weight ^b (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	90 ± 4	329 ± 8	239 ± 7	
7.5	10/10	92 ± 4	321 ± 8	228 ± 8	98
15	10/10	91 ± 5	332 ± 4	241 ± 4	101
30	10/10	92 ± 4	309 ± 6*	217 ± 4*	94
60	10/10	90 ± 3	307 ± 6*	217 ± 6*	93
120	10/10	92 ± 4	277 ± 9**	185 ± 9**	84
Female					
0	10/10	87 ± 3	191 ± 4	104 ± 4	
7.5	10/10	89 ± 3	196 ± 3	107 ± 4	102
15	10/10	87 ± 3	191 ± 3	103 ± 4	100
30	10/10	88 ± 3	187 ± 3	99 ± 4	98
60	10/10	88 ± 3	192 ± 2	104 ± 3	100
120	10/10	88 ± 3	182 ± 3	93 ± 3	95

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Number of animals surviving at 14 weeks/number initially in group

^b Weights and weight changes are given as mean ± standard error.

concentrations and alkaline phosphatase activity of 120 mg/kg rats could suggest an initial, compound-related, decreased food intake that ameliorated with further compound administration and would be supported by the decreased body weights.

No biologically significant organ weight changes were observed (Table G2). There were no significant differences in sperm motility or vaginal cytology parameters between dosed and vehicle control rats (Tables H1 and H2).

The incidences of epithelial hyperplasia, degeneration, and chronic active inflammation of the forestomach in 120 mg/kg males and females were significantly greater than those in the vehicle controls (Table 5). The severity of hyperplasia was mild to moderate and was characterized by two- to fourfold thickenings of the epithelium, mostly in a diffuse pattern. The increased thickness was primarily due to increased layers of squamous cells and keratin (Plates 1 and 2) as well as irregular downgrowths of the basal cell layer. Degenerative changes of hyperplastic epithelium were found in some rats and were composed of staining pallor and intracytoplasmic hydropic change. Inflammatory changes, more severe in

males than females, consisted of intraepithelial neutrophils (microabscesses) or mixed inflammatory cell infiltrates beneath the epithelium.

The incidences of atrophy, osteofibrosis, and exudate of the nose in 120 mg/kg males were significantly increased (Table 5). Atrophy was characterized by minimal to moderate flattening and disorganization of the olfactory epithelium in the middle and posterior nasal sections. Osteofibrosis of the underlying turbinate bones, characterized by proliferation of fibroblast-like periosteal cells, loss of bone, and slightly increased overall thickness of the turbinate, accompanied the atrophy. Exudate in several males consisted of inflammatory cell debris in the nasal cavity lumen. In contrast to male rats, nasal lesions in females were limited to acute necrosis in one 60 mg/kg and two 120 mg/kg females.

Dose Selection Rationale: Based on the increased incidences and severities of forestomach lesions and decreased body weight gains in male rats administered 120 mg/kg and the absence of any effects in rats that received 60 mg/kg, the highest dose selected for the 2-year study in rats was 90 mg/kg.

TABLE 5
Incidences of Selected Nonneoplastic Lesions in Rats in the 14-Week Gavage Study of 2,4-Hexadienal

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Male						
Stomach, Forestomach ^a	10	10	10	10	9	10
Epithelium, Hyperplasia ^b	0	0	0	0	0	10** (2.9) ^c
Epithelium, Degeneration	0	0	0	0	0	8** (1.5)
Inflammation, Chronic Active	0	0	0	0	0	10** (2.9)
Nose	10	10	10	10	10	10
Olfactory Epithelium, Atrophy	0	0	0	0	0	8** (2.3)
Turbinates, Osteofibrosis	0	0	0	0	0	8** (2.3)
Exudate	0	0	0	0	0	5* (1.2)
Female						
Stomach, Forestomach	10	10	10	10	10	10
Epithelium, Hyperplasia	0	0	0	0	0	10** (2.4)
Epithelium, Degeneration	0	0	0	0	0	4* (1.8)
Inflammation, Chronic Active	0	0	0	0	0	5* (2.0)
Nose	10	10	10	10	10	10
Olfactory Epithelium, Necrosis	0	0	0	0	1 (2.0)	2 (2.0)

* 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 tissue examined microscopically

^b Number of animals with lesion

^c Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

2-YEAR STUDY

Survival

Estimates of 2-year survival probabilities for male and female rats are shown in Table 6 and in the Kaplan-Meier survival curves (Figure 1). Survival of all dosed groups of rats was similar to that of the vehicle control groups.

Body Weights and Clinical Findings

Mean body weights of 90 mg/kg males were less than those of the vehicle controls after week 27 (Figure 2; Tables 7 and 8). There were no clinical findings related to 2,4-hexadienal administration.

TABLE 6
Survival of Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Male				
Animals initially in study	50	50	50	50
Accidental deaths ^a	1	2	1	5
Moribund	10	11	13	10
Natural deaths	2	2	3	5
Animals surviving to study termination	37	35	33	30
Percent probability of survival at end of study ^b	76	73	67	68
Mean survival (days) ^c	693	682	685	666
Survival analysis ^d	P=0.411	P=0.979	P=0.465	P=0.537
Female				
Animals initially in study	50	50	50	50
Accidental deaths	0	1	1	6
Moribund	12	9	6	9
Natural deaths	1	1	2	4
Animals surviving to study termination	37	39 ^e	41	31
Percent probability of survival at end of study	74	80	84	71
Mean survival (days)	697	701	700	659
Survival analysis	P=0.704	P=0.638N	P=0.383N	P=0.847

^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 lower mortality in a dose group is indicated by N.

^e Includes one animal that died during the last week of the study

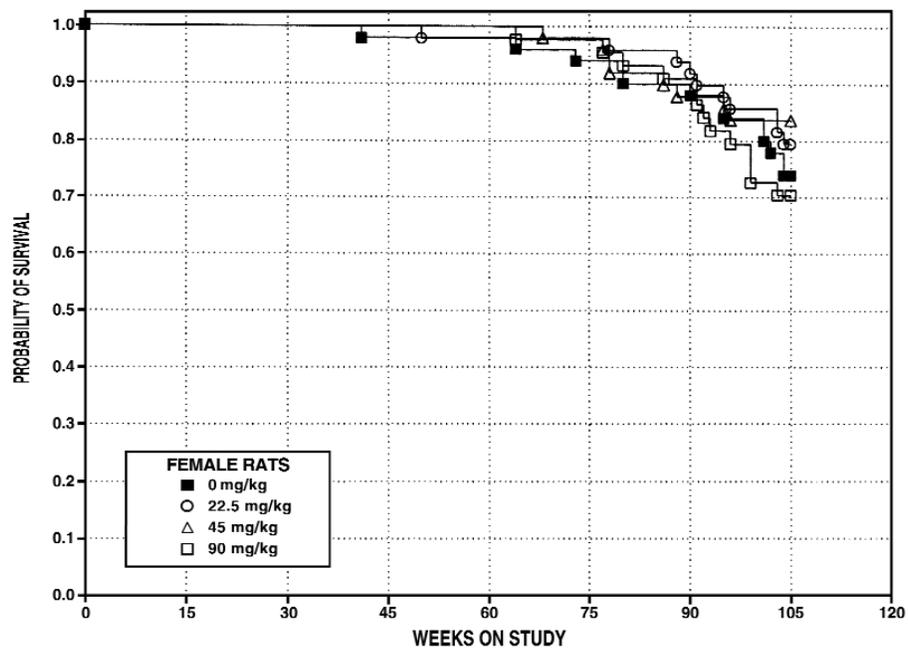
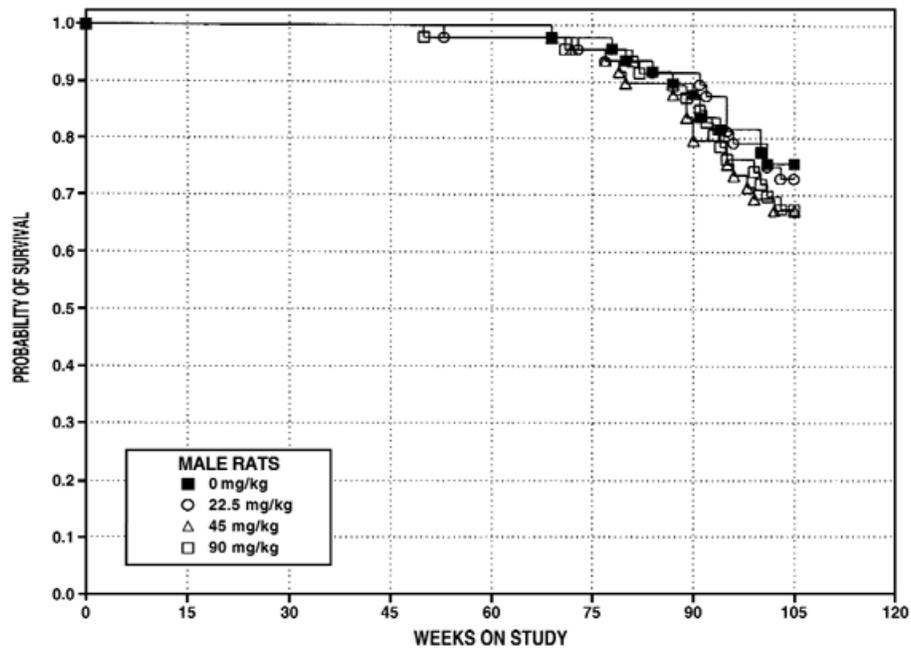


FIGURE 1
Kaplan-Meier Survival Curves for Male and Female Rats
Administered 2,4-Hexadienal by Gavage for 2 Years

TABLE 7
Mean Body Weights and Survival of Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

Weeks on Study	0 mg/kg		22.5 mg/kg			45 mg/kg			90 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	80	50	78	98	50	79	99	50	80	100	50
3	163	50	164	100	50	165	101	50	162	99	50
7	255	50	257	101	50	258	101	50	250	98	50
11	310	50	311	100	50	311	100	50	298	96	50
15	349	50	349	100	50	349	100	50	334	96	50
19	377	50	377	100	50	379	100	50	361	96	50
23	400	50	399	100	50	400	100	50	381	95	49
27	417	50	414	99	50	417	100	50	396	95	49
31	433	50	428	99	49	431	100	50	408	94	49
35	448	50	443	99	49	445	99	50	421	94	49
39	462	49	456	99	49	459	99	50	434	94	49
43	474	49	466	98	49	470	99	50	442	93	49
47	482	49	473	98	49	481	100	50	452	94	49
51	492	49	485	99	48	489	100	50	458	93	48
55	493	49	487	99	47	490	99	49	459	93	46
59	502	49	494	98	47	500	100	49	467	93	46
63	503	49	498	99	47	504	100	49	470	93	46
67	506	49	499	99	47	504	100	49	468	93	46
71	511	48	497	97	47	503	99	48	469	92	45
75	510	48	501	98	46	503	99	47	468	92	45
79	509	47	500	98	45	493	97	46	464	91	45
83	506	46	498	98	45	496	98	44	461	91	42
87	506	45	502	99	44	501	99	43	464	92	42
91	507	41	500	99	43	503	99	39	458	90	39
95	502	40	498	99	39	501	100	37	457	91	35
99	495	40	488	99	38	492	100	34	447	90	34
103	482	37	488	101	35	490	102	33	445	92	30
Mean for weeks											
1-13	202		203	100		203	100		198	98	
14-52	433		429	99		432	100		409	94	
53-103	502		496	99		498	99		461	92	

TABLE 8
Mean Body Weights and Survival of Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal

Weeks on Study	0 mg/kg		22.5 mg/kg			45 mg/kg			90 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	79	50	79	101	50	80	102	50	81	102	50
4	132	50	131	99	50	130	99	50	132	100	50
8	165	50	164	100	50	163	99	50	166	101	50
12	182	50	181	99	50	179	98	50	180	99	50
16	192	50	190	99	50	190	99	50	191	99	50
20	200	50	198	99	50	197	99	50	200	100	50
24	208	50	208	100	50	208	100	50	208	100	50
28	214	50	212	99	50	212	99	50	213	99	50
32	219	50	218	99	50	216	99	50	217	99	50
36	226	50	224	99	50	221	98	50	224	99	50
40	231	50	229	99	50	229	99	50	228	99	50
44	239	49	237	99	50	236	99	50	235	99	50
48	244	49	241	99	50	241	99	50	236	97	50
52	251	49	249	99	48	245	98	50	241	96	49
56	258	49	255	99	48	253	98	50	247	96	45
60	268	49	265	99	48	264	99	50	256	96	44
64	276	49	276	100	48	274	99	50	265	96	44
68	283	48	282	100	48	279	99	50	270	95	43
72	288	48	287	100	48	283	98	48	275	95	43
76	293	47	292	100	48	288	98	48	281	96	43
80	294	47	295	100	47	292	99	45	281	96	42
84	299	45	297	99	47	294	98	45	285	95	41
88	306	45	303	99	47	299	98	44	294	96	40
92	310	44	309	100	44	303	98	43	295	95	37
96	311	42	309	99	43	304	98	42	297	95	35
100	309	42	307	99	42	306	99	41	298	96	32
104	315	38	306	97	40	309	98	41	297	94	31
Mean for weeks											
1-13	140		139	99		138	99		140	100	
14-52	222		221	100		220	99		219	99	
53-104	293		291	99		288	98		280	96	

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of mononuclear cell leukemia and neoplasms and nonneoplastic lesions of the forestomach, oral cavity, testis, spleen, adrenal medulla, pancreas, and nose. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix A for male rats and Appendix B for female rats.

Forestomach: The incidences of squamous cell papilloma of the forestomach occurred with positive trends in males and females (Tables 9, A3, and B3). The incidences of squamous cell papilloma in 45 and 90 mg/kg males and females were significantly greater than those in the vehicle controls. The incidences of this lesion in dosed males and in 45 and 90 mg/kg females exceeded the historical ranges in controls (all routes) given NTP-2000 diet or corn oil vehicle controls given NIH-07 diet (Tables 9, A4, and B4). Multiple papillomas were commonly observed in the forestomach of 90 mg/kg male rats. Squamous cell carcinomas were observed in a 45 mg/kg male rat and in two 90 mg/kg male rats. Although not significantly increased, the incidences of squamous cell carcinoma in these groups exceeded that in historical controls. Papillomas were exophytic papillary proliferations of well-differentiated squamous epithelium supported by branched fibrovascular stromal cores (Plate 3). Carcinomas in male rats demonstrated endophytic cords or nests of atypical squamous cells extending into the submucosa (Plate 4) from ulcerated areas of proliferative epithelium.

Several nonneoplastic lesions in the forestomach of males and females were related to 2,4-hexadienal administration. The incidences of epithelial hyperplasia were significantly increased in all dosed groups. Most 45 and 90 mg/kg rats had hyperplastic change of mild to moderate severity. Hyperplasia was characterized by focally extensive to diffuse thickenings of all layers of the squamous epithelium. The spinous cell layers were increased by two- to four-fold and the basal cell layer was variably thickened by irregular downgrowths. Hyperplasia is considered a potential precursor lesion to neoplasia in

the forestomach. Incidences of inflammation and cyst were significantly greater in 90 mg/kg male rats than in vehicle controls. Mild to moderate inflammation was diagnosed in some animals in association with hyperplastic or neoplastic change but was not a particularly prominent component of the proliferative process. Cysts were located in the submucosa and lined by well-differentiated squamous epithelium and filled with keratin. These were interpreted to be the results of downward growths of benign hyperplastic epithelium.

Oral Cavity: Single incidences of squamous cell papilloma or of squamous cell carcinoma of the tongue were observed in most treated groups of males (vehicle control, 0/50; 22.5 mg/kg, 1/50; 45 mg/kg, 1/50; 90 mg/kg, 0/50; Table A1) and females (0/50, 0/49, 2/50, 1/50; Table B1). Although no squamous cell neoplasms of the tongue occurred in male or female vehicle controls, squamous cell papillomas of the oral mucosa occurred in one male and one female vehicle control rat. When the incidences of squamous cell papilloma or carcinoma of the tongue and oral mucosa were combined, there was no clear dose-related trend in males (1/50, 1/50, 2/50, 1/50) or females (1/50, 0/50, 2/50, 1/50). Therefore, neoplasms of the tongue or oral cavity in general were not considered related to 2,4-hexadienal administration.

Testis: There was a positive trend in the incidence of testis interstitial cell adenoma in male rats, and the incidence in 90 mg/kg males was significantly increased (41/50, 45/50, 45/50, 46/50; Table A3). However, interstitial cell neoplasms occur at a high and variable rate in male F344/N rats. The vehicle control incidence of 82% is at the lower end of the range of historical controls (all routes) given the NTP-2000 diet [535/609 (86.4% ± 9.1%), range 72%-98%] or corn oil gavage controls given NIH-07 diet [350/396 (88.3% ± 6.0%), range 76%-94%], and the 92% incidence in the 90 mg/kg group is at the higher end of these historical ranges. The increased incidences of testis interstitial cell adenomas in treated males were therefore not considered biologically significant.

Spleen: There was a dose-related increase in the incidences of splenic pigmentation in male rats (7/50, 9/50, 18/50, 20/50; Table A5); severity ranged from mild to marked (average severity: 2.4, 2.3, 2.3, 2.6). The pigment appeared as brown granules in the cytoplasm of red

TABLE 9
Incidences of Neoplasms and Nonneoplastic Lesions of the Forestomach in Rats
in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Male				
Number Necropsied	50	50	50	50
Epithelium, Cyst ^a	0	0	1 (3.0)	5* (3.0) ^b
Epithelium, Hyperplasia	3 (1.0)	19** (1.3)	42** (1.9)	50** (2.9)
Inflammation, Chronic Active	0	0	1 (2.0)	6* (2.7)
Squamous Cell Papilloma, Multiple	0	0	0	11**
Squamous Cell Papilloma (includes multiple) ^c				
Overall rate ^d	0/50 (0%)	3/50 (6%)	10/50 (20%)	29/50 (58%)
Adjusted rate ^e	0.0%	6.9%	23.2%	67.0%
Terminal rate ^f	0/37 (0%)	3/35 (9%)	9/33 (27%)	23/30 (77%)
First incidence (days)	— ^h	729 (T)	691	574
Poly-3 test ^g	P<0.001	P=0.114	P<0.001	P<0.001
Squamous Cell Carcinoma	0	0	1	2
Squamous Cell Papilloma or Carcinoma ^c	0	3	11**	29**
Female				
Number Necropsied	50	50	50	50
Epithelium, Hyperplasia	2 (3.0)	16** (1.1)	37** (1.4)	41** (2.5)
Squamous Cell Papilloma, Multiple	0	0	1	1
Squamous Cell Papilloma (includes multiple) ⁱ				
Overall rate	0/50 (0%)	1/50 (2%)	5/50 (10%)	17/50 (34%)
Adjusted rate	0.0%	2.2%	11.0%	41.9%
Terminal rate	0/37 (0%)	1/39 (3%)	5/41 (12%)	15/31 (48%)
First incidence (days)	—	729 (T)	729 (T)	691
Poly-3 test	P<0.001	P=0.503	P=0.031	P<0.001

* Significantly different (P≤0.05) from the vehicle control group by the Poly-3 test

** P≤0.01

(T) Terminal sacrifice

^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 2-year studies with controls given NTP-2000 diet (mean ± standard deviation): 2/609 (0.3% ± 0.7%), range 0%-2%; with corn oil vehicle controls given NIH-07 diet: 2/402 (0.5% ± 0.9%), range 0%-2%

^d Number of animals with neoplasm per number of animals necropsied

^e Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^f Observed incidence at terminal kill

^g Beneath the vehicle control incidence are the P values 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 Not applicable; no neoplasms in animal group

ⁱ Historical incidence for NTP-2000 diet: 0/659; for NIH-07 diet: 2/401 (0.5% ± 0.9%); range 0%-2%

pulp macrophages and was morphologically consistent with hemosiderin.

Adrenal Medulla: There was a positive trend in the incidences of malignant pheochromocytoma in male rats (vehicle control, 0/50; 22.5 mg/kg, 1/49; 45 mg/kg, 1/50; 90 mg/kg, 4/49; Table A3), and the incidence in 90 mg/kg males was greater than that in the vehicle controls. This incidence slightly exceeded the range in historical controls (all routes) given the NTP-2000 diet [10/607 (1.7% ± 1.4%), range 0%-4%] or corn oil gavage controls given the NIH-07 diet [8/401 (2.0% ± 1.9%), range 0%-6%]. Malignant pheochromocytoma of the adrenal medulla is part of a morphologic and biologic continuum that includes hyperplasia, benign pheochromocytoma, and complex pheochromocytoma. The incidences of these proliferative lesions of the adrenal medulla did not exhibit significant treatment-related trends, either individually or combined, and therefore the slight increase in malignant neoplasms was not considered related to 2,4-hexadienal administration.

Other Organs: For male rats, vehicle control incidences of pancreatic acinar cell adenoma (10/50, 20%)

exceeded the range of historical controls (all routes) given the NTP-2000 diet [10/607 (1.5% ± 1.4%), range 0%-4%]. The vehicle control incidence of mononuclear cell leukemia in male rats (11/50, 22%) fell below the corresponding historical control range [300/609 (47.3% ± 10.5%), range 32%-68%]. The incidences of both of these neoplasms were similar in dosed and vehicle control groups (pancreatic acinar cell adenoma: 10/50, 5/50, 9/50, 6/50; Table A3; mononuclear cell leukemia: 11/50, 14/50, 9/50, 17/50; Table A3). The differences between the incidences in this study and the concurrent and historical control rates for these two neoplasms were likely related to the corn oil vehicle, which has been shown to increase the incidences of pancreatic acinar cell neoplasms and to reduce the incidences of mononuclear cell leukemia in male F344/N rats (Haseman *et al.*, 1985; Haseman and Rao, 1992).

In the 14-week study, atrophy of the nasal olfactory epithelium was observed in male rats exposed to 120 mg/kg. In the 2-year study, two males in the 90 mg/kg group had similar findings (Table A5). However, due to the low incidence, the relationship to treatment was uncertain.

MICE**16-DAY STUDY**

One male and one female in the 240 mg/kg groups died on day 7 or 10; one female in the 3 mg/kg group died on day 4 of a suspected dosing accident (Table 10). Female mice in the 240 mg/kg group lost weight during the study (Table 10). Clinical findings included lethargy and ruffled fur in 240 mg/kg males and females, and one female from this group had convulsions. One 3 mg/kg female was lethargic, had nasal and eye discharge, and a seizure.

No biologically significant differences in organ weights were observed. Marked ulceration and/or necrosis of the

forestomach were present in all 240 mg/kg mice (data not shown), in many cases associated with grossly visible thickening of the stomach wall and/or adhesions between the stomach, liver, and spleen. In 80 mg/kg mice necrotic change of the forestomach was absent and the primary lesion was minimal to mild epithelial hyperplasia and hyperkeratosis. No forestomach effect was seen microscopically in the 27 mg/kg groups.

Dose Selection Rationale: Based on the early deaths and increased incidences and severities of forestomach lesions in 240 mg/kg males and females and decreased body weight gain of 240 mg/kg females, the highest dose selected for the 14-week study in mice was 120 mg/kg.

TABLE 10
Survival and Body Weights of Mice in the 16-Day Gavage Study of 2,4-Hexadienal

Dose (mg/kg)	Survival ^a	Mean Body Weight ^b (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	22.5 ± 0.8	25.2 ± 0.3	2.7 ± 0.7	
3	5/5	22.9 ± 0.9	25.1 ± 0.7	2.2 ± 0.3	100
9	5/5	23.2 ± 0.4	25.7 ± 0.4	2.5 ± 0.3	102
27	5/5	23.1 ± 0.5	25.9 ± 0.5	2.8 ± 0.5	103
80	5/5 ^c	22.8 ± 0.5	24.6 ± 0.3	1.8 ± 0.4	98
240	4/5 ^c	23.1 ± 0.7	24.5 ± 0.3	0.9 ± 0.6	97
Female					
0	5/5	18.2 ± 0.7	19.9 ± 0.7	1.7 ± 0.2	
3	4/5 ^d	18.4 ± 0.6	19.8 ± 0.5	2.0 ± 0.2	99
9	5/5	18.2 ± 0.6	20.1 ± 0.6	1.8 ± 0.4	101
27	5/5	19.2 ± 0.4	21.2 ± 0.4	2.0 ± 0.3	106
80	5/5 ^e	18.1 ± 0.6	20.2 ± 0.6	2.1 ± 0.2	102
240	4/5 ^e	18.2 ± 0.4	17.9 ± 0.9	-0.1 ± 0.7**	90

** Significantly different ($P \leq 0.01$) from the vehicle control group by Dunnett's test

^a Number of animals surviving at 16 days/number initially in group

^b Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.

^c Day of death: 7

^d Day of death: 4

^e Day of death: 10

14-WEEK STUDY

One vehicle control male and one 60 mg/kg male died during week 1, and one vehicle control male died during week 13; all three deaths were due to dosing accidents (Table 11). Mean body weights of males and females were similar to those of the vehicle controls throughout the study. Clinical findings included salivation during week 7 of the study and anal wetness in 60 and 120 mg/kg males and 120 mg/kg females beginning at week 9 or 10 of the study.

Hematology data for mice are listed in Table F2; there were no chemical-related or biologically relevant differences between the dosed and vehicle control groups.

Kidney weights of 60 and 120 mg/kg males, absolute and relative liver weights of 60 mg/kg males and females, and relative liver weights of all dosed groups of females were significantly greater than those of the vehicle controls (Table G4). There were no significant differences in sperm motility or vaginal cytology parameters between dosed and vehicle control males or females (Tables H3 and H4).

A significant increase in the incidence of epithelial hyperplasia of the forestomach occurred in 120 mg/kg females (Table 12). Hyperplasia in this group was a minimal to mild change composed of focal thickening of the squamous epithelium, often in a papillary pattern.

TABLE 11
Survival and Body Weights of Mice in the 14-Week Gavage Study of 2,4-Hexadienal

Dose (mg/kg)	Survival ^a	Mean Body Weight ^b (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	8/10 ^c	23.6 ± 0.3	38.8 ± 0.8	15.5 ± 0.7	
7.5	10/10	23.3 ± 0.3	38.2 ± 1.2	14.9 ± 1.2	98
15	10/10	23.5 ± 0.3	40.9 ± 0.8	17.4 ± 0.6	105
30	10/10	23.2 ± 0.4	38.2 ± 0.8	15.0 ± 0.8	99
60	9/10 ^d	23.8 ± 0.3	38.4 ± 1.1	14.8 ± 1.0	99
120	10/10	23.4 ± 0.3	37.6 ± 0.7	14.2 ± 0.5	97
Female					
0	10/10	19.0 ± 0.2	33.6 ± 0.6	14.6 ± 0.5	
7.5	10/10	18.9 ± 0.3	34.1 ± 1.2	15.2 ± 1.0	102
15	10/10	19.2 ± 0.3	33.6 ± 0.9	14.4 ± 0.6	100
30	10/10	18.7 ± 0.5	32.5 ± 1.7	13.9 ± 1.3	97
60	10/10	19.2 ± 0.3	33.2 ± 0.7	14.0 ± 0.7	99
120	10/10	18.5 ± 0.2	32.8 ± 1.1	14.3 ± 0.9	98

^a Number of animals surviving at 14 weeks/number initially in group

^b Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.

^c Week of death: 1, 13

^d Week of death: 1

The basal cell proliferation as seen in rats was not evident in mice. There was no appreciable inflammatory reaction in the forestomach of dosed mice.

Similar to findings in the 14-week rat study, nasal lesions were restricted to the olfactory region located in the middle and posterior nasal cavity, and the dorsal meatus was the most commonly affected site. The incidences of minimal to mild olfactory epithelial necrosis were significantly greater in 120 mg/kg mice than in the vehicle controls (Table 12); this lesion was characterized by cell swelling and nuclear pyknosis and karyorrhexis.

Although the incidence of olfactory epithelial atrophy was significantly increased in 120 mg/kg males, the lesion was judged to be of mild to moderate severity and consisted of decreased thickness and disorganization of the olfactory epithelium.

Dose Selection Rationale: Because there were no effects on survival or body weights of 120 mg/kg mice in the 14-week study, and histopathologic lesions at this dose were not sufficiently severe to limit chronic dose setting, the highest dose selected for the 2-year study in mice was 120 mg/kg.

TABLE 12
Incidences of Selected Nonneoplastic Lesions in Mice in the 14-Week Gavage Study of 2,4-Hexadienal

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Male						
Stomach, Forestomach ^a	10	10	10	10	9	10
Epithelium, Hyperplasia ^b	2 (2.0) ^c	0	0	0	0	3 (1.3)
Nose	10	10	10	10	10	10
Olfactory Epithelium, Necrosis	0	0	0	0	0	5* (1.2)
Olfactory Epithelium, Atrophy	0	0	0	0	0	4* (2.5)
Female						
Stomach, Forestomach	10	10	10	10	10	10
Epithelium, Hyperplasia	0	0	0	0	1 (1.0)	5* (1.4)
Nose	10	10	10	10	10	10
Olfactory Epithelium, Necrosis	0	0	0	0	0	5* (1.6)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Fisher exact test

^a Number of animals with tissue examined microscopically

^b Number of animals with lesion

^c Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

2-YEAR STUDY

Survival

Estimates of 2-year survival probabilities for male and female mice are shown in Table 13 and in the Kaplan-Meier survival curves (Figure 3). Survival of all dosed groups of mice was similar to that of the vehicle control groups.

Body Weights and Clinical Findings

The mean body weights of all dosed groups were generally similar to those of the vehicle control groups throughout the study (Tables 14 and 15; Figure 4). There were no clinical findings related to 2,4-hexadienal administration.

TABLE 13
Survival of Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Male				
Animals initially in study	50	50	50	50
Moribund	3	3	3	8
Natural deaths	3	8	3	3
Animals surviving to study termination	44	39	44	39 ^a
Percent probability of survival at end of study ^b	88	78	88	78
Mean survival (days) ^c	721	694	714	698
Survival analysis ^d	P=0.387	P=0.237	P=1.000	P=0.253
Female				
Animals initially in study	50	50	50	50
Accidental death ^e	0	0	0	1
Missing ^e	0	1	0	0
Moribund	2	8	8	4
Natural deaths	6	4	5	6 ^f
Animals surviving to study termination	42	37	37	39 ^f
Percent probability of survival at end of study	84	76	74	80
Mean survival (days)	715	708	709	702
Survival analysis	P=0.776	P=0.447	P=0.327	P=0.751

^a Includes one animal that died during the last week of the study

^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.

^e Censored from survival analyses

^f Includes two animals that died during the last week of the study

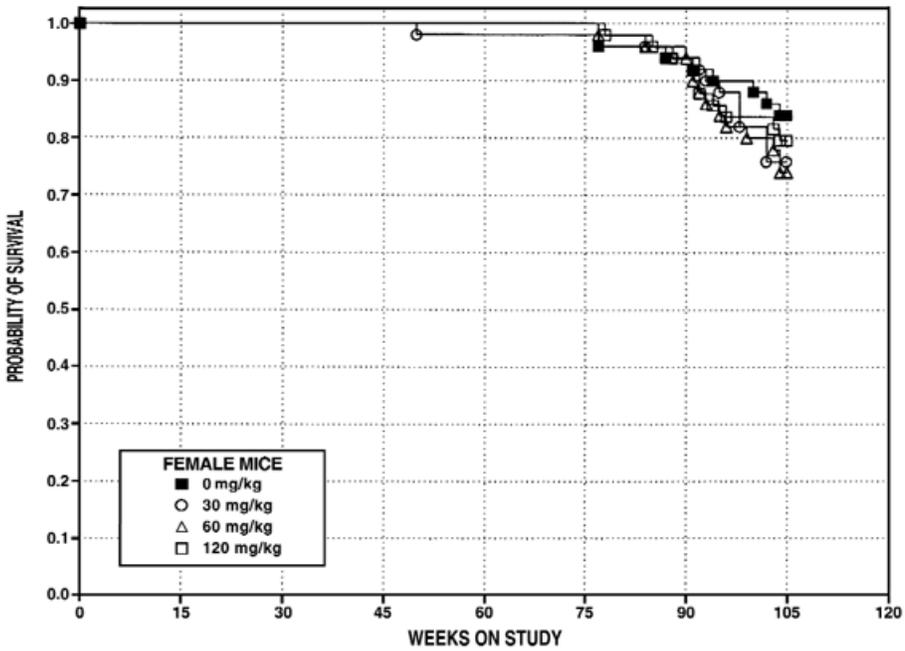
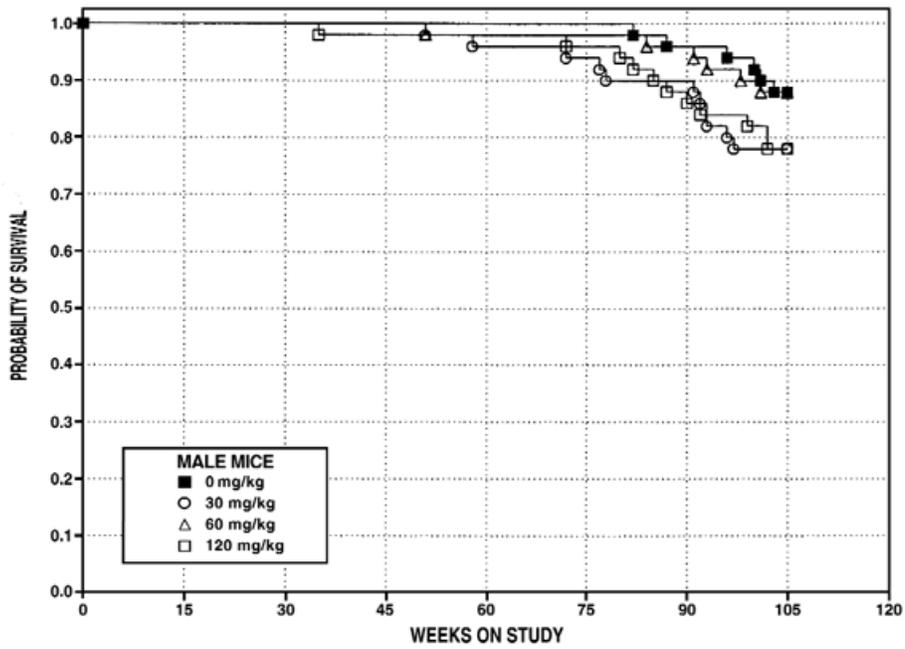


FIGURE 3
Kaplan-Meier Survival Curves for Male and Female Mice
Administered 2,4-Hexadienal by Gavage for 2 Years

TABLE 14
Mean Body Weights and Survival of Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

Weeks on Study	0 mg/kg		30 mg/kg			60 mg/kg			120 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	22.1	50	21.9	99	50	22.1	100	50	22.3	101	50
3	25.6	50	25.7	100	50	25.9	101	50	26.0	102	50
7	30.8	50	30.8	100	50	30.9	100	50	31.1	101	50
11	35.6	50	35.7	100	50	35.7	100	50	36.0	101	50
15	39.7	50	39.7	100	50	40.2	101	50	40.2	101	50
19	43.8	50	44.3	101	50	44.6	102	50	44.9	103	50
23	46.9	50	47.2	101	50	47.5	101	50	47.8	102	50
27	48.8	50	49.0	100	50	49.2	101	50	49.4	101	50
31	50.1	50	50.4	101	50	50.6	101	50	51.0	102	50
35	50.8	50	50.9	100	50	51.5	101	50	52.0	102	49
39	52.2	50	52.3	100	50	53.0	102	50	53.3	102	49
43	52.9	50	53.1	100	50	53.2	101	50	53.7	102	49
47	54.1	50	53.7	99	50	54.3	100	50	54.5	101	49
51	54.8	50	54.7	100	49	55.1	101	49	55.4	101	49
55	55.3	50	54.5	99	49	55.1	100	49	55.5	100	49
59	56.0	50	55.9	100	48	55.9	100	49	56.0	100	49
63	56.6	50	55.9	99	48	56.7	100	49	56.6	100	49
67	56.9	50	56.9	100	48	57.0	100	49	57.1	100	49
71	56.7	50	56.2	99	48	57.1	101	49	56.3	99	49
75	57.3	50	56.8	99	47	57.9	101	49	57.4	100	48
79	57.3	50	57.1	100	45	58.1	101	49	57.4	100	48
83	57.0	49	56.1	98	45	58.0	102	49	57.4	101	46
87	56.5	48	55.9	99	45	58.2	103	48	57.9	103	44
91	56.1	48	55.6	99	44	57.3	102	48	57.1	102	43
95	54.6	48	54.3	100	41	57.1	105	46	55.2	101	42
99	53.5	47	54.6	102	39	56.0	105	45	53.8	101	41
103	52.9	44	52.9	100	39	53.2	101	44	51.5	97	39
Mean for weeks											
1-13	28.5		28.5	100		28.7	101		28.9	101	
14-52	49.4		49.5	100		49.9	101		50.2	102	
53-103	55.9		55.6	99		56.7	101		56.1	100	

TABLE 15
Mean Body Weights and Survival of Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

Weeks on Study	0 mg/kg		30 mg/kg			60 mg/kg			120 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	18.2	50	18.0	99	50	17.9	98	50	17.8	98	50
3	21.2	50	20.9	99	50	21.0	99	50	20.8	98	50
7	25.3	50	25.2	100	50	25.3	100	50	24.8	98	50
11	29.0	50	28.9	100	50	27.8	96	50	28.8	99	50
15	33.5	50	32.7	98	50	32.7	98	50	32.5	97	50
19	37.3	50	37.6	101	50	38.5	103	50	37.8	101	50
23	41.2	50	41.7	101	50	41.8	102	50	40.9	99	50
27	44.3	50	45.5	103	50	45.9	104	50	43.8	99	50
31	47.6	50	48.3	102	50	48.3	102	50	46.5	98	50
35	50.3	50	50.9	101	50	51.6	103	50	49.1	98	49
39	53.4	50	54.1	101	50	54.0	101	50	51.9	97	49
43	56.4	50	56.4	100	50	56.8	101	50	54.0	96	49
47	59.3	50	59.5	100	50	57.5	97	50	54.6	92	49
51	61.4	50	60.5	99	49	59.9	98	50	57.2	93	49
55	62.3	50	61.6	99	49	61.1	98	50	59.2	95	49
59	64.0	50	63.1	99	49	62.5	98	50	62.1	97	49
63	64.2	50	63.1	98	49	62.9	98	50	62.2	97	49
67	63.5	50	62.2	98	49	62.0	98	50	62.5	98	49
71	63.4	50	61.5	97	49	62.3	98	50	62.4	98	49
75	62.5	50	60.9	97	49	61.4	98	50	62.4	100	49
79	62.0	48	60.3	97	49	60.6	98	49	62.3	101	48
83	60.9	48	58.6	96	49	58.7	96	49	60.1	99	48
87	60.8	47	59.0	97	48	58.7	97	48	60.1	99	47
91	59.2	46	57.2	97	47	56.4	95	45	58.2	98	45
95	56.9	45	55.6	98	44	54.1	95	42	57.2	101	42
99	54.0	45	54.3	101	41	53.2	99	40	55.8	103	41
103	52.2	43	53.6	103	37	52.4	100	39	54.6	105	40
Mean for weeks											
1-13	23.4		23.3	100		23.0	98		23.1	99	
14-52	48.5		48.7	100		48.7	100		46.8	96	
53-103	60.5		59.3	98		58.9	97		59.9	99	

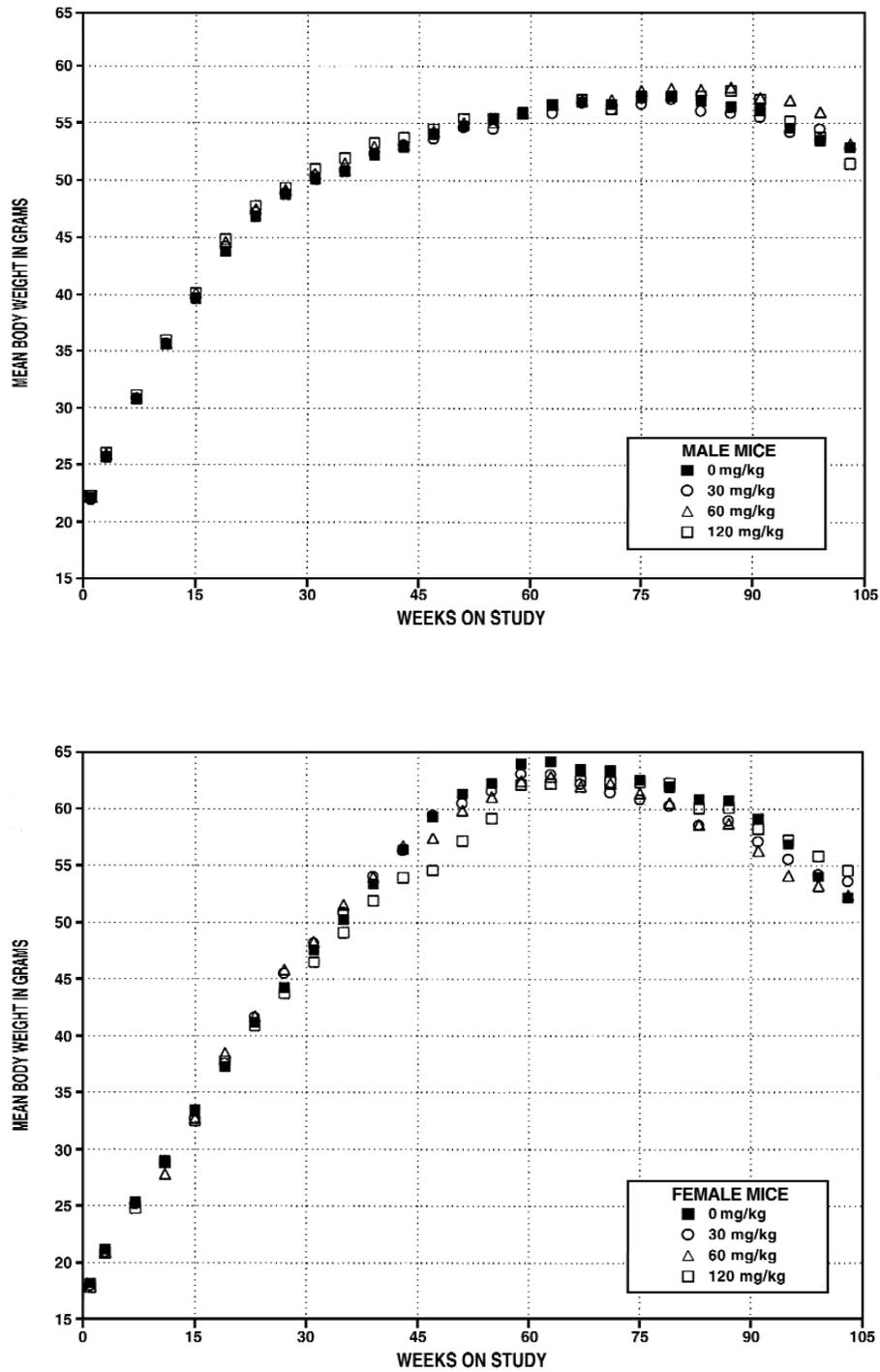


FIGURE 4
Growth Curves for Male and Female Mice
Administered 2,4-Hexadienal by Gavage for 2 Years

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms and nonneoplastic lesions of the forestomach, oral cavity, and nose. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix C for male mice and Appendix D for female mice.

Forestomach: The incidences of squamous cell papilloma and squamous cell papilloma or carcinoma (combined) occurred with positive trends in male and female mice (Tables 16, C3, and D3). In 120 mg/kg males, the incidences of squamous cell papilloma and of squamous cell papilloma or carcinoma (combined) were significantly increased. The incidences of these lesions in 60 and 120 mg/kg females and the incidence of carcinoma alone in 120 mg/kg females were significantly greater than those in the vehicle controls. The incidences of squamous cell papilloma and squamous cell papilloma

or carcinoma (combined) in all dosed groups of males and in 60 and 120 mg/kg females generally exceeded the historical ranges for these neoplasms in controls (all routes) given NTP-2000 diet and for corn oil vehicle controls given NIH-07 diet (Tables 16, C4a, and D4). The incidences of squamous cell carcinoma in 120 mg/kg males and females also exceeded both historical ranges. Neoplasms in mice had morphologic features similar to those described in rats. Papillomas were exophytic papillary proliferations of well-differentiated squamous epithelium supported by branched fibrovascular stromal cores (Plate 5). Carcinomas demonstrated a clearly endophytic growth component with cords of atypical squamous epithelial cells extending into the submucosa (Plate 6). The two carcinomas in 120 mg/kg male mice were small lesions found microscopically in areas of ulcerated proliferative epithelium. Carcinomas in females were often large, grossly visible masses, and microscopically there was transmural invasion of malignant cells (Plate 7). Metastases to various organs (mesentery, pancreas, esophagus, ovary, and lymph nodes) occurred in three of the seven females from the 120 mg/kg group with forestomach carcinoma (Table D1).

TABLE 16
Incidences of Neoplasms and Nonneoplastic Lesions of the Forestomach in Mice
in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Male				
Number Necropsied	50	50	50	50
Epithelium, Hyperplasia, Squamous ^a	14 (1.7) ^b	7 (1.6)	9 (2.0)	26** (1.8)
Ulcer	2 (1.5)	0	3 (1.7)	10* (2.0)
Squamous Cell Papilloma, Multiple	1	1	0	2
Squamous Cell Papilloma (includes multiple) ^c				
Overall Rate ^d	2/50 (4%)	4/50 (8%)	5/50 (10%)	8/50 (16%)
Adjusted Rate ^e	4.1%	8.9%	10.5%	17.6%
Terminal Rate ^f	2/44 (5%)	4/39 (10%)	5/44 (11%)	8/39 (21%)
First Incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test ^g	P=0.022	P=0.301	P=0.210	P=0.035
Squamous Cell Carcinoma ^h	0	1	0	2
Squamous Cell Papilloma or Carcinoma ⁱ				
Overall Rate	2/50 (4%)	4/50 (8%)	5/50 (10%)	10/50 (20%)
Adjusted Rate	4.1%	8.9%	10.5%	22.0%
Terminal Rate	2/44 (5%)	4/39 (10%)	5/44 (11%)	10/39 (26%)
First Incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.004	P=0.301	P=0.210	P=0.009

TABLE 16
Incidences of Neoplasms and Nonneoplastic Lesions of the Forestomach in Mice
in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Female				
Number Necropsied	50	49	50	50
Epithelium, Hyperplasia, Squamous	4 (1.8)	8 (1.5)	12* (1.8)	31** (1.8)
Squamous Cell Papilloma, Multiple	0	0	2	2
Squamous Cell Papilloma ^j (includes multiple)				
Overall rate	2/50 (4%)	2/49 (4%)	11/50 (22%)	13/50 (26%)
Adjusted rate	4.2%	4.4%	23.5%	28.2%
Terminal rate	2/42 (5%)	2/37 (5%)	10/37 (27%)	13/39 (33%)
First incidence (days)	729 (T)	729 (T)	672	729 (T)
Poly-3 test	P<0.001	P=0.679	P=0.006	P<0.001
Squamous Cell Carcinoma ^k	0	0	0	7**
Squamous Cell Papilloma or Carcinoma ^l				
Overall rate	2/50 (4%)	2/49 (4%)	11/50 (22%)	18/50 (36%)
Adjusted rate	4.2%	4.4%	23.5%	38.6%
Terminal rate	2/42 (5%)	2/37 (5%)	10/37 (27%)	16/39 (41%)
First incidence (days)	729 (T)	729 (T)	672	614
Poly-3 test	P<0.001	P=0.679	P=0.006	P<0.001

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Poly-3 test

** $P \leq 0.01$

(T) Terminal sacrifice

^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 2-year studies with controls given NTP-2000 diet (mean \pm standard deviation): 10/659 (1.8% \pm 1.9%), range, 0%-6%; with corn oil vehicle controls given NIH-07 diet: 19/464 (4.1% \pm 1.7%), range 2%-6%

^d Number of animals with neoplasm per number of animals necropsied

^e Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^f Observed incidence at terminal kill

^g Beneath the vehicle control incidence are the P values 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 for NTP-2000 diet: 1/659 (0.2% \pm 0.6%), range 0%-2%; for NIH-07 diet: 3/464 (0.7% \pm 1.0%), range 0%-2%

ⁱ Historical incidence for NTP-2000 diet: 11/659 (2.0% \pm 2.0%), range 0%-6%; for NIH-07 diet: 22/464 (4.7% \pm 2.0%), range 2%-8%

^j Historical incidence for NTP-2000 diet: 9/659 (1.4% \pm 2.0%), range 0%-6%; for NIH-07 diet: 19/463 (4.1% \pm 3.5%), range 0%-10%

^k Historical incidence for NTP-2000 diet: 1/659 (0.2% \pm 0.6%), range 0%-2%; for NIH-07 diet: 0/463

^l Historical incidence for NTP-2000 diet: 10/659 (1.6% \pm 1.9%), range 0%-6%; for NIH-07 diet: 19/463 (4.1% \pm 3.5%), range 0%-10%

Nonneoplastic forestomach lesions seen in the forestomach related to chemical administration were squamous epithelial hyperplasia in males and females and ulcers in males (Table 16). Hyperplasia was minimal to mild in severity and the incidences of this lesion were significantly increased in 120 mg/kg males and females and in 60 mg/kg females. This change, manifested as focal to diffuse thickening of the squamous epithelium, is considered a potential precursor to squamous cell papilloma and carcinoma in the forestomach. Ulceration, which was significantly increased in 120 mg/kg males, was seen as focal losses of epithelium, usually affecting papillomas or hyperplastic epithelium. Slight inflammation was occasionally associated with the proliferative effects or ulceration in males, but the incidences were not significantly increased.

Oral Cavity: Two male mice in the 120 mg/kg group had gross masses of the tongue which were diagnosed microscopically as squamous cell carcinoma (Table C1). Although not significantly increased relative to the vehicle controls, this incidence exceeded historical incidences in controls (all routes) given NTP-2000 diet or corn oil gavage controls given NIH-07 diet (Table C4b). This incidence also exceeded the historical control range for squamous cell papilloma and carcinoma (combined) at this site in male controls in both historical databases. Considering the induction of squamous neoplasms in the forestomach and the oral exposure due to gavage administration, the increased incidence of this uncommon neoplasm in the oral cavity of 120 mg/kg male mice may have been related to 2,4-hexadienal administration.

Nose: Although lesions of the nasal olfactory epithelium were observed in the 120 mg/kg groups of the 14-week study, no treatment-related olfactory changes were found in mice administered the same dose for 2 years.

OXIDATIVE STRESS AND DNA ADDUCTS

In the forestomach of male and female rats, there were statistically significant dose-related positive trends in the concentrations of reduced, oxidized, and total glutathione (Table L1). There were no statistically significant changes in malondialdehyde concentrations in the forestomach of male or female rats (Table L2).

In the determination of cyclic DNA adducts, there was an increase in the concentration of Cro-dG 2 in the rat

forestomach (Table M2). Increases in adduct levels were not observed in rat liver or mouse forestomach (Tables M1 and M3).

GENETIC TOXICOLOGY

2,4-Hexadienal was tested at two laboratories for induction of mutations in three strains of *Salmonella typhimurium* (Table E1). Neither laboratory detected mutagenic activity in strains TA98 or TA1535, with or without Aroclor 1254-induced rat or hamster liver S9 enzymes. At one laboratory, significant responses were seen in strain TA100 without S9 and in the presence of 5%, 10%, or 30% S9 from rat and hamster liver. At the second laboratory, results in TA100 in the absence of S9 were negative; a positive response was noted with 30% hamster and 30% rat liver S9. Additional concentrations of S9 were not tested in this second laboratory study. Both laboratories tested similar concentrations of 2,4-hexadienal. Strain TA100 mutates via base pair substitution. Results of acute tests with 2,4-hexadienal for induction of micronuclei in bone marrow polychromatic erythrocytes of male rats (Table E2) and male mice (Table E3) were judged to be inconclusive. Each of the initial trials, one in rats and one in mice, gave an indication of an effect. In the mouse study, trend analysis of the response over the dose range of 40 to 160 mg/kg 2,4-hexadienal yielded a P value of 0.024, which is significant. However, no individual groups were significantly elevated over the concurrent vehicle control group. In the rat study, the trend test P value was 0.017, which is also significant. As with the study in male mice, none of the mean values for the individual groups of treated rats differed significantly from the concurrent control group value. Because no repeat testing was performed to confirm the response in either rats or mice, the results in both bone marrow micronucleus tests were judged to be inconclusive. No increases in the frequencies of micronucleated normochromatic (mature) erythrocytes were seen in peripheral blood samples obtained from male or female mice after 14 weeks of exposure to 2,4-hexadienal (7.5 to 120 mg/kg) by gavage (Table E4).

In summary, 2,4-hexadienal induced gene mutations in *S. typhimurium*, but it did not conclusively affect erythrocyte micronucleus frequencies, an indirect indicator of numerical or chromosomal damage, in rats or mice after acute or subchronic administration.

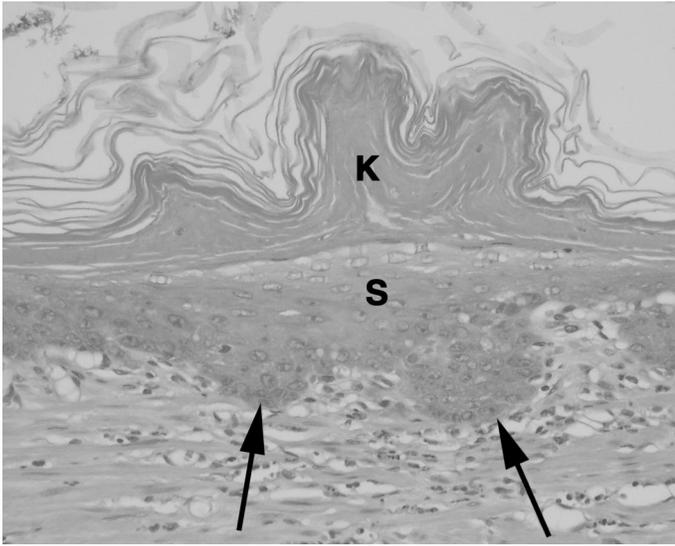


PLATE 1

Forestomach of a male F344/N rat exposed to 120 mg/kg 2,4-hexadienal by gavage for 14 weeks. There is irregular thickening (hyperplasia) of the spinous cell (S) and keratin (K) layers as well as downward projections of the basal cell layer (arrows). Mild inflammation is present beneath the epithelium. H&E; 190x□

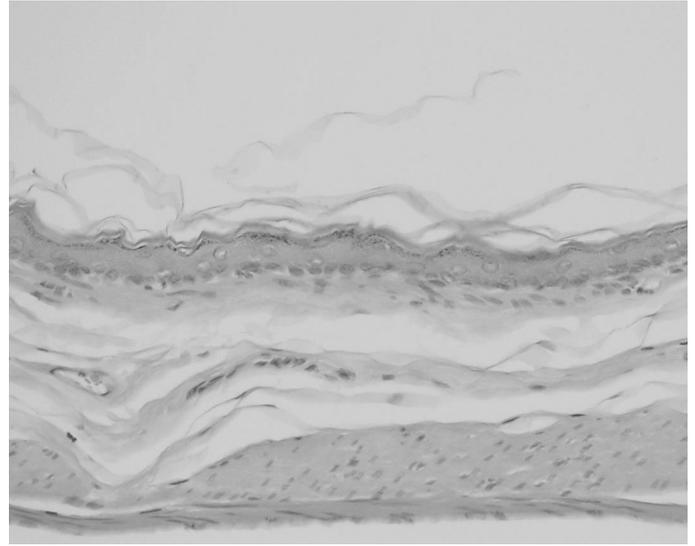


PLATE 2

Forestomach of a vehicle control male F344/N rat from the 14-week gavage study of 2,4-hexadienal. Note the normal 2-3 cell layer thickness of the squamous epithelium. H&E; 190x□

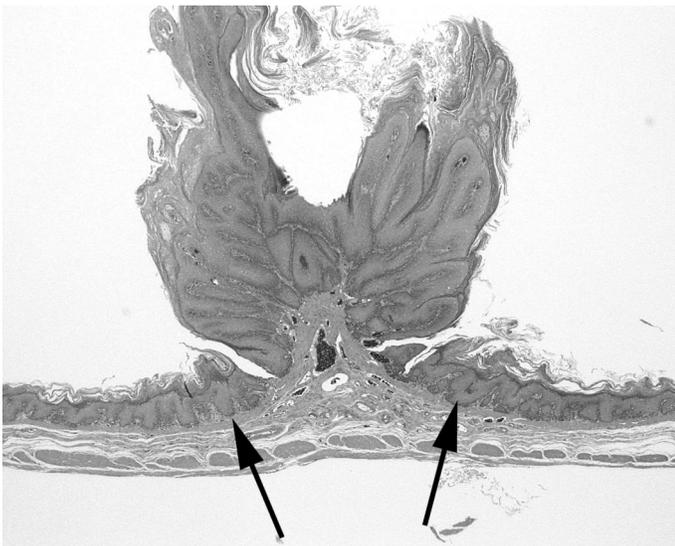


PLATE 3

Squamous papilloma of the forestomach in a male F344/N rat exposed to 90 mg/kg 2,4-hexadienal by gavage for 2 years. The papilloma consists of projections of well-differentiated squamous epithelium resting on arborized fibrovascular cores. Note the epithelial hyperplasia at the margins of the papilloma (arrows). H&E; 22x□

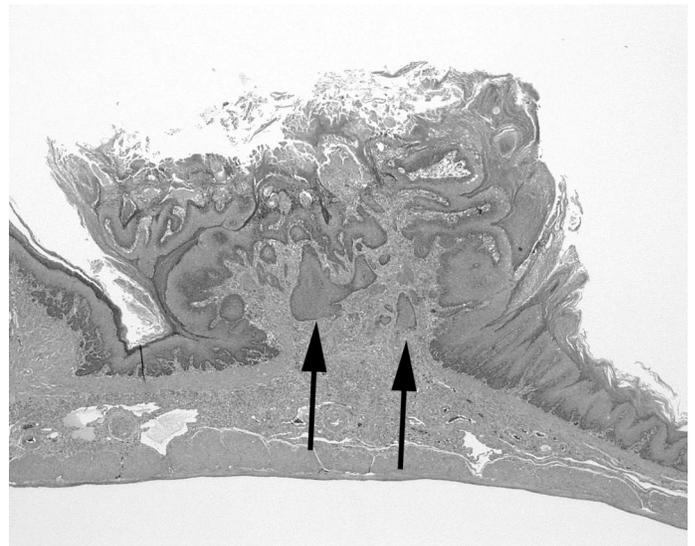


PLATE 4

Squamous carcinoma of the forestomach in a male F344/N rat exposed to 90 mg/kg 2,4-hexadienal by gavage for 2 years. The carcinoma is characterized by endophytic extension of nests of neoplastic cells into the submucosa (arrows) from proliferative squamous epithelium lining the mucosal surface. H&E; 22x□



PLATE 5
 Forestomach of a female B6C3F₁ mouse exposed to 120 mg/kg 2,4-hexadienal by gavage for 2 years. An exophytic squamous papilloma projects into the lumen. H&E; 22x□

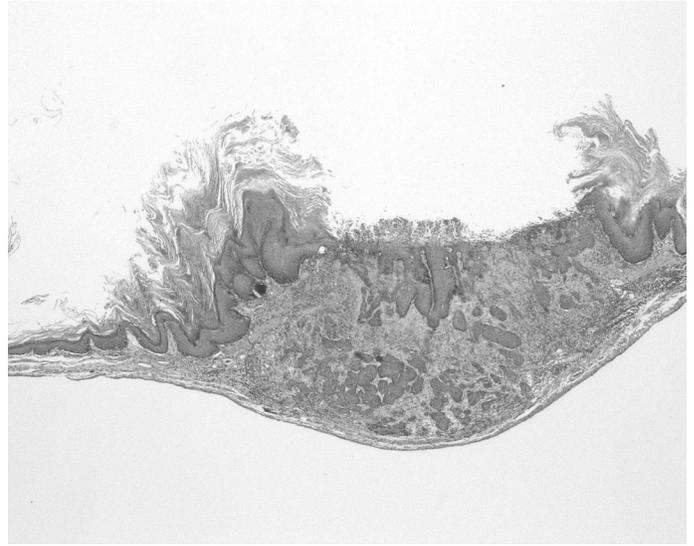


PLATE 6
 Squamous carcinoma of the forestomach in a female B6C3F₁ mouse exposed to 120 mg/kg 2,4-hexadienal by gavage for 2 years. The wall is thickened due to invasion by irregular nests of neoplastic cells and associated inflammation. The surface of the tumor is ulcerated. H&E; 22x□

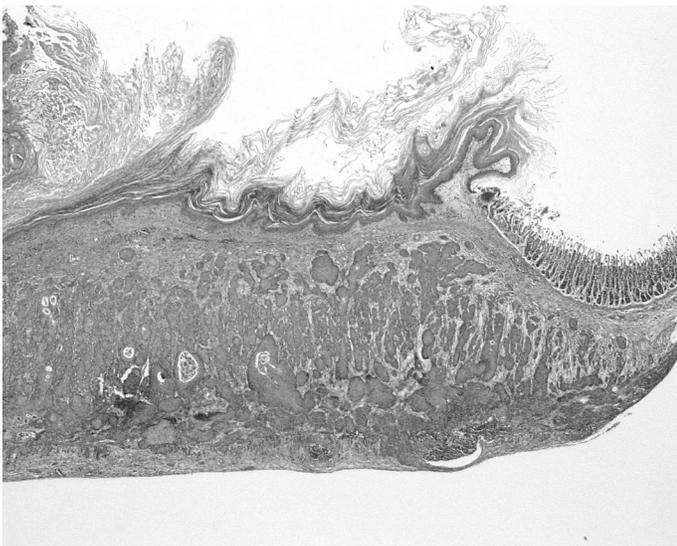


PLATE 7
 Squamous carcinoma of the forestomach in a female B6C3F₁ mouse exposed to 120 mg/kg 2,4-hexadienal by gavage for 2 years. Compared to the tumor in Plate 6, there is more extensive transmurial invasion by neoplastic cells in this animal. The neoplasm is located near the junction between the forestomach and glandular stomach (on right). H&E; 22x□

DISCUSSION AND CONCLUSIONS

The National Cancer Institute nominated 2,4-hexadienal for study because of the potential for carcinogenicity based on its α,β -unsaturated aldehyde structure and the potential link between exposure to lipid peroxidation products in the diet and human malignancies. 2,4-Hexadienal was evaluated for toxicity and carcinogenicity in 16-day, 14-week, and 2-year gavage studies in male and female F344/N rats and B6C3F₁ mice.

The primary effect associated with administration of 2,4-hexadienal was an increase in the incidences of hyperplasia, squamous cell papilloma, and squamous cell carcinoma in the forestomach of rats and mice. Rodents, unlike humans, have a forestomach, where food is temporarily stored before digestion begins in the glandular stomach. Rodents and humans have an oral cavity and esophagus which, like the rodent forestomach, are lined by squamous epithelium. However, exposure of the forestomach to ingested material is generally longer than that of the oral cavity or esophagus.

While spontaneous neoplasms of the forestomach are rare in rats and mice, the forestomach is a relatively common target tissue in NTP rodent bioassays. The pathogenesis of neoplasm development in the forestomach is likely as varied as it is in any other site. Some chemicals are very irritating to the forestomach, causing ulcerative lesions and inflammation followed by proliferative responses of hyperplasia and neoplasia. In the 16-day studies, severe ulcerative and necrotizing lesions of the forestomach were observed in 240 mg/kg rats and mice. Ulcerative lesions of lesser severity and hyperplasia occurred in 80 mg/kg rats. Hyperplasia occurred in mice administered 80 mg/kg; necrotic change of the forestomach was not seen in these animals. No forestomach effects occurred in 27 mg/kg rats or mice. In the 14-week studies, minimal to moderate epithelial hyperplasia was observed in male and female rats and mice administered 120 mg/kg. Inflammation was a component of the hyperplastic process in rats but not in mice at this dose concentration. The no-observed-adverse-effect level in the 14-week studies was 60 mg/kg. In the 2-year studies, the incidences of

squamous cell neoplasms of the forestomach were significantly increased at doses as low as 45 mg/kg in rats, and there were three squamous cell papillomas in male rats administered 22.5 mg/kg. Increased incidences of forestomach neoplasms occurred in 60 and 120 mg/kg male and female mice. Therefore, the forestomach carcinogenic effect following administration of 2,4-hexadienal for 2 years occurred at lower doses than those at which an obvious irritative or inflammatory effect was observed in the 16-day and 14-week studies.

Hyperplasia is recognized as a potential precursor lesion to neoplasia in the rodent forestomach, and two morphologic types of forestomach hyperplasia have been described. Squamous cell hyperplasia is characterized by proliferation and thickening of the suprabasal spinous cell layer, often in association with a thickened keratin layer. Basal cell hyperplasia consists of downward (endophytic) proliferation of the basal cells. The two types of forestomach hyperplasia may occur concurrently, as shown in the 2,4-hexadienal studies in which a basal cell component of the hyperplastic process was clearly evident in rats. Studies with the prototype forestomach carcinogen butylated hydroxyanisole suggest that basal hyperplasia may be more relevant to neoplastic progression (Tatematsu *et al.*, 1991). However, hyperplastic responses in the forestomach are often reversible and do not necessarily progress to neoplasia. The hyperplastic response of the rodent forestomach and the relationships between its various morphologic expressions, underlying regenerative or mitogenic stimuli, and potential to progress to neoplasia are clearly complex and poorly understood issues requiring further study.

In addition to forestomach neoplasms, squamous cell carcinoma of the oral cavity (tongue) occurred in two 120 mg/kg male mice. Because this is a rare spontaneous neoplasm in mice, it may have been chemically induced. 2,4-Hexadienal on the end of the gavage needle may have been deposited in the oral cavity during the gavage procedure, or alternatively, there may have been some regurgitation of gavaged material. In NTP

studies, neoplastic responses of the upper alimentary tract (squamous papillomas and/or squamous cell carcinomas) are most common in the forestomach (with the gavage route disproportionately represented), followed by the oral cavity and esophagus. Of the three NTP studies in which there was a tumorigenic response in the esophagus, responses were also observed in the oral cavity and forestomach (NTP 1986, 1993, 1996).

Little is known about the mechanisms of toxicity of 2,4-hexadienal. There are at least two possible pathways by which 2,4-hexadienal may act to induce neoplasms: 2,4-hexadienal may interact directly with the target tissue macromolecules causing gene mutations, or 2,4-hexadienal may promote injury by reactive oxygen species following glutathione depletion and/or stimulation of inflammation processes. The reactive oxygen species may interact with target tissue DNA, causing genetic damage. Either pathway may eventually lead to carcinogenesis.

Mutagenicity studies showed that 2,4-hexadienal is mutagenic in *Salmonella typhimurium* in the absence of liver S9 activation enzymes, indicating that 2,4-hexadienal may be a direct-acting alkylating agent to cellular nucleophilic groups which does not require metabolic activation (Marnett *et al.*, 1985; Appendix E). Frankel *et al.* (1987) demonstrated that 2,4-hexadienal interacted with calf thymus DNA *in vitro*. Eder *et al.* (1993) reported that the chemical induced DNA strand breaks in 1,210 mouse leukemia cells as measured with the alkaline elution technique and postulated that the effect was probably due to the formation of 1,N²-cyclic adducts and 7,8-cyclic adducts with deoxyguanosine.

Lipid peroxidation generates a complex variety of secondary peroxidation products such as *n*-alkanals, *trans*-2-alkenals, *trans,trans*- and *cis,trans*-alka-2,4-dienals, 4-hydroxy-*trans*-2-alkenals, and malondialdehyde (Claxon *et al.*, 1994; Haywood *et al.*, 1995). Many of these products are reactive electrophiles and have been shown to react covalently with nucleophilic sites on DNA bases forming adducts, i.e., 2'-deoxyguanosine (dG) and 2'-deoxyadenosine (dA) (Douki and Ames, 1994; Doerge *et al.*, 1998). They are involved in the formation of modified DNA bases such as the malondialdehyde-guanine adducts, the 1,N²-propano adducts, and the ethano adducts (Eder and Hoffman, 1993; Bartsch, 1999; Cadet *et al.*, 1999). The propano- and malondialdehyde-derived adducts are formed from α,β -unsaturated

aldehydes or enols produced by peroxidation of lipids. The ethano adducts are products of reactions with chloroacetaldehyde, 1-substituted oxiranes, and the epoxides of enols (Chung *et al.*, 1999). The adducts may exhibit miscoded base-pairing properties and could be involved in the mutagenic and carcinogenic effects (Bartsch, 1999).

Acr-dG 3, Cro-dG 1, and Cro-dG 2 are 1,N²-propanodeoxyguanosine adducts which form from the interaction of enals from lipid peroxidation with DNA (Chung *et al.*, 1999). The adducts are similar to those characterized by Eder and Hoffman (1993) though the nomenclatures are different. The adducts represent DNA lesions caused by lipid peroxidation and are pro-mutagenic and may be involved in carcinogenesis (Chung *et al.*, 1999). In an NTP study, Acr-dG 3, Cro-dG 1, and Cro-dG2 adduct levels in liver (rats only) and forestomach tissues from rats and mice exposed to 2,4-hexadienal for 14 weeks were determined by a ³²P-postlabeling technique (Appendix M). The results showed that in the rat forestomach, Cro-dG 2 adduct levels were significantly greater in tissue from dosed animals than in tissue from vehicle controls; no significant difference in the concentration of Acr-dG 3 adduct levels was seen between the dosed and vehicle control tissue samples. These data suggest that treatment with 2,4-hexadienal may increase cyclic adduct formation in rat forestomach DNA. However, in the rat liver and mouse forestomach samples, no increase in Acr-dG 3, Cro-dG 1, or Cro-dG2 adduct concentrations were detected in samples from dosed animals. This may be related to the tissue glutathione concentrations observed (Appendix L) as GSH is an antioxidant and an effective scavenger of enals, a sustained GSH depletion is likely needed to cause an increase in Acr-dG and Cro-dG adduct levels.

Reactive oxygen species have been shown to be cytotoxic, injuring cells through damage to DNA. Alka-2,4-dienals may initiate lipid peroxidation and oxidative stress (Poulsen *et al.*, 1998). Gavage administration of 2,4-hexadienal to rats and mice may initiate an auto-oxidation process in the biological system. The events in the forestomach may include generation of reactive oxygen species and impairment of the antioxidant defense system. The process may allow the reactive oxygen species generated to interact with the forestomach DNA causing damage. The process may also depress the repair enzyme functions, allowing the

accumulation of damaged DNA that leads to mutagenesis and carcinogenesis (Breimer, 1991; Yamamoto *et al.*, 1992; Demple and Harrison, 1994). Interaction between reactive oxygen species and cellular membranes leads to the formation of malondialdehyde at the interaction site. The malondialdehyde levels reflect the amount of free radicals generated and the extent of membrane lipid peroxidation. Ingested lipid oxidation products have been reported to cause increased excretion of mutagens, cellular injury to the liver and kidneys, increased cell proliferation in the gastrointestinal tract, and other nonspecific tissue injury and irritation effects (Poulsen *et al.*, 1998). Attempts to measure reactive oxygen species with the thiobarbituric acid technique did not show increased formation of malondialdehyde in the forestomach of the 2,4-hexadienal treated rats (Appendix L). The thiobarbituric acid technique has been used extensively to determine malondialdehyde levels (Buege and Aust, 1978; Wasowicz *et al.*, 1993), which reflect the amount of reactive oxygen species being generated and the extent of membrane lipid peroxidation. However, the thiobarbituric acid technique is not considered a very good indicator for quantitation of reactive oxygen species *in vivo* as malondialdehyde can be removed by the mitochondrial aldehyde-metabolizing system and can also be excreted (Horton and Fairhurst, 1987).

Glutathione functions as a reactive oxygen species scavenger in the antioxidant systems against oxidative stress. Glutathione is conjugated to an electrophilic site of a broad range of potentially toxic and carcinogenic compounds including fatty acid oxidation products (Eaton and Bammler, 1999), thereby decreasing their reactivity with cellular macromolecules (Armstrong, 1997). The glutathione conjugating reaction is catalyzed by the glutathione *S*-transferases. When the cellular antioxidant defense systems are overwhelmed, the effects of free radicals are manifested (Gogvadze and Zhukova, 1991). Depletion of glutathione enhances the lipid peroxidation initiated by reactive oxygen species (Yoshikawa *et al.*, 1997). Peters *et al.* (1993) reported that low levels of glutathione *S*-transferases and glutathione correlate well with increased risk for developing cytogenetic damage and tumors. During the scavenging of reactive oxygen species, glutathione is oxidized to oxidized glutathione (GSSG) and forms glutathione-protein mixed disulfides (Enomoto *et al.*, 2001). Oxidative stress may be reflected by a decline in the reduced glutathione level. Depletion of reduced glutathione (GSH) in the presence of high GSSG concentrations was reported related to

several pathophysiologies and diseases (Schulz *et al.*, 2000). There was a significant reduction of the GSH/GSSG ratio in male rats at 4 hours postdosing (Appendix L). The concentration of GSH increased significantly in males at 1 and 4 hours after dosing and in females at 4 and 24 hours after dosing. The concentration of GSSG increased significantly in males at all three timepoints and in females at 4 and 24 hours postdosing. The concentration of GSH + GSSG increased significantly in males at 4 hours after dosing and in females at 4 and 24 hours postdosing. The increases in GSH and GSSG values, as well as in total glutathione levels (GSH + GSSG) suggested a high consumption of reduced glutathione. This may indicate oxidative stress and consequent induction of GSH synthesis by an up regulation mechanism (Mates, 2000). It is possible that at longer durations (e.g., 13 weeks or 2 years) the antioxidant system may not be able to cope with the potential oxidative stress induced by 2,4-hexadienal due to accumulation of highly reactive oxygen species or depletion of GSH through direct reaction with 2,4-hexadienal. This may ultimately lead to increased incidences of stomach neoplasms. Further studies are needed to confirm this hypothesis.

Accumulation of oxidative DNA damage may be a significant causative factor in carcinogenesis. Measurements of 8-hydroxydeoxyguanosine (8-OH-dG) have been used as a sensitive marker of oxidative stress in carcinogenesis. 8-OH-dG is produced by hydroxylation in the C-8 position of deoxyguanosine residues in DNA by reactive oxygen species, especially the hydroxyl radical. Increased 8-OH-dG in the target organs has been found in mouse lung carcinogenesis (Nagashima *et al.*, 1995; Ichinose *et al.*, 1997), rat liver carcinogenesis (Randerath *et al.*, 1997), and mouse hepatocarcinogenesis (Dahlhaus *et al.*, 1995). The present studies did not include measurements of 8-OH-dG following 2,4-hexadienal administration. It may be informative to measure 8-OH-dG to affirm that 2,4-hexadienal administration induces oxidative stress in the forestomach.

CONCLUSIONS

Under the conditions of these 2-year gavage studies, there was *clear evidence of carcinogenic activity** of 2,4-hexadienal in male and female F344/N rats and male and female B6C3F₁ mice based on increased incidences of squamous cell neoplasms of the forestomach. The

occurrence of squamous cell carcinoma of the oral cavity (tongue) in male B6C3F₁ mice may have been related to the administration of 2,4-hexadienal.

Hyperplasia of the forestomach in male and female rats and mice was associated with administration of 2,4-hexadienal.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 11. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 13.

REFERENCES

- The Aldrich Library of Infrared Spectra* (1981). 3rd ed. (C.J. Pouchert, Ed.). Aldrich Chemical Company, Inc., Milwaukee.
- The Aldrich Library of NMR Spectra* (1983). 2nd ed., Vol. 5. Aldrich Chemical Company, Inc., Milwaukee, WI.
- Aldrich's 1991-1992 Flavors and Fragrances Catalog* (1991). Aldrich Chemical Company, Inc., Milwaukee, WI.
- Alexander, J.C., Valli, V.E., and Chanin, B.E. (1987). Biological observations from feeding heated corn oil and heated peanut oil to rats. *J. Toxicol. Environ. Health*, **21**, 295-309.
- Armstrong, R.N. (1997). Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chem. Res. Toxicol.* **10**, 2-18.
- Ashby, J., and Tennant, R.W. (1991). Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the U.S. NTP. *Mutat. Res.* **257**, 229-306.
- 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.
- Bartsch, H. (1999). Keynote address: Exocyclic adducts as new risk markers for DNA damage in man. *IARC Sci. Publ.* **150**, 1-16.
- Bedoukian, P.Z. (1985). The alkadienals. *Perfumer Flavorist* **9**, 25-26.
- Bieler, G.S., and Williams, R.L. (1993). Ratio estimates, the delta method, and quantal response tests for increased carcinogenicity. *Biometrics* **49**, 793-801.
- 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, L.H. (1991). Repair of DNA damage induced by reactive oxygen species. *Free Radic. Res. Commun.* **14**, 159-171.
- Buege, J.A., and Aust, D.S. (1978). Microsomal lipid peroxidation. *Methods Enzymol.* **52**, 302-310.
- Cadet, J., Carvalho, V.M., Onuki, J., Douki, T., Medeiros, M.H.G., and Di Mascio, P.D. (1999). Purine DNA adducts of 4,5-dioxovaleric acid and 2,4-decadienal. *IARC Sci. Publ.* **150**, 103-113.
- Casanova-Schmitz, M., and Heck, H.D'A. (1983). Effects of formaldehyde exposure on the extractability of DNA from proteins in the rat nasal mucosa. *Toxicol. Appl. Pharmacol.* **70**, 121-132.
- Chung, F.L., Nath, R.G., Nagao, M., Nishikawa, A., Zhou, G.D., and Randerath, K. (1999). Endogenous formation and significance of 1,N2-propanodeoxyguanosine adducts. *Mutat. Res.* **424**, 71-81.
- Claxton, A.W.D., Hawkes, G.E., Richardson, D.P., Naughton, D.P., Haywood, R.M., Chander, C.L., Atherton, M., Lynch, E.J., and Grootveld, M.C. (1994). Generation of lipid peroxidation products in culinary oils and fats during episodes of thermal stress: A high field 1H NMR study. *FEBS Lett.* **355**, 81-90.
- Code of Federal Regulations (CFR) **21**, Part 58.
- Cox, D.R. (1972). Regression models and life-tables. *J. R. Stat. Soc.* **B34**, 187-220.

- Crawford, B.D. (1985). Perspectives on the somatic mutation model of carcinogenesis. In *Advances in Modern Environmental Toxicology. Mechanisms and Toxicity of Chemical Carcinogens and Mutagens* (M.A. Mehlman, W.G. Flamm, and R.J. Lorentzen, Eds.), pp. 13-59. Princeton Scientific Publishing Co., Inc., Princeton, NJ.
- Crawford, L., and Wheeler, E.L. (1983). Microsomal P-450 induction by some secondary products from thermal oxidation of dietary lipids: Epidermal hyperplasia, mutagenicity and cytochrome P-450. *Cancer Lett.* **21**, 211-217.
- Crook, T.R., Souhami, R.L., and McLean, A.E.M. (1986). Cytotoxicity, DNA cross-linking, and single strand breaks induced by activated cyclophosphamide and acrolein in human leukemia cells. *Cancer Res.* **46**, 5029-5034.
- Dahlhaus, M., Almstadt, E., Henschke, P., Luttgert, S., and Appel, K.E. (1995). Induction of 8-hydroxy-2-deoxyguanosine and single-strand breaks in DNA of V79 cells by tetrachloro-p-hydroquinone. *Mutat. Res.* **329**, 29-36.
- Demple, B., and Harrison, L. (1994). Repair of oxidative damage to DNA: Enzymology and biology. *Ann. Rev. Biochem.* **63**, 915-948.
- Dianzani, M.U. (1982). Biochemical effects of saturated and unsaturated aldehydes. In *Free Radicals, Lipid Peroxidation and Cancer* (D.C.H. McBrien and T.F. Slater, Eds.), pp. 129-158. Academic Press, New York.
- Dixon, W.J., and Massey, F.J., Jr. (1951). *Introduction to Statistical Analysis*, 1st ed., pp. 145-147. McGraw-Hill Book Company, Inc., New York.
- Doerge, D.R., Yi, P., Churchwell, M.I., Preece, S.W., Langridge, J., and Fu, P.P. (1998). Mass spectrometric analysis of 2-deoxyribonucleoside and 2'-deoxyribonucleotide adducts with aldehydes derived from lipid peroxidation. *Rapid Commun. Mass Spectrom.* **12**, 1665-1672.
- Douki, T., and Ames, B.N. (1994). An HPLC-EC assay for 1,N2-propano adducts of 2'-deoxyguanosine with 4-hydroxynonenal and other alpha,beta-unsaturated aldehydes. *Chem. Res. Toxicol.* **7**, 511-518.
- Dumdei, B.E., Kenny, D.V., Shepson, P.B., Kleindienst, T.E., Nero, C.M., Cupitt, L.T., and Claxton, L.D. (1988). MS/MS analysis of the products of toluene photooxidation and measurement of their mutagenic activity. *Environ. Sci. Technol.* **22**, 1493-1498.
- Dunn, O.J. (1964). Multiple comparisons using rank sums. *Technometrics* **6**, 241-252.
- Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* **50**, 1096-1121.
- Eaton, D.L., and Bammler, T.K. (1999). Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol. Sci.* **49**, 156-164.
- Eder, E., and Hoffman, C. (1993). Identification and characterization of deoxyguanosine adducts of mutagenic beta-alkyl-substituted acrolein congeners. *Chem. Res. Toxicol.* **6**, 486-494.
- Eder, E., Deininger, C., Neudecker, T., and Deininger, D. (1992). Mutagenicity of beta-alkyl substituted acrolein congeners in the Salmonella typhimurium strain TA100 and genotoxicity testing in the SOS chromotest. *Environ. Mol. Mutagen.* **19**, 338-345.
- Eder, E., Scheckenbach, S., Deininger, C., and Hoffman, C. (1993). The possible role of alpha,beta-unsaturated carbonyl compounds in mutagenesis and carcinogenesis. *Toxicol. Lett.* **67**, 87-103.
- Enomoto, A., Itoh, K., Nagayoshi, E., Haruta, J., Kimura, T., O'Connor, T., Harada, T., and Yamamoto, M. (2001). High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. *Toxicol. Sci.* **59**, 169-177.
- Esterbauer, H. (1982). Free radicals, lipid peroxidation, and cancer. In *Aldehydic Products of Lipid Peroxidation* (D.C.H. McBrien and T.F. Slater, Eds.), pp. 101-112. Academic Press, London.
- Esterbauer, H. (1985). Lipid peroxidation products: Formation, chemical properties, and biological activities. In *Free Radicals in Liver Injury* (G. Poli, K.H. Cheeseman, M.U. Dianzani, and T.F. Slater, Eds.), pp. 29-47. IRL Press, Oxford.

- Ferrario, J.B., Lawler, G.C., DeLeon, I.R., and Laseter, J.L. (1985). Volatile organic pollutants in biota and sediments of Lake Pontchartrain. *Bull. Environ. Contam. Toxicol.* **34**, 246-255.
- Florin, I., Rutberg, L., Curvall, M., and Enzell, C.R. (1980). Screening of tobacco smoke constituents for mutagenicity using the Ames' test. *Toxicology* **18**, 219-232.
- Ford, R.A., Letizia, C., and Api, A.M. (1988). Monographs on fragrance raw materials: Trans, trans-2,4-hexadienal. *Food Chem. Toxicol.* **26**, 337-338.
- Frankel, E.N., Neff, W.E., Brooks, D.D., and Fujimoto, K. (1987). Fluorescence formation from the interaction of DNA with lipid oxidation degradation products. *Biochim. Biophys. Acta* **919**, 239-244.
- Gogvadze, V.G., and Zhukova, A.A. (1991). The role of lipid peroxidation products in cumene hydroperoxide-induced Ca²⁺ efflux from mitochondria. *FEBS Lett.* **287**, 139-141.
- Grootveld, M., Atherton, M.D., Sheerin, A.N., Hawkes, J., Blake, D.R., Richens, T.E., Silwood, C.J., Lynch, E., and Claxson, A.W. (1998). In vivo absorption, metabolism, and urinary excretion of alpha,beta-unsaturated aldehydes in experimental animals. Relevance to the development of cardiovascular diseases by the dietary ingestion of thermally stressed polyunsaturated-rich culinary oils. *J. Clin. Invest.* **101**, 1210-1218.
- Growcock, F.B., Frenier, W.W., and Andreozzi, P.A. (1989). Inhibition of steel corrosion in HCl by derivatives of cinnamaldehyde. Part II. Structure-activity correlations. *Corrosion* **45**, 1007-1015.
- Gueldner, R.C., Wilson, D.M., and Heidt, A.R. (1985). Volatile compounds inhibiting *Aspergillus flavus*. *J. Agric. Food Chem.* **33**, 411-413.
- Hageman, G., Verhagen, H., Schutte, B., and Kleinjans, J. (1991). Biological effects of short-term feeding to rats of repeatedly used deep-frying fats in relation to fat mutagen content. *Food Chem. Toxicol.* **29**, 689-698.
- Haseman, J.K., and Rao, G.N. (1992). Effects of corn oil, time-related changes, and inter-laboratory variability on tumor occurrence in control Fischer 344 (F344/N) rats. *Toxicol. Pathol.* **20**, 52-60.
- Haseman, J.K., Huff, J.E., Rao, G.N., Arnold, J.E., Boorman, G.A., and McConnell, E.E. (1985). Neoplasms observed in untreated and corn oil gavage control groups of F344/N rats and (C57BL/6N × C3H/HeN)F₁ (B6C3F₁) mice. *JNCI* **75**, 975-984.
- Haywood, R.M., Claxson, A.W., Hawkes, G.E., Richardson, D.P., Naughton, D.P., Coumbarides, G., Hawkes, J., Lynch, E.J., and Grootveld, M.C. (1995). Detection of aldehydes and their conjugated hydroperoxydiene precursors in thermally-stressed culinary oils and fats: Investigations using high resolution proton NMR spectroscopy. *Free Radic. Res.* **22**, 441-482.
- Heil, T.P., and Lindsay, R.C. (1988). Volatile compounds in flavor-tainted fish from the Upper Wisconsin River. *J. Environ. Sci. Health* **23**, 489-512.
- Hollander, M., and Wolfe, D.A. (1973). *Nonparametric Statistical Methods*, pp. 120-123. John Wiley and Sons, New York.
- Horton, A.A., and Fairhurst, S. (1987). Lipid peroxidation and mechanisms of toxicity. *Critical Rev. Toxicol.* **18**, 27-79.
- Ichinose, T., Yajima, Y., Nagashima, M., Takenoshita, S., Nagamachi, Y., and Sagai, M. (1997). Lung carcinogenesis and formation of 8-hydroxydeoxyguanosine in mice by diesel exhaust particles. *Carcinogenesis* **18**, 185-192.
- Imai, K., Yoshimura, S., Hashimoto, K., and Boorman, G.A. (1991). Effects of dietary restriction on age-associated pathological changes in Fischer 344 rats. In *Biological Effects of Dietary Restriction* (L. Fishbein, Ed.) ILSI monograph, pp. 87-98. Springer-Verlag, New York.
- Integrated Laboratory Systems (ILS) (1990). *Micronucleus Data Management and Statistical Analysis Software, Version 1.4*. ILS, P.O. Box 13501, Research Triangle Park, NC 27707.

- Izaki, Y., Yoshikawa, S., and Uchiyama, M. (1984). Effect of ingestion of thermally oxidized frying oil on peroxidative criteria in rats. *Lipids* **5**, 324-331.
- Jenkins, F.P., and Robinson, J.A. (1975). Serum biochemical changes in rats deprived of food or water for 24 h. *Proc. Nutr. Soc.* **34**, 37A.
- Jonckheere, A.R. (1954). A distribution-free *k*-sample test against ordered alternatives. *Biometrika* **41**, 133-145.
- Jorgensen, B.M., Agerbo, P., Jensen, B., Borresen, T., and Holmer, G. (1992). Inhibition of microsomal glucose 6-phosphatase by unsaturated aliphatic aldehydes and ketones. *Chem. Biol. Interact.* **81**, 209-218.
- Kanazawa, K., Kanazawa, E., and Natake, M. (1985). Uptake of secondary autoxidation products of linoleic acid by the rat. *Lipids* **20**, 412-419.
- Kanazawa, K., Ashida, H., Minamoto, S., and Natake, M. (1986). The effect of orally administered secondary autoxidation products of linoleic acid on the activity of detoxifying enzymes in the rat liver. *Biochim. Biophys. Acta* **879**, 36-43.
- Kaneko, J.J., Ed. (1989). *Clinical Biochemistry of Domestic Animals*, 4th ed. Academic Press, Inc., San Diego.
- Kaplan, E.L., and Meier, P. (1958). Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* **53**, 457-481.
- Kawakami, M., and Shibamoto, T. (1991). The volatile constituents of piled tea: Toyama kurocha. *Agric. Biol. Chem.* **55**, 1839-1847.
- Kaye, C.M. (1973). Biosynthesis of mercapturic acids from allyl alcohol, allyl esters, and acrolein. *Biochem. J.* **134**, 1093-1101.
- Keller, C.L., Balaban, S.M., Hickey, C.S., and DiFate, V.G. (1983). Sorbic acid. In *Kirk-Othmer Encyclopedia of Chemical Technology* (M. Grayson and D. Eckroth, Eds.), 3rd ed., Vol. 21., p. 406. John Wiley and Sons, Inc., New York.
- Kline, S.A., Xiang, Q., Goldstein, B.D., and Witz, G. (1993). Reaction of (E,E)-muconaldehyde and its aldehydic metabolites, (E,E)-6-oxohexadienoic acid and (E,E)-6-hydroxyhexa-2,4-dienal, with glutathione. *Chem Res. Toxicol.* **6**, 578-583.
- Kosugi, H., Kato, T., and Kikugawa, K. (1988). Formation of a red pigment by a two-step 2-thiobarbituric acid reaction of alka-2,4-dienals. Potential products of lipid oxidation. *Lipids* **23**, 1024-1031.
- Lancaster Synthesis, Inc. (1991). *Lancaster Catalogue 1991/92*. MTM Research Chemicals, Windham, NH.
- Latriano, L., Witz, G., Goldstein, B.D., and Jeffrey, A.M. (1989). Chromatographic and spectrophotometric characterization of adducts formed during the reaction of trans,trans-muconaldehyde with 14C-deoxyguanosine 5'-phosphate. *Environ. Health Perspect.* **82**, 249-251.
- 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.
- McGirr, L.G., Hadley, M., and Draper, H.H. (1985). Identification of N-alpha-acetyl-epsilon-(2-propenal) lysine as a urinary metabolite of malondialdehyde. *J. Biol. Chem.* **260**, 15,427-15,431.
- MacGregor, J.T., Wehr, C.M., Henika, P.R., and Shelby, M.D. (1990). The *in vivo* erythrocyte micronucleus test: Measurement at steady state increases assay efficiency and permits integration with toxicity studies. *Fundam. Appl. Toxicol.* **14**, 513-522.
- McMahon, R.E. (1982). Alcohols, aldehydes, and ketones. In *Metabolic Basis of Detoxication: Metabolism of Functional Groups* (W.B. Jakoby, J.R. Bend, and J. Caldwell, Eds.). Academic Press, New York.
- Marnett, L.J., Hurd, H.K., Hollstein, M.C., Levin, D.E., Esterbauer, H., and Ames, B.N. (1985). Naturally occurring carbonyl compounds are mutagens in Salmonella tester strain TA104. *Mutat. Res.* **148**, 25-34.

- 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.
- Material Safety Data Sheet (MSDS) (1992). Aldrich Chemical Company, Inc., Milwaukee, WI.
- Mates, M. (2000). Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology* **153**, 83-104.
- Miller, J.A., and Miller, E.C. (1977). Ultimate chemical carcinogens as reactive mutagenic electrophiles. In *Origins of Human Cancer* (H.H. Hiatt, J.D. Watson, and J.A. Winsten, Eds.), pp. 605-627. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Morrison, D.F. (1976). *Multivariate Statistical Methods*, 2nd ed., pp. 170-179. McGraw-Hill Book Company, New York.
- Nagashima, M., Kasai, H., Yokota, J., Nagamachi, Y., Ichinose, T., and Sagai, M. (1995). Formation of an oxidative DNA damage, 8-hydroxydeoxyguanosine, in mouse lung DNA after intratracheal instillation of diesel exhaust particles and effects of high dietary fat and beta-carotene on this process. *Carcinogenesis* **16**, 1441-1445.
- National Toxicology Program (NTP) (1986). Toxicology and Carcinogenesis Studies of Dimethylvinyl Chloride (1-Chloro-2-methylpropene) (CAS No. 513-37-1) in F344/N Rats and B6C3F₁ Mice (Gavage Studies). Technical Report Series No. 316. NIH Publication No. 86-2572. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.
- National Toxicology Program (NTP) (1992). Technical Protocol for Sperm Morphology and Vaginal Cytology Evaluations in Toxicity Testing for Rats and Mice, 10/31/82 version (updated August 1992). Research Triangle Park, NC.
- National Toxicology Program (NTP) (1993). Toxicology and Carcinogenesis Studies of 2,3-Dibromo-1-propanol (CAS No. 96-13-9) in F344/N Rats and B6C3F₁ Mice (Dermal Studies). Technical Report Series No. 400. NIH Publication No. 94-2855. 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 2,2-Bis(bromomethyl)-1,3-propanediol (FR-1138®) (CAS No. 3296-90-0) in F344/N Rats and B6C3F₁ Mice (Feed Studies). Technical Report Series No. 452. NIH Publication No. 96-3368. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.
- Peters, W.H., Roelofs, H.M., Hectors, M.P., Nagengast, F.M., and Jansen, J.B. (1993). Glutathione and glutathione S-transferases in Barrett's epithelium. *Br. J. Cancer* **6**, 1413-1417.
- Pettersson, B., Curvall, M., and Enzell, C.R. (1980). Effects of tobacco smoke compounds on the noradrenaline induced oxidative metabolism in isolated brown fat cells. *Toxicology* **18**, 1-15.
- Piegorsch, W.W., and Bailer, A.J. (1997). *Statistics for Environmental Biology and Toxicology*, Section 6.3.2. Chapman and Hall, London.
- Pilotti, A., Ancker, K., Arrhenius, E., and Enzell, C. (1975). Effects of tobacco and tobacco smoke constituents on cell multiplication in vitro. *Toxicology* **5**, 49-52.
- 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.

- Poulsen, H.E., Priem, H., and Loft, S. (1998). Role of oxidative DNA damage in cancer initiation and promotion. *Eur. J. Cancer Prev.* **7**, 9-16.
- Przybylski, R., and Hougen, F.W. (1989). Simple method for estimation of volatile carbonyl compounds in edible oils and fried potato chips. *J. Am. Oil Chem. Soc.* **66**, 1465-1468.
- Randerath, K., Zhou, G.D., Monk, S.A., and Randerath, E. (1997). Enhanced levels in neonatal rat liver of 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-hydroxydeoxyguanosine), a major mutagenic oxidative DNA lesion. *Carcinogenesis* **18**, 1419-1421.
- Rao, G.N. (1996). New diet (NTP-2000) for rats in the National Toxicology Program toxicity and carcinogenicity studies. *Fundam. Appl. Toxicol.* **32**, 102-108.
- Rao, G.N. (1997). New nonpurified diet (NTP-2000) for rodents in the National Toxicology Program's toxicology and carcinogenesis studies. *J. Nutr.* **127**, 842s-846s.
- Registry of Toxic Effects of Chemical Substances (RTECS)* [database online] (1992): National Institute for Occupational Safety and Health; 1971 to present. Updated quarterly. Available from the National Library of Medicine, Bethesda, MD.
- Righetti, A.B., and Kaplan, M.M. (1971). The origin of the serum alkaline phosphatase in normal rats. *Biochim. Biophys. Acta.* **230**, 504-509.
- Schatz-Kornbrust, E., Goldstein, B.D., Witz, G (1991). The binding of trans, trans-mucoaldehyde to deoxyguanosine and calf thymus DNA. *Toxicologist* **11**, 61.
- Schoenfeld, H.A., and Witz, G. (2000). Structure-activity relationships in the induction of DNA-protein cross-links by hematotoxic ring-opened benzene metabolites and related compounds in HL60 cells. *Toxicol. Lett.* **116**, 79-88.
- Schulz, J.B., Lindenau, J., Seyfried, J., and Dichgans, J. (2000). Glutathione, oxidative stress and neurodegeneration. *Eur. J. Biochem.* **267**, 4904-4911.
- Selke, E. and Rohwedder, W.K. (1983). Volatile components from trilinolenin heated in air. *J. Am. Oil Chem. Soc.* **60**, 1853-1858.
- Shelby, M.D. (1988). The genetic toxicity of human carcinogens and its implications. *Mutat. Res.* **204**, 3-15.
- Shelby, M.D., and Witt, K.L. (1995). Comparison of results from mouse bone marrow chromosome aberration and micronucleus tests. *Environ. Mol. Mutagen.* **25**, 302-313.
- Shelby, M.D., and Zeiger, E. (1990). Activity of human carcinogens in the Salmonella and rodent bone-marrow cytogenetics tests. *Mutat. Res.* **234**, 257-261.
- Shelby, M.D., Erexson, G.L., Hook, G.J., and Tice, R.R. (1993). Evaluation of a three-exposure mouse bone marrow micronucleus protocol: Results with 49 chemicals. *Environ. Mol. Mutagen.* **21**, 160-179.
- Sherman, P.D., Jr., and Kavasmaneck, P.R. (1980). Ethanol. In *Kirk-Othmer Encyclopedia of Chemical Technology* (M. Grayson and D. Eckroth, Eds.), 3rd ed., Vol. 9, p. 355. John Wiley and Sons, Inc., New York.
- Shirley, E. (1977). A non-parametric equivalent of Williams' test for contrasting increasing dose levels of a treatment. *Biometrics* **33**, 386-389.
- Snyder, J.M., Frankel, E.N., and Selke, E. (1985). Capillary gas chromatographic analyses of headspace volatiles from vegetable oils. *J. Am. Oil Chem. Soc.* **62**, 1675-1679.
- STN International (STN) (1992). Chemical Abstracts Service databases (file CA). STN International, Columbus, OH.
- Straus, D.S. (1981). Somatic mutation, cellular differentiation, and cancer causation. *JNCI* **67**, 233-241.
- Sturmer, D.M. and Diehl, D.R. (1982). Polymethine dyes. In *Kirk-Othmer Encyclopedia of Chemical Technology*, (M. Grayson and D. Eckroth, Eds.), 3rd ed., Vol. 18, p. 866. John Wiley and Sons, Inc., New York

- Suzuki, J., and Bailey, M.E. (1985). Direct sampling capillary GLC analysis of flavor volatiles from ovine fat. *J. Agric. Food Chem.* **33**, 343-347.
- Takeoka, G.R., Giintert, M., Flath, R.A., Wurz, R.E., and Jennings, W. (1986). Volatile constituents of kiwi fruit (*Actinidia chinensis* Planch). *J. Agric. Food Chem.* **34**, 576-578.
- Tanaka, T. (1979). Skin damage and its prevention from lipoperoxide. *Vitamins* **53**, 577-586.
- Tanaka, T., and Hayakawa, R. (1986). Lipid peroxides in cosmetic products and their effect to irritate the skin. *J. Clin. Biochem. Nutr.* **1**, 201-207.
- Tarone, R.E. (1975). Tests for trend in life table analysis. *Biometrika* **62**, 679-682.
- Tatematsu, M., Ogawa, K., Mutai, M., Aoki, T., Hoshiya, T., and Ito, N. (1991). Rapid regression of squamous cell hyperplasia and slow regression of basal cell hyperplasia in the forestomach of F344 rats treated with N-methyl-N'-nitro-N-nitrosoguanidine and/or butylated hydroxyanisole. *Cancer Res.* **51**, 318-323.
- Tennant, R.W., Margolin, B.H., Shelby, M.D., Zeiger, E., Haseman, J.K., Spalding, J., Caspary, W., Resnick, M., Stasiewicz, S., Anderson, B., and Minor, R. (1987). Prediction of chemical carcinogenicity in rodents from *in vitro* genetic toxicity assays. *Science* **236**, 933-941.
- Thelestam, M., Curvall, M., and Enzell, C.R. (1980). Effect of tobacco smoke on the plasma membrane of cultured human lung fibroblasts. *Toxicology* **15**, 203-217.
- Tice, R.R., Erexson, G.L., and Shelby, M.D. (1990). The induction of micronucleated polychromatic erythrocytes in mice using single and multiple treatments. *Mutat. Res.* **234**, 187-193.
- Troller, J.A., and Olsen, R.A. (1967). Derivatives of sorbic acid as food preservatives. *J. Food Sci.* **32**, 228-231.
- Udupi, V., Goldstein, B.D., and Witz, G. (1994). Interaction of trans,trans-muconaldehyde with bovine serum albumin. *Arch. Biochem. Biophys.* **310**, 385-391.
- U.S. Environmental Protection Agency (USEPA) (2000). Toxic Substances Control Act Chemical Substance Inventory. Office of Toxic Substances. Washington, D.C.
- Urbany, D. (1992). State cancer rate linked to lifestyles. *Gazette Newspapers*, March 4, 1992, p. A-10. Bethesda, MD.
- Wasowicz, W., Neve, J., and Peretz, A. (1993). Optimized steps in fluorometric determination of thio-barbituric acid-reactive substances in serum: Importance of extraction pH and influence of sample preservation and storage. *Clin. Chem.* **39**, 2522-2526.
- Weeks, W.W., Chaplin, J.F., and Campbell, C.R. (1989). Capillary chromatography: Evaluation of volatiles from flue-cured tobacco varieties. *J. Agric. Food Chem.* **37**, 1038-1045.
- White, P.J., and Hammond, E.G. (1983). Quantification of carbonyl compounds in oxidized fats as trichlorophenylhydrazones. *J. Am. Oil Chem. Sec.* **60**, 1769-1773.
- Williams, D.A. (1971). A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biometrics* **27**, 103-117.
- Williams, D.A. (1972). The comparison of several dose levels with a zero dose control. *Biometrics* **28**, 519-531.
- Witt, K.L., Knapton, A., Wehr, C.M., Hook, G.J., Mirsalis, J., Shelby, M.D., and MacGregor, J.T. (2000). Micronucleated erythrocyte frequency in peripheral blood of B6C3F₁ mice from short-term, prechronic, and chronic studies of the NTP Carcinogenesis Bioassay Program. *Environ. Mol. Mutagen.* **36**, 163-194.
- Witz, G. (1989). Biological interactions of alpha,beta-unsaturated aldehydes. *Free Radic. Biol. Med.* **7**, 333-349.
- Witz, G., Lawrie, N.J., Amoruso, M.A., and Goldstein, B.D. (1987). Inhibition by reactive aldehydes of superoxide anion radical production from stimulated polymorphonuclear leukocytes and pulmonary alveolar macrophages. Effects on cellular sulfhydryl groups and NADPH oxidase activity. *Biochem. Pharmacol.* **36**, 721-726.
- Witz, G., Zhang, Z., and Goldstein, B.D. (1996). Reactive ring-opened metabolites in benzene hematotoxicity. *Environ. Health Perspect.* **104**, 1195-1199.
- Wright, D.H., and Harris, N.D. (1985). Effect of nitrogen and potassium fertilization on tomato flavor. *J. Agric. Food Chem.* **33**, 355-358.

- Yamamoto, F., Kasai, H., Bessho, T., Chung, M.H., Inoue, H., Ohtsuka, E., Hori, T., and Nishimura, S. (1992). Ubiquitous presence in mammalian cells of enzymatic activity specifically cleaving 8-hydroxyguanine-containing DNA. *Jpn. J. Cancer Res.* **83**, 351-357.
- Yoshikawa, T., Minamiyama, Y., Ichikawa, H., Takahashi, S., Naito, Y., and Kondo, M. (1997). Role of lipid peroxidation and antioxidants in gastric mucosal injury induced by the hypoxanthine-xanthine oxidase system in rats. *Free Radic. Biol. Med.* **23**, 243-250.
- Zeiger, E., Haseman, J.K., Shelby, M.D., Margolin, B.H., and Tennant, R.W. (1990). Evaluation of four in vitro genetic toxicity tests for predicting rodent carcinogenicity: Confirmation of earlier results with 41 additional chemicals. *Environ. Mol. Mutagen.* **16** (Suppl. 18), 1-14.
- Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., and Mortelmans, K. (1992). Salmonella mutagenicity tests: V. Results from the testing of 311 chemicals. *Environ. Mol. Mutagen.* **19** (Suppl. 21), 2-141.
- Zeringue, H.J., and McCormick, S.P. (1989). Relationships between cotton leaf-derived volatiles and growth of *A. flavus*. *J. Am. Oil Chem. Soc.* **66**, 581-585.

APPENDIX A
SUMMARY OF LESIONS IN MALE RATS
IN THE 2-YEAR GAVAGE STUDY
OF 2,4-HEXADIENAL

TABLE A1	Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal	72
TABLE A2	Individual Animal Tumor Pathology of Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal	76
TABLE A3	Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal	100
TABLE A4	Historical Incidence of Forestomach Neoplasms in Control Male F344/N Rats	105
TABLE A5	Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal	106

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths	1	2	1	5
Moribund	10	11	13	10
Natural deaths	2	2	3	5
Survivors				
Terminal sacrifice	37	35	33	30
Animals examined microscopically	50	50	50	50
Alimentary System				
Intestine large, colon	(48)	(49)	(50)	(50)
Carcinoma			1 (2%)	
Intestine large, cecum	(50)	(50)	(50)	(50)
Intestine small, duodenum	(50)	(50)	(50)	(50)
Intestine small, jejunum	(49)	(50)	(50)	(47)
Intestine small, ileum	(49)	(49)	(50)	(50)
Liver	(50)	(50)	(50)	(50)
Hepatocellular carcinoma		1 (2%)		
Histiocytic sarcoma	1 (2%)		1 (2%)	1 (2%)
Mesentery	(13)	(8)	(19)	(17)
Hemangiosarcoma				1 (6%)
Oral mucosa	(1)	(1)	(1)	(1)
Squamous cell carcinoma				1 (100%)
Squamous cell papilloma	1 (100%)		1 (100%)	
Pancreas	(50)	(50)	(50)	(50)
Acinus, adenoma	10 (20%)	5 (10%)	9 (18%)	6 (12%)
Salivary glands	(50)	(50)	(50)	(50)
Schwannoma malignant	1 (2%)			
Stomach, forestomach	(50)	(50)	(50)	(50)
Lipoma	1 (2%)			
Squamous cell carcinoma			1 (2%)	2 (4%)
Squamous cell papilloma		3 (6%)	10 (20%)	18 (36%)
Squamous cell papilloma, multiple				11 (22%)
Stomach, glandular	(50)	(50)	(50)	(50)
Tongue	(2)	(1)	(2)	(1)
Squamous cell carcinoma			1 (50%)	
Squamous cell papilloma		1 (100%)		
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Schwannoma malignant				1 (2%)

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Adenoma	1 (2%)			
Adrenal medulla	(50)	(49)	(50)	(49)
Pheochromocytoma malignant		1 (2%)	1 (2%)	4 (8%)
Pheochromocytoma complex		1 (2%)	1 (2%)	
Pheochromocytoma benign	7 (14%)	6 (12%)	7 (14%)	7 (14%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Adenoma	4 (8%)	6 (12%)	5 (10%)	1 (2%)
Carcinoma		2 (4%)		1 (2%)
Pituitary gland	(50)	(50)	(50)	(50)
Pars distalis, adenoma	12 (24%)	16 (32%)	15 (30%)	19 (38%)
Thyroid gland	(50)	(50)	(50)	(50)
Bilateral, C-cell, adenoma		1 (2%)	1 (2%)	
C-cell, adenoma	4 (8%)	4 (8%)	5 (10%)	6 (12%)
C-cell, carcinoma	1 (2%)	1 (2%)		4 (8%)
Follicular cell, carcinoma	1 (2%)		1 (2%)	1 (2%)
General Body System				
Peritoneum	(4)	(1)	(1)	
Genital System				
Preputial gland	(49)	(50)	(50)	(50)
Adenoma	1 (2%)	4 (8%)		1 (2%)
Carcinoma	3 (6%)	3 (6%)	5 (10%)	1 (2%)
Prostate	(49)	(47)	(50)	(50)
Ventral, adenoma	2 (4%)	2 (4%)	3 (6%)	3 (6%)
Seminal vesicle	(50)	(50)	(50)	(50)
Testes	(50)	(50)	(50)	(50)
Bilateral, interstitial cell, adenoma	35 (70%)	36 (72%)	32 (64%)	40 (80%)
Interstitial cell, adenoma	6 (12%)	9 (18%)	13 (26%)	6 (12%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Lymph node	(21)	(27)	(29)	(28)
Deep cervical, carcinoma, metastatic, Zymbal's gland				1 (4%)
Mediastinal, carcinoma, metastatic, thyroid gland		1 (4%)		
Pancreatic, histiocytic sarcoma			1 (3%)	
Lymph node, mandibular	(8)	(1)	(3)	(2)
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Spleen	(50)	(50)	(50)	(50)
Histiocytic sarcoma		1 (2%)		
Pheochromocytoma malignant, metastatic, adrenal medulla				1 (2%)
Thymus	(50)	(49)	(50)	(49)

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Integumentary System				
Mammary gland	(48)	(45)	(46)	(44)
Carcinoma	2 (4%)			
Fibroadenoma	3 (6%)	1 (2%)	1 (2%)	2 (5%)
Skin	(50)	(50)	(50)	(50)
Basal cell adenoma			1 (2%)	
Basal cell carcinoma				1 (2%)
Histiocytic sarcoma	1 (2%)		1 (2%)	1 (2%)
Keratoacanthoma	3 (6%)	2 (4%)	1 (2%)	1 (2%)
Neural crest tumor	1 (2%)			1 (2%)
Squamous cell papilloma	1 (2%)		1 (2%)	
Trichoepithelioma	1 (2%)			
Pinna, neural crest tumor		1 (2%)		1 (2%)
Sebacous gland, adenoma			1 (2%)	1 (2%)
Subcutaneous tissue, fibroma	4 (8%)	3 (6%)	6 (12%)	5 (10%)
Subcutaneous tissue, fibrosarcoma	3 (6%)	1 (2%)	1 (2%)	
Subcutaneous tissue, hemangiopericytoma		1 (2%)		
Subcutaneous tissue, hemangiosarcoma			1 (2%)	
Subcutaneous tissue, lipoma	1 (2%)		1 (2%)	
Subcutaneous tissue, schwannoma malignant		1 (2%)		
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Osteosarcoma		1 (2%)	1 (2%)	1 (2%)
Skeletal muscle	(1)	(6)	(1)	(3)
Sarcoma		1 (17%)		
Nervous System				
Brain	(50)	(50)	(50)	(50)
Astrocytoma malignant				1 (2%)
Spinal cord	(2)	(4)	(1)	(2)
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	1 (2%)		4 (8%)	1 (2%)
Alveolar/bronchiolar carcinoma			3 (6%)	1 (2%)
Carcinoma, metastatic, preputial gland		1 (2%)		
Carcinoma, metastatic, Zymbal's gland	1 (2%)			1 (2%)
Histiocytic sarcoma	1 (2%)		1 (2%)	1 (2%)
Osteosarcoma, metastatic, bone		1 (2%)		
Pheochromocytoma malignant, metastatic, adrenal medulla				1 (2%)
Squamous cell carcinoma			1 (2%)	
Special Senses System				
Zymbal's gland	(2)	(1)		(2)
Adenoma	1 (50%)			
Carcinoma	1 (50%)	1 (100%)		2 (100%)

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Histiocytic sarcoma	1 (2%)			
Renal tubule, carcinoma			1 (2%)	
Transitional epithelium, carcinoma		1 (2%)		
Urinary bladder	(50)	(50)	(50)	(50)
Systemic Lesions				
Multiple organs ^b	(50)	(50)	(50)	(50)
Histiocytic sarcoma	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Leukemia mononuclear	11 (22%)	14 (28%)	9 (18%)	17 (34%)
Mesothelioma malignant	4 (8%)	1 (2%)	1 (2%)	
Neoplasm Summary				
Total animals with primary neoplasms ^c	50	48	50	47
Total primary neoplasms	128	132	147	170
Total animals with benign neoplasms	48	47	49	46
Total benign neoplasms	99	100	117	128
Total animals with malignant neoplasms	21	23	24	30
Total malignant neoplasms	28	31	30	40
Total animals with metastatic neoplasms	1	3	1	2
Total metastatic neoplasms	1	3	3	4
Total animals with uncertain neoplasms- benign or malignant	1	1		2
Total uncertain neoplasms	1	1		2

^a Number of animals examined microscopically at the site and the number of animals with neoplasm

^b Number of animals with any tissue examined microscopically

^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A2
Individual Animal Tumor Pathology of Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal: 22.5 mg/kg

Number of Days on Study	7 7	
	3 3	
	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 2 2	
Carcass ID Number	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0	Total Tissues/Tumors
	5 5 5 7 7 7 7 7 8 8 8 8 9 9 9 0 5 5 6 6 6 7 7 6 6	
	4 5 6 1 5 7 8 9 0 2 3 5 6 8 9 0 7 9 6 7 8 2 4 0 2	
Respiratory System		
Lung	+ +	50
Carcinoma, metastatic, preputial gland		1
Osteosarcoma, metastatic, bone		1
Nose	+ +	50
Trachea	+ +	50
Special Senses System		
Eye		2
Zymbal's gland		1
Carcinoma		1
Urinary System		
Kidney	+ +	50
Transitional epithelium, carcinoma		1
Urinary bladder	+ +	50
Systemic Lesions		
Multiple organs	+ +	50
Histiocytic sarcoma		1
Leukemia mononuclear		14
Mesothelioma malignant		1

TABLE A3
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate ^a	7/50 (14%)	6/49 (12%)	7/50 (14%)	7/49 (14%)
Adjusted rate ^b	15.5%	14.1%	15.9%	17.2%
Terminal rate ^c	5/37 (14%)	6/34 (18%)	5/33 (15%)	5/29 (17%)
First incidence (days) ^d	587	729 (T)	536	691
Poly-3 test	P=0.437	P=0.546N	P=0.593	P=0.529
Adrenal Medulla: Malignant Pheochromocytoma				
Overall rate	0/50 (0%)	1/49 (2%)	1/50 (2%)	4/49 (8%)
Adjusted rate	0.0%	2.4%	2.3%	9.8%
Terminal rate	0/37 (0%)	1/34 (3%)	1/33 (3%)	3/29 (10%)
First incidence (days) ^e	—	729 (T)	729 (T)	496
Poly-3 test	P=0.014	P=0.491	P=0.493	P=0.050
Adrenal Medulla: Benign, Complex, or Malignant Pheochromocytoma				
Overall rate	7/50 (14%)	8/49 (16%)	9/50 (18%)	11/49 (22%)
Adjusted rate	15.5%	18.7%	20.3%	26.6%
Terminal rate	5/37 (14%)	7/34 (21%)	6/33 (18%)	8/29 (28%)
First incidence (days)	587	671	536	496
Poly-3 test	P=0.118	P=0.454	P=0.373	P=0.155
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	1/50 (2%)	0/50 (0%)	4/50 (8%)	1/50 (2%)
Adjusted rate	2.2%	0.0%	9.1%	2.4%
Terminal rate	1/37 (3%)	0/35 (0%)	3/33 (9%)	1/30 (3%)
First incidence (days)	729 (T)	—	364	729 (T)
Poly-3 test	P=0.400	P=0.505N	P=0.173	P=0.744
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	0/50 (0%)	0/50 (0%)	3/50 (6%)	1/50 (2%)
Adjusted rate	0.0%	0.0%	7.0%	2.4%
Terminal rate	0/37 (0%)	0/35 (0%)	3/33 (9%)	1/30 (3%)
First incidence (days)	—	— ^f	729 (T)	729 (T)
Poly-3 test	P=0.214	—	P=0.112	P=0.485
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	1/50 (2%)	0/50 (0%)	6/50 (12%)	2/50 (4%)
Adjusted rate	2.2%	0.0%	13.7%	4.8%
Terminal rate	1/37 (3%)	0/35 (0%)	5/33 (15%)	2/30 (7%)
First incidence (days)	729 (T)	—	364	729 (T)
Poly-3 test	P=0.184	P=0.505N	P=0.053	P=0.473
Mammary Gland: Fibroadenoma				
Overall rate	3/50 (6%)	1/50 (2%)	1/50 (2%)	2/50 (4%)
Adjusted rate	6.7%	2.3%	2.3%	4.8%
Terminal rate	3/37 (8%)	1/35 (3%)	0/33 (0%)	0/30 (0%)
First incidence (days)	729 (T)	729 (T)	620	655
Poly-3 test	P=0.486N	P=0.313N	P=0.315N	P=0.530N
Mammary Gland: Fibroadenoma or Carcinoma				
Overall rate	5/50 (10%)	1/50 (2%)	1/50 (2%)	2/50 (4%)
Adjusted rate	11.2%	2.3%	2.3%	4.8%
Terminal rate	4/37 (11%)	1/35 (3%)	0/33 (0%)	0/30 (0%)
First incidence (days)	704	729 (T)	620	655
Poly-3 test	P=0.199N	P=0.107N	P=0.108N	P=0.244N

TABLE A3
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Pancreas: Adenoma				
Overall rate	10/50 (20%)	5/50 (10%)	9/50 (18%)	6/50 (12%)
Adjusted rate	22.3%	11.5%	20.8%	14.5%
Terminal rate	8/37 (22%)	5/35 (14%)	8/33 (24%)	6/30 (20%)
First incidence (days)	699	729 (T)	686	729 (T)
Poly-3 test	P=0.326N	P=0.140N	P=0.537N	P=0.257N
Pancreatic Islets: Adenoma				
Overall rate	4/50 (8%)	6/50 (12%)	5/50 (10%)	1/50 (2%)
Adjusted rate	8.9%	13.6%	11.6%	2.4%
Terminal rate	3/37 (8%)	3/35 (9%)	5/33 (15%)	1/30 (3%)
First incidence (days)	554	659	729 (T)	729 (T)
Poly-3 test	P=0.141N	P=0.356	P=0.469	P=0.207N
Pancreatic Islets: Adenoma or Carcinoma				
Overall rate	4/50 (8%)	7/50 (14%)	5/50 (10%)	2/50 (4%)
Adjusted rate	8.9%	15.8%	11.6%	4.8%
Terminal rate	3/37 (8%)	4/35 (11%)	5/33 (15%)	2/30 (7%)
First incidence (days)	554	659	729 (T)	729 (T)
Poly-3 test	P=0.226N	P=0.248	P=0.469	P=0.379N
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	12/50 (24%)	16/50 (32%)	15/50 (30%)	19/50 (38%)
Adjusted rate	26.0%	35.3%	33.8%	44.0%
Terminal rate	8/37 (22%)	10/35 (29%)	10/33 (30%)	12/30 (40%)
First incidence (days)	542	537	560	574
Poly-3 test	P=0.055	P=0.227	P=0.277	P=0.055
Preputial Gland: Adenoma				
Overall rate	1/49 (2%)	4/50 (8%)	0/50 (0%)	1/50 (2%)
Adjusted rate	2.3%	9.2%	0.0%	2.4%
Terminal rate	1/37 (3%)	4/35 (11%)	0/33 (0%)	1/30 (3%)
First incidence (days)	729 (T)	729 (T)	—	729 (T)
Poly-3 test	P=0.355N	P=0.177	P=0.503N	P=0.748
Preputial Gland: Carcinoma				
Overall rate	3/49 (6%)	3/50 (6%)	5/50 (10%)	1/50 (2%)
Adjusted rate	6.7%	6.8%	11.5%	2.4%
Terminal rate	1/37 (3%)	1/35 (3%)	3/33 (9%)	0/30 (0%)
First incidence (days)	259	671	620	566
Poly-3 test	P=0.313N	P=0.651	P=0.338	P=0.332N
Preputial Gland: Adenoma or Carcinoma				
Overall rate	4/49 (8%)	7/50 (14%)	5/50 (10%)	2/50 (4%)
Adjusted rate	8.9%	15.9%	11.5%	4.8%
Terminal rate	2/37 (5%)	5/35 (14%)	3/33 (9%)	1/30 (3%)
First incidence (days)	259	671	620	566
Poly-3 test	P=0.218N	P=0.246	P=0.479	P=0.371N
Prostate Gland: Adenoma				
Overall rate	2/49 (4%)	2/47 (4%)	3/50 (6%)	3/50 (6%)
Adjusted rate	4.6%	4.9%	7.0%	7.3%
Terminal rate	2/36 (6%)	2/33 (6%)	3/33 (9%)	3/30 (10%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.352	P=0.671	P=0.494	P=0.475

TABLE A3
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Skin: Keratoacanthoma				
Overall rate	3/50 (6%)	2/50 (4%)	1/50 (2%)	1/50 (2%)
Adjusted rate	6.7%	4.6%	2.3%	2.4%
Terminal rate	3/37 (8%)	2/35 (6%)	1/33 (3%)	1/30 (3%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.214N	P=0.510N	P=0.318N	P=0.333N
Skin: Squamous Cell Papilloma or Keratoacanthoma				
Overall rate	4/50 (8%)	2/50 (4%)	2/50 (4%)	1/50 (2%)
Adjusted rate	9.0%	4.6%	4.7%	2.4%
Terminal rate	4/37 (11%)	2/35 (6%)	2/33 (6%)	1/30 (3%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.151N	P=0.348N	P=0.354N	P=0.202N
Skin: Squamous Cell Papilloma, Keratoacanthoma, Trichoepithelioma, Basal Cell Adenoma, or Basal Cell Carcinoma				
Overall rate	5/50 (10%)	2/50 (4%)	3/50 (6%)	2/50 (4%)
Adjusted rate	11.2%	4.6%	7.0%	4.8%
Terminal rate	5/37 (14%)	2/35 (6%)	3/33 (9%)	2/30 (7%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.233N	P=0.225N	P=0.376N	P=0.248N
Skin (Subcutaneous Tissue): Fibroma				
Overall rate	4/50 (8%)	3/50 (6%)	6/50 (12%)	5/50 (10%)
Adjusted rate	8.9%	6.8%	13.7%	11.9%
Terminal rate	3/37 (8%)	2/35 (6%)	5/33 (15%)	3/30 (10%)
First incidence (days)	587	643	503	636
Poly-3 test	P=0.293	P=0.514N	P=0.349	P=0.454
Skin (Subcutaneous Tissue): Fibrosarcoma				
Overall rate	3/50 (8%)	1/50 (2%)	1/50 (2%)	0/50 (0%)
Adjusted rate	6.7%	2.3%	2.3%	0.0%
Terminal rate	3/37 (8%)	0/35 (0%)	1/33 (3%)	0/30 (0%)
First incidence (days)	729 (T)	704	729 (T)	—
Poly-3 test	P=0.078	P=0.313N	P=0.318	P=0.133
Skin (Subcutaneous Tissue): Fibroma or Fibrosarcoma				
Overall rate	7/50 (14%)	4/50 (8%)	7/50 (14%)	5/50 (10%)
Adjusted rate	15.5%	9.1%	16.0%	11.9%
Terminal rate	6/37 (16%)	2/35 (6%)	6/33 (18%)	3/30 (10%)
First incidence (days)	587	643	503	636
Poly-3 test	P=0.474N	P=0.274N	P=0.589	P=0.431N
Stomach (Forestomach): Squamous Cell Papilloma				
Overall rate	0/50 (0%)	3/50 (6%)	10/50 (20%)	29/50 (58%)
Adjusted rate	0.0%	6.9%	23.2%	67.0%
Terminal rate	0/37 (0%)	3/35 (9%)	9/33 (27%)	23/30 (77%)
First incidence (days)	—	729 (T)	691	574
Poly-3 test	P<0.001	P=0.114	P<0.001	P<0.001
Stomach (Forestomach): Squamous Cell Papilloma or Squamous Cell Carcinoma				
Overall rate	0/50 (0%)	3/50 (6%)	11/50 (22%)	29/50 (58%)
Adjusted rate	0.0%	6.9%	25.5%	67.0%
Terminal rate	0/37 (0%)	3/35 (9%)	10/33 (30%)	23/30 (77%)
First incidence (days)	—	729 (T)	691	574
Poly-3 test	P<0.001	P=0.114	P<0.001	P<0.001

TABLE A3
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Testes: Adenoma				
Overall rate	41/50 (82%)	45/50 (90%)	45/50 (90%)	46/50 (92%)
Adjusted rate	87.1%	96.0%	94.3%	99.2%
Terminal rate	33/37 (89%)	35/35 (100%)	32/33 (97%)	30/30 (100%)
First incidence (days)	479	509	503	496
Poly-3 test	P=0.012	P=0.098	P=0.175	P=0.015
Thyroid Gland (C-cell): Adenoma				
Overall rate	4/50 (8%)	5/50 (10%)	6/50 (12%)	6/50 (12%)
Adjusted rate	9.0%	11.4%	13.8%	14.4%
Terminal rate	4/37 (11%)	4/35 (11%)	4/33 (12%)	5/30 (17%)
First incidence (days)	729 (T)	634	663	620
Poly-3 test	P=0.261	P=0.490	P=0.353	P=0.327
Thyroid Gland (C-cell): Carcinoma				
Overall rate	1/50 (2%)	1/50 (2%)	0/50 (0%)	4/50 (8%)
Adjusted rate	2.2%	2.3%	0.0%	9.6%
Terminal rate	1/37 (3%)	1/35 (3%)	0/33 (0%)	3/30 (10%)
First incidence (days)	729 (T)	729 (T)	—	651
Poly-3 test	P=0.055	P=0.756	P=0.507N	P=0.158
Thyroid Gland (C-cell): Adenoma or Carcinoma				
Overall rate	5/50 (10%)	6/50 (12%)	6/50 (12%)	9/50 (18%)
Adjusted rate	11.2%	13.7%	13.8%	21.4%
Terminal rate	5/37 (14%)	5/35 (14%)	4/33 (12%)	7/30 (23%)
First incidence (days)	729 (T)	634	663	620
Poly-3 test	P=0.115	P=0.489	P=0.482	P=0.158
All Organs: Mononuclear Cell Leukemia				
Overall rate	11/50 (22%)	14/50 (28%)	9/50 (18%)	17/50 (34%)
Adjusted rate	23.5%	30.4%	20.2%	38.5%
Terminal rate	5/37 (14%)	8/35 (23%)	4/33 (12%)	8/30 (27%)
First incidence (days)	554	371	483	350
Poly-3 test	P=0.101	P=0.303	P=0.448N	P=0.091
All Organs: Malignant Mesothelioma				
Overall rate	4/50 (8%)	1/50 (2%)	1/50 (2%)	0/50 (0%)
Adjusted rate	8.9%	2.3%	2.3%	0.0%
Terminal rate	3/37 (8%)	1/35 (3%)	0/33 (0%)	0/30 (0%)
First incidence (days)	699	729 (T)	624	—
Poly-3 test	P=0.036N	P=0.186N	P=0.188N	P=0.071N
All Organs: Benign Neoplasms				
Overall rate	48/50 (96%)	47/50 (94%)	49/50 (98%)	46/50 (92%)
Adjusted rate	97.9%	99.5%	99.4%	99.2%
Terminal rate	36/37 (97%)	35/35 (100%)	33/33 (100%)	30/30 (100%)
First incidence (days)	479	509	364	496
Poly-3 test	P=0.467	P=0.591	P=0.592	P=0.638
All Organs: Malignant Neoplasms				
Overall rate	21/50 (42%)	23/50 (46%)	24/50 (48%)	30/50 (60%)
Adjusted rate	42.8%	49.1%	51.4%	65.7%
Terminal rate	10/37 (27%)	14/35 (40%)	13/33 (39%)	18/30 (60%)
First incidence (days)	259	371	483	350
Poly-3 test	P=0.014	P=0.339	P=0.261	P=0.019

TABLE A3
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
All Organs: Benign or Malignant Neoplasms				
Overall rate	50/50 (100%)	48/50 (96%)	50/50 (100%)	47/50 (94%)
Adjusted rate	100.0%	99.7%	100.0%	99.5%
Terminal rate	37/37 (100%)	35/35 (100%)	33/33 (100%)	30/30 (100%)
First incidence (days)	259	371	364	350
Poly-3 test	P=0.940N	P=1.000N	—	P=1.000N

(T) Terminal sacrifice

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, lung, pancreas, pancreatic islets, pituitary gland, preputial gland, prostate gland, testis, and thyroid gland; for other tissues, denominator is number of animals necropsied.

^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the vehicle control incidence are the P values 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. A negative trend or a lower incidence in a dosed group is indicated by N.

^e Not applicable; no neoplasms in animal group

^f Value of statistic cannot be computed.

TABLE A4
Historical Incidence of Forestomach Neoplasms in Control Male F344/N Rats

Study	Incidence in Controls		
	Squamous Cell Papilloma	Squamous Cell Carcinoma	Squamous Cell Papilloma or Squamous Cell Carcinoma
Historical Incidence in Controls Given NTP-2000 Diet^a			
Citral (feed)	0/100	0/100	0/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	0/50	0/50	0/50
Indium phosphide (inhalation)	0/50	0/50	0/50
60-Hz Magnetic fields (whole body exposure)	1/100	0/100	1/100
Methacrylonitrile (gavage)	0/50	0/50	0/50
Naphthalene (inhalation)	0/49	0/49	0/49
<i>o</i> -Nitrotoluene (feed)	0/60	0/60	0/60
<i>p</i> -Nitrotoluene (feed)	0/50	0/50	0/50
Sodium nitrite (drinking water)	1/50	0/50	1/50
Vanadium pentoxide (inhalation)	0/50	0/50	0/50
Overall Historical Incidence in Controls Given NTP-2000 Diet			
Total	2/609 (0.3%)	0/609	2/609 (0.3%)
Mean ± standard deviation	0.3% ± 0.7%		0.3% ± 0.7%
Range	0%-2%		0%-2%
Historical Incidence in Corn Oil Gavage Controls Given NIH-07 Diet at Southern Research Institute^b			
Salicylazosulfapyridine	1/50	0/50	1/50
Theophylline	0/50	0/50	0/50
Overall Historical Incidence in Corn Oil Gavage Controls Given NIH-07 Diet			
Total	2/402 (0.5%)	0/402	2/402 (0.5%)
Mean ± standard deviation	0.5% ± 0.9%		0.5% ± 0.9%
Range	0%-2%		0%-2%

^a Data as of January 17, 2001

^b Data as of December 21, 1999

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths	1	2	1	5
Moribund	10	11	13	10
Natural deaths	2	2	3	5
Survivors				
Terminal sacrifice	37	35	33	30
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(49)	(48)	(50)
Hemorrhage				1 (2%)
Perforation	1 (2%)	1 (2%)	1 (2%)	
Intestine large, cecum	(50)	(50)	(50)	(50)
Edema			1 (2%)	
Liver	(50)	(50)	(50)	(50)
Basophilic focus	33 (66%)	32 (64%)	31 (62%)	23 (46%)
Clear cell focus	7 (14%)	6 (12%)	8 (16%)	7 (14%)
Degeneration, cystic	1 (2%)	1 (2%)		
Eosinophilic focus	4 (8%)	9 (18%)	10 (20%)	5 (10%)
Hemorrhage	1 (2%)	1 (2%)		
Hepatodiaphragmatic nodule	2 (4%)	7 (14%)	3 (6%)	6 (12%)
Infiltration cellular, mixed cell	5 (10%)	2 (4%)	1 (2%)	3 (6%)
Mixed cell focus	7 (14%)	7 (14%)	10 (20%)	2 (4%)
Bile duct, hyperplasia	25 (50%)	34 (68%)	39 (78%)	15 (30%)
Centrilobular, necrosis	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Hepatocyte, vacuolization cytoplasmic	1 (2%)	9 (18%)	6 (12%)	1 (2%)
Kupffer cell, pigmentation		1 (2%)		1 (2%)
Mesentery	(13)	(8)	(19)	(17)
Accessory spleen	2 (15%)	1 (13%)	1 (5%)	2 (12%)
Hemorrhage	1 (8%)			1 (6%)
Fat, necrosis	11 (85%)	7 (88%)	17 (89%)	14 (82%)
Oral mucosa	(1)	(1)	(1)	(1)
Hyperplasia		1 (100%)		
Pancreas	(50)	(50)	(50)	(50)
Atrophy	16 (32%)	14 (28%)	7 (14%)	13 (26%)
Cyst	2 (4%)	3 (6%)	2 (4%)	2 (4%)
Acinus, cytoplasmic alteration	3 (6%)	2 (4%)	3 (6%)	2 (4%)
Acinus, hyperplasia, focal	17 (34%)	13 (26%)	18 (36%)	12 (24%)
Salivary glands	(50)	(50)	(50)	(50)
Atrophy	3 (6%)	1 (2%)		1 (2%)
Stomach, forestomach	(50)	(50)	(50)	(50)
Edema		2 (4%)	2 (4%)	
Erosion				1 (2%)
Inflammation, chronic active			1 (2%)	6 (12%)
Ulcer	1 (2%)			
Epithelium, cyst			1 (2%)	5 (10%)
Epithelium, hyperplasia	3 (6%)	19 (38%)	42 (84%)	50 (100%)

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

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Alimentary System (continued)				
Stomach, glandular	(50)	(50)	(50)	(50)
Edema			1 (2%)	
Erosion	1 (2%)	1 (2%)		2 (4%)
Ulcer	1 (2%)	1 (2%)	1 (2%)	
Tongue	(2)	(1)	(2)	(1)
Hyperplasia				1 (100%)
Epithelium, hyperplasia	2 (100%)		1 (50%)	
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	36 (72%)	40 (80%)	42 (84%)	40 (80%)
Inflammation, suppurative				1 (2%)
Thrombosis	1 (2%)			
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Accessory adrenal cortical nodule	18 (36%)	21 (42%)	17 (34%)	17 (34%)
Angiectasis	1 (2%)	1 (2%)	1 (2%)	2 (4%)
Degeneration, fatty	12 (24%)	8 (16%)	10 (20%)	11 (22%)
Hyperplasia, diffuse	1 (2%)		2 (4%)	1 (2%)
Hyperplasia, focal	1 (2%)	2 (4%)	6 (12%)	6 (12%)
Hypertrophy, focal	5 (10%)	3 (6%)	5 (10%)	8 (16%)
Metaplasia, osseous		1 (2%)		
Adrenal medulla	(50)	(49)	(50)	(49)
Hyperplasia	7 (14%)	10 (20%)	6 (12%)	5 (10%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Hyperplasia	2 (4%)			
Pituitary gland	(50)	(50)	(50)	(50)
Cyst				1 (2%)
Pars distalis, angiectasis	3 (6%)	4 (8%)	1 (2%)	1 (2%)
Pars distalis, cyst	4 (8%)	1 (2%)	6 (12%)	8 (16%)
Pars distalis, hyperplasia				2 (4%)
Pars distalis, hyperplasia, focal	8 (16%)	8 (16%)	9 (18%)	9 (18%)
Pars intermedia, angiectasis			2 (4%)	2 (4%)
Pars intermedia, cyst			1 (2%)	4 (8%)
Thyroid gland	(50)	(50)	(50)	(50)
C-cell, hyperplasia	26 (52%)	16 (32%)	18 (36%)	10 (20%)
Follicle, cyst	1 (2%)		2 (4%)	
Follicular cell, hyperplasia			1 (2%)	
General Body System				
None				
Genital System				
Epididymis	(50)	(50)	(50)	(50)
Atypia cellular	35 (70%)	36 (72%)	28 (56%)	37 (74%)
Preputial gland	(49)	(50)	(50)	(50)
Cyst	1 (2%)	1 (2%)	1 (2%)	2 (4%)
Inflammation, chronic	21 (43%)	21 (42%)	31 (62%)	24 (48%)

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Genital System (continued)				
Prostate	(49)	(47)	(50)	(50)
Inflammation, chronic	15 (31%)	17 (36%)	17 (34%)	13 (26%)
Epithelium, hyperplasia				3 (6%)
Epithelium, ventral, degeneration	3 (6%)			1 (2%)
Epithelium, ventral, hyperplasia	12 (24%)	9 (19%)	11 (22%)	10 (20%)
Ventral, inflammation	3 (6%)			1 (2%)
Testes	(50)	(50)	(50)	(50)
Germinal epithelium, atrophy	8 (16%)	9 (18%)	11 (22%)	5 (10%)
Interstitial cell, hyperplasia	8 (16%)	7 (14%)	7 (14%)	5 (10%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hyperplasia	4 (8%)	3 (6%)	6 (12%)	4 (8%)
Lymph node	(21)	(27)	(29)	(28)
Deep cervical, hyperplasia, lymphoid				1 (4%)
Iliac, pigmentation		1 (4%)		
Mediastinal, ectasia	1 (5%)			1 (4%)
Mediastinal, hemorrhage	3 (14%)	1 (4%)	4 (14%)	
Mediastinal, hyperplasia, lymphoid	5 (24%)	4 (15%)	4 (14%)	4 (14%)
Mediastinal, pigmentation	3 (14%)		5 (17%)	
Pancreatic, ectasia			1 (3%)	2 (7%)
Pancreatic, hemorrhage	8 (38%)	9 (33%)	11 (38%)	11 (39%)
Pancreatic, hyperplasia, lymphoid	8 (38%)	14 (52%)	15 (52%)	16 (57%)
Pancreatic, pigmentation	12 (57%)	18 (67%)	22 (76%)	23 (82%)
Lymph node, mandibular	(8)	(1)	(3)	(2)
Ectasia	1 (13%)		1 (33%)	
Hyperplasia, lymphoid	1 (13%)			
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Ectasia		2 (4%)		1 (2%)
Hemorrhage	14 (28%)	8 (16%)	5 (10%)	4 (8%)
Hyperplasia, lymphoid	16 (32%)	9 (18%)	22 (44%)	22 (44%)
Pigmentation	43 (86%)	32 (64%)	31 (62%)	21 (42%)
Spleen	(50)	(50)	(50)	(50)
Fibrosis	2 (4%)		1 (2%)	1 (2%)
Hematopoietic cell proliferation	20 (40%)	20 (40%)	22 (44%)	14 (28%)
Pigmentation	7 (14%)	9 (18%)	18 (36%)	20 (40%)
Lymphoid follicle, atrophy				3 (6%)
Lymphoid follicle, hyperplasia		1 (2%)	1 (2%)	1 (2%)
Thymus	(50)	(49)	(50)	(49)
Hemorrhage	1 (2%)	1 (2%)		2 (4%)
Integumentary System				
Mammary gland	(48)	(45)	(46)	(44)
Cyst			1 (2%)	
Hyperplasia	13 (27%)	12 (27%)	14 (30%)	10 (23%)
Skin	(50)	(50)	(50)	(50)
Cyst epithelial inclusion			2 (4%)	2 (4%)
Foreign body	1 (2%)			
Granuloma			1 (2%)	
Hyperkeratosis				1 (2%)
Inflammation, chronic	1 (2%)			
Necrosis	1 (2%)			
Ulcer				2 (4%)
Epidermis, hyperplasia	1 (2%)			1 (2%)

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Cranium, osteopetrosis	1 (2%)			
Nervous System				
Brain	(50)	(50)	(50)	(50)
Compression	7 (14%)	3 (6%)	6 (12%)	3 (6%)
Hemorrhage	1 (2%)		1 (2%)	
Hydrocephalus	3 (6%)	2 (4%)	1 (2%)	3 (6%)
Necrosis	1 (2%)		1 (2%)	
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Congestion	1 (2%)	1 (2%)	2 (4%)	3 (6%)
Edema	3 (6%)	1 (2%)	1 (2%)	5 (10%)
Foreign body	1 (2%)			
Hemorrhage	4 (8%)	3 (6%)	1 (2%)	4 (8%)
Infiltration cellular, histiocyte	33 (66%)	25 (50%)	19 (38%)	27 (54%)
Inflammation, granulomatous				1 (2%)
Inflammation, suppurative		1 (2%)		1 (2%)
Metaplasia, osseous	1 (2%)		2 (4%)	1 (2%)
Alveolar epithelium, hyperplasia	7 (14%)	4 (8%)	5 (10%)	6 (12%)
Alveolar epithelium, hyperplasia, focal			1 (2%)	
Nose	(50)	(50)	(50)	(50)
Foreign body	5 (10%)	6 (12%)	5 (10%)	2 (4%)
Inflammation, chronic	9 (18%)	10 (20%)	12 (24%)	8 (16%)
Olfactory epithelium, atrophy				2 (4%)
Respiratory epithelium, hyperplasia	2 (4%)	7 (14%)	7 (14%)	1 (2%)
Respiratory epithelium, metaplasia, squamous		3 (6%)	2 (4%)	
Trachea	(50)	(50)	(50)	(50)
Epithelium, hyperplasia		1 (2%)		
Special Senses System				
Eye	(1)	(2)		(3)
Cataract	1 (100%)	2 (100%)		2 (67%)
Hemorrhage				1 (33%)
Inflammation, chronic				1 (33%)
Retina, degeneration	1 (100%)	2 (100%)		1 (33%)
Harderian gland			(1)	(1)
Pigmentation			1 (100%)	

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Cyst			1 (2%)	1 (2%)
Glomerulosclerosis				1 (2%)
Hydronephrosis	1 (2%)	1 (2%)		
Infarct		1 (2%)		
Inflammation, chronic	3 (6%)	3 (6%)	2 (4%)	2 (4%)
Nephropathy	28 (56%)	22 (44%)	19 (38%)	19 (38%)
Renal tubule, cytoplasmic alteration				2 (4%)
Renal tubule, dilatation	1 (2%)	1 (2%)	2 (4%)	
Renal tubule, necrosis	1 (2%)	1 (2%)		
Renal tubule, pigmentation			1 (2%)	3 (6%)
Transitional epithelium, hyperplasia		2 (4%)		1 (2%)
Urinary bladder	(50)	(50)	(50)	(50)
Edema		1 (2%)		
Hemorrhage	1 (2%)	2 (4%)		1 (2%)
Inflammation, chronic		1 (2%)		
Inflammation, suppurative	1 (2%)			
Transitional epithelium, hyperplasia		2 (4%)		

APPENDIX B
SUMMARY OF LESIONS IN FEMALE RATS
IN THE 2-YEAR GAVAGE STUDY
OF 2,4-HEXADIENAL

TABLE B1	Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal	113
TABLE B2	Individual Animal Tumor Pathology of Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal	116
TABLE B3	Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal	134
TABLE B4	Historical Incidence of Forestomach Neoplasms in Control Female F344/N Rats	138
TABLE B5	Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal	139

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths		1	1	6
Moribund	12	9	6	9
Natural deaths	1	1	2	4
Survivors				
Died last week of study		1		
Terminal sacrifice	37	38	41	31
Animals examined microscopically	50	50	50	50
Alimentary System				
Intestine large, colon	(50)	(50)	(49)	(50)
Intestine large, rectum	(49)	(50)	(50)	(49)
Intestine large, cecum	(50)	(50)	(50)	(50)
Intestine small, duodenum	(50)	(49)	(50)	(50)
Leiomyoma			1 (2%)	
Liver	(50)	(50)	(50)	(50)
Mesentery	(13)	(16)	(19)	(15)
Oral mucosa	(1)			
Squamous cell papilloma	1 (100%)			
Pancreas	(50)	(50)	(50)	(50)
Acinus, adenoma	1 (2%)			1 (2%)
Salivary glands	(50)	(50)	(50)	(50)
Stomach, forestomach	(50)	(49)	(50)	(50)
Squamous cell papilloma		1 (2%)	4 (8%)	16 (32%)
Squamous cell papilloma, multiple			1 (2%)	1 (2%)
Stomach, glandular	(50)	(49)	(50)	(50)
Tongue	(1)		(2)	(1)
Squamous cell carcinoma			1 (50%)	
Squamous cell papilloma			1 (50%)	1 (100%)
Tooth	(1)			
Odontogenic tumor	1 (100%)			
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Adenoma	1 (2%)	1 (2%)		
Adrenal medulla	(50)	(49)	(50)	(50)
Pheochromocytoma malignant	1 (2%)			1 (2%)
Pheochromocytoma complex				1 (2%)
Pheochromocytoma benign	1 (2%)	5 (10%)	5 (10%)	1 (2%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Adenoma	1 (2%)	1 (2%)	1 (2%)	
Carcinoma		1 (2%)		
Pituitary gland	(50)	(50)	(50)	(50)
Pars distalis, adenoma	19 (38%)	26 (52%)	22 (44%)	20 (40%)
Pars intermedia, adenoma	1 (2%)		1 (2%)	

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Endocrine System (continued)				
Thyroid gland	(50)	(50)	(50)	(50)
Bilateral, C-cell, adenoma	1 (2%)			
C-cell, adenoma	11 (22%)	6 (12%)	7 (14%)	6 (12%)
C-cell, carcinoma	1 (2%)	1 (2%)		1 (2%)
Follicular cell, carcinoma	1 (2%)		1 (2%)	
General Body System				
Peritoneum	(1)			
Genital System				
Clitoral gland	(50)	(50)	(50)	(50)
Adenoma	5 (10%)	8 (16%)	11 (22%)	7 (14%)
Carcinoma	2 (4%)	1 (2%)	3 (6%)	3 (6%)
Ovary	(50)	(50)	(50)	(50)
Thecoma malignant		1 (2%)		
Uterus	(50)	(50)	(50)	(50)
Adenoma				1 (2%)
Carcinoma	1 (2%)			
Deciduoma benign				1 (2%)
Polyp stromal	14 (28%)	11 (22%)	9 (18%)	13 (26%)
Sarcoma stromal			1 (2%)	
Vagina	(1)	(3)	(1)	(1)
Sarcoma		1 (33%)		
Sarcoma stromal, metastatic, uterus			1 (100%)	
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Lymph node	(29)	(30)	(30)	(38)
Fibrosarcoma, metastatic, skin				1 (3%)
Deep cervical, carcinoma, metastatic, thyroid gland		1 (3%)		
Lymph node, mandibular	(6)	(2)	(5)	(1)
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Spleen	(50)	(50)	(50)	(50)
Thymus	(48)	(47)	(50)	(50)
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Adenoma	2 (4%)			
Carcinoma	2 (4%)	3 (6%)		1 (2%)
Fibroadenoma	22 (44%)	28 (56%)	22 (44%)	18 (36%)
Schwannoma benign				1 (2%)
Skin	(49)	(49)	(50)	(50)
Basal cell adenoma			1 (2%)	
Fibrous histiocytoma			1 (2%)	
Keratoacanthoma	1 (2%)			
Neural crest tumor			1 (2%)	
Pinna, neural crest tumor			1 (2%)	
Subcutaneous tissue, fibroma	2 (4%)		1 (2%)	2 (4%)
Subcutaneous tissue, fibrosarcoma		1 (2%)		1 (2%)
Subcutaneous tissue, schwannoma malignant			2 (4%)	

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Osteosarcoma		1 (2%)		
Nervous System				
Brain	(50)	(50)	(50)	(50)
Astrocytoma malignant	2 (4%)			
Glioma malignant			1 (2%)	
Oligodendroglioma malignant		1 (2%)		
Spinal cord	(3)	(1)		
Astrocytoma malignant, metastatic, brain	1 (33%)			
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	3 (6%)	1 (2%)	1 (2%)	
Alveolar/bronchiolar carcinoma			1 (2%)	
Special Senses System				
Zymbal's gland		(1)		
Carcinoma		1 (100%)		
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Renal tubule, carcinoma	1 (2%)			
Urinary bladder	(50)	(50)	(50)	(50)
Papilloma		1 (2%)		
Systemic Lesions				
Multiple organs ^b	(50)	(50)	(50)	(50)
Leukemia mononuclear	10 (20%)	11 (22%)	5 (10%)	16 (32%)
Mesothelioma malignant	1 (2%)			
Neoplasm Summary				
Total animals with primary neoplasms ^c	48	48	48	44
Total primary neoplasms	109	112	106	113
Total animals with benign neoplasms	45	45	43	44
Total benign neoplasms	86	89	88	89
Total animals with malignant neoplasms	18	19	14	21
Total malignant neoplasms	22	23	16	24
Total animals with metastatic neoplasms	1	1	1	1
Total metastatic neoplasms	1	1	1	1
Total animals with uncertain neoplasms-				
benign or malignant	1		2	
Total uncertain neoplasms	1		2	

^a Number of animals examined microscopically at the site and the number of animals with neoplasm

^b Number of animals with any tissue examined microscopically

^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE B2
Individual Animal Tumor Pathology of Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal: Vehicle Control

Number of Days on Study	7 7	
	3 3	
	3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 4 4 4 4 4 5 5 5 5 5	
Carcass ID Number	2 2	Total
	2 2 2 2 2 3 3 3 3 3 3 4 4 4 0 4 4 4 5 0 0 1 1 1 2	Tissues/
	3 4 7 8 9 0 1 2 3 4 5 2 3 4 2 6 8 9 0 4 5 7 8 9 0	Tumors
Special Senses System		
Eye		1
Urinary System		
Kidney	+ +	50
Renal tubule, carcinoma		1
Urinary bladder	+ +	50
Systemic Lesions		
Multiple organs	+ +	50
Leukemia mononuclear		10
Mesothelioma malignant		1

TABLE B2
Individual Animal Tumor Pathology of Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal: 90 mg/kg

Number of Days on Study	3	3	3	3	3	4	4	5	5	6	6	6	6	6	6	6	6	6	7	7	7	7	7	7	7		
	4	6	6	6	6	0	4	3	6	0	3	3	3	5	6	9	9	9	2	2	2	2	2	2	2		
	6	3	4	4	4	5	5	6	0	1	1	6	8	1	6	0	1	1	1	9	9	9	9	9	9		
Carcass ID Number	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3		
	9	6	6	8	9	5	8	6	9	9	6	9	7	5	8	7	5	7	8	5	5	5	6	8	8		
	5	9	7	1	7	5	4	6	4	2	5	6	1	3	5	6	8	5	2	6	7	9	0	6	7		
Hematopoietic System																											
Bone marrow	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Lymph node				+				+	+	+	+			+	+			+	+	+	+		+	+	+	+	
Fibrosarcoma, metastatic, skin																											
Lymph node, mandibular	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	
Lymph node, mesenteric	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Spleen	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Thymus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Integumentary System																											
Mammary gland	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Carcinoma																											
Fibroadenoma								X			X			X	X	X			X		X	X			X		
Schwannoma benign																											
Skin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Subcutaneous tissue, fibroma																											
Subcutaneous tissue, fibrosarcoma																											
Musculoskeletal System																											
Bone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Nervous System																											
Brain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Respiratory System																											
Lung	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Nose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Trachea	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Special Senses System																											
Eye																											
Urinary System																											
Kidney	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Urinary bladder	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Systemic Lesions																											
Multiple organs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Leukemia mononuclear												X	X		X	X	X	X							X		

TABLE B3
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate ^a	1/50 (2%)	5/49 (10%)	5/50 (10%)	1/50 (2%)
Adjusted rate ^b	2.2%	11.1%	10.9%	2.4%
Terminal rate ^c	1/37 (3%)	4/38 (11%)	4/41 (10%)	0/31 (0%)
First incidence (days) ^d	729 (T)	719	659	536
Poly-3 test	P=0.493N	P=0.099	P=0.103	P=0.738
Adrenal Medulla: Benign, Complex, or Malignant Pheochromocytoma				
Overall rate	2/50 (4%)	5/49 (10%)	5/50 (10%)	3/50 (6%)
Adjusted rate	4.4%	11.1%	10.9%	7.3%
Terminal rate	2/37 (5%)	4/38 (11%)	4/41 (10%)	1/31 (3%)
First incidence (days)	729 (T)	719	659	536
Poly-3 test	P=0.446	P=0.213	P=0.219	P=0.457
Clitoral Gland: Adenoma				
Overall rate	5/50 (10%)	8/50 (16%)	11/50 (22%)	7/50 (14%)
Adjusted rate	11.0%	17.3%	24.0%	17.3%
Terminal rate	5/37 (14%)	7/39 (18%)	10/41 (24%)	6/31 (19%)
First incidence (days)	729 (T)	610	602	691
Poly-3 test	P=0.242	P=0.290	P=0.087	P=0.302
Clitoral Gland: Carcinoma				
Overall rate	2/50 (4%)	1/50 (2%)	3/50 (6%)	3/50 (6%)
Adjusted rate	4.4%	2.2%	6.6%	7.4%
Terminal rate	0/37 (0%)	1/39 (3%)	3/41 (7%)	2/31 (7%)
First incidence (days)	704	729 (T)	729 (T)	691
Poly-3 test	P=0.251	P=0.497N	P=0.500	P=0.448
Clitoral Gland: Adenoma or Carcinoma				
Overall rate	7/50 (14%)	9/50 (18%)	14/50 (28%)	10/50 (20%)
Adjusted rate	15.4%	19.4%	30.5%	24.6%
Terminal rate	5/37 (14%)	8/39 (21%)	13/41 (32%)	8/31 (26%)
First incidence (days)	704	610	602	691
Poly-3 test	P=0.135	P=0.408	P=0.069	P=0.212
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	3/50 (6%)	1/50 (2%)	1/50 (2%)	0/50 (0%)
Adjusted rate	6.6%	2.2%	2.2%	0.0%
Terminal rate	2/37 (5%)	1/39 (3%)	1/41 (2%)	0/31 (0%)
First incidence (days)	704	729 (T)	729 (T)	— ^e
Poly-3 test	P=0.082N	P=0.302N	P=0.305N	P=0.141N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	3/50 (6%)	1/50 (2%)	2/50 (4%)	0/50 (0%)
Adjusted rate	6.6%	2.2%	4.4%	0.0%
Terminal rate	2/37 (5%)	1/39 (3%)	2/41 (5%)	0/31 (0%)
First incidence (days)	704	729 (T)	729 (T)	—
Poly-3 test	P=0.123N	P=0.302N	P=0.500N	P=0.141N
Mammary Gland: Fibroadenoma				
Overall rate	22/50 (44%)	28/50 (56%)	22/50 (44%)	18/50 (36%)
Adjusted rate	46.6%	59.3%	48.1%	42.5%
Terminal rate	16/37 (43%)	23/39 (59%)	21/41 (51%)	12/31 (39%)
First incidence (days)	511	544	659	445
Poly-3 test	P=0.249N	P=0.148	P=0.524	P=0.432N

TABLE B3
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Mammary Gland: Fibroadenoma or Adenoma				
Overall rate	23/50 (46%)	28/50 (56%)	22/50 (44%)	18/50 (36%)
Adjusted rate	48.7%	59.3%	48.1%	42.5%
Terminal rate	17/37 (46%)	23/39 (59%)	21/41 (51%)	12/31 (39%)
First incidence (days)	511	544	659	445
Poly-3 test	P=0.197N	P=0.201	P=0.560N	P=0.355N
Mammary Gland: Carcinoma				
Overall rate	2/50 (4%)	3/50 (6%)	0/50 (0%)	1/50 (2%)
Adjusted rate	4.4%	6.5%	0.0%	2.5%
Terminal rate	0/37 (0%)	2/39 (5%)	0/41 (0%)	1/31 (3%)
First incidence (days)	704	719	—	729 (T)
Poly-3 test	P=0.260N	P=0.505	P=0.237N	P=0.542N
Mammary Gland: Adenoma or Carcinoma				
Overall rate	3/50 (6%)	3/50 (6%)	0/50 (0%)	1/50 (2%)
Adjusted rate	6.6%	6.5%	0.0%	2.5%
Terminal rate	1/37 (3%)	2/39 (5%)	0/41 (0%)	1/31 (3%)
First incidence (days)	704	719	—	729 (T)
Poly-3 test	P=0.147N	P=0.658N	P=0.119N	P=0.349N
Mammary Gland: Fibroadenoma, Adenoma, or Carcinoma				
Overall rate	24/50 (48%)	30/50 (60%)	22/50 (44%)	18/50 (36%)
Adjusted rate	50.7%	63.6%	48.1%	42.5%
Terminal rate	17/37 (46%)	25/39 (64%)	21/41 (51%)	12/31 (39%)
First incidence (days)	511	544	659	445
Poly-3 test	P=0.123N	P=0.143	P=0.483N	P=0.287N
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	19/50 (38%)	26/50 (52%)	22/50 (44%)	20/50 (40%)
Adjusted rate	40.6%	55.2%	47.2%	47.8%
Terminal rate	12/37 (32%)	21/39 (54%)	19/41 (46%)	15/31 (48%)
First incidence (days)	560	610	602	560
Poly-3 test	P=0.399	P=0.111	P=0.330	P=0.318
Skin (Subcutaneous Tissue): Fibroma or Fibrosarcoma				
Overall rate	2/50 (4%)	1/50 (2%)	2/50 (4%)	3/50 (6%)
Adjusted rate	4.4%	2.2%	4.4%	7.3%
Terminal rate	1/37 (3%)	0/39 (0%)	1/41 (2%)	2/31 (7%)
First incidence (days)	664	722	659	536
Poly-3 test	P=0.274	P=0.497N	P=0.693N	P=0.452
Stomach (Forestomach): Squamous Cell Papilloma				
Overall rate	0/50 (0%)	1/50 (2%)	5/50 (10%)	17/50 (34%)
Adjusted rate	0.0%	2.2%	11.0%	41.9%
Terminal rate	0/37 (0%)	1/39 (3%)	5/41 (12%)	15/31 (48%)
First incidence (days)	—	729 (T)	729 (T)	691
Poly-3 test	P<0.001	P=0.503	P=0.031	P<0.001
Thyroid Gland (C-cell): Adenoma				
Overall rate	12/50 (24%)	6/50 (12%)	7/50 (14%)	6/50 (12%)
Adjusted rate	26.5%	13.0%	15.4%	14.8%
Terminal rate	12/37 (32%)	4/39 (10%)	7/41 (17%)	5/31 (16%)
First incidence (days)	729 (T)	671	729 (T)	690
Poly-3 test	P=0.155N	P=0.086N	P=0.149N	P=0.144N

TABLE B3
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Thyroid Gland (C-cell): Adenoma or Carcinoma				
Overall rate	13/50 (26%)	7/50 (14%)	7/50 (14%)	7/50 (14%)
Adjusted rate	28.7%	15.2%	15.4%	17.3%
Terminal rate	13/37 (35%)	5/39 (13%)	7/41 (17%)	6/31 (19%)
First incidence (days)	729 (T)	671	729 (T)	690
Poly-3 test	P=0.159N	P=0.093N	P=0.100N	P=0.160N
Uterus: Stromal Polyp				
Overall rate	14/50 (28%)	11/50 (22%)	9/50 (18%)	13/50 (26%)
Adjusted rate	29.9%	23.2%	19.2%	30.9%
Terminal rate	11/37 (30%)	8/39 (21%)	7/41 (17%)	11/31 (36%)
First incidence (days)	445	346	476	363
Poly-3 test	P=0.491	P=0.306N	P=0.168N	P=0.553
Uterus: Stromal Polyp or Stromal Sarcoma				
Overall rate	14/50 (28%)	11/50 (22%)	10/50 (20%)	13/50 (26%)
Adjusted rate	29.9%	23.2%	21.2%	30.9%
Terminal rate	11/37 (30%)	8/39 (21%)	7/41 (17%)	11/31 (36%)
First incidence (days)	445	346	476	363
Poly-3 test	P=0.475	P=0.306N	P=0.231N	P=0.553
All Organs: Mononuclear Cell Leukemia				
Overall rate	10/50 (20%)	11/50 (22%)	5/50 (10%)	16/50 (32%)
Adjusted rate	21.5%	23.8%	10.8%	38.2%
Terminal rate	5/37 (14%)	8/39 (21%)	3/41 (7%)	10/31 (32%)
First incidence (days)	554	671	536	601
Poly-3 test	P=0.069	P=0.495	P=0.131N	P=0.067
All Organs: Benign Neoplasms				
Overall rate	45/50 (90%)	45/50 (90%)	43/50 (86%)	44/50 (88%)
Adjusted rate	92.8%	91.6%	89.8%	95.3%
Terminal rate	34/37 (92%)	35/39 (90%)	38/41 (93%)	30/31 (97%)
First incidence (days)	445	346	476	363
Poly-3 test	P=0.374	P=0.567N	P=0.433N	P=0.461
All Organs: Malignant Neoplasms				
Overall rate	18/50 (36%)	19/50 (38%)	14/50 (28%)	21/50 (42%)
Adjusted rate	38.1%	39.7%	29.0%	49.1%
Terminal rate	10/37 (27%)	12/39 (31%)	8/41 (20%)	13/31 (42%)
First incidence (days)	511	347	476	536
Poly-3 test	P=0.223	P=0.519	P=0.234N	P=0.200

TABLE B3
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
All Organs: Benign or Malignant Neoplasms				
Overall rate	47/50 (94%)	48/50 (96%)	48/50 (96%)	44/50 (88%)
Adjusted rate	95.8%	96.0%	97.4%	95.3%
Terminal rate	35/37 (95%)	37/39 (95%)	40/41 (98%)	30/31 (97%)
First incidence (days)	445	346	476	363
Poly-3 test	P=0.571N	P=0.676	P=0.551	P=0.663N

(T) Terminal sacrifice

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, clitoral gland, lung, pituitary gland, and thyroid gland; for other tissues, denominator is number of animals necropsied.

^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the vehicle control incidence are the P values 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. A negative trend or a lower incidence in a dosed group is indicated by N.

^e Not applicable; no neoplasms in animal group

TABLE B4
Historical Incidence of Forestomach Neoplasms in Control Female F344/N Rats

Study	Incidence in Controls		
	Squamous Cell Papilloma	Squamous Cell Carcinoma	Squamous Cell Papilloma or Squamous Cell Carcinoma
Historical Incidence in Controls Given NTP-2000 Diet^a			
Citral (feed)	0/100	0/100	0/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	0/50	0/50	0/50
Indium phosphide (inhalation)	0/50	0/50	0/50
60-Hz Magnetic fields (whole body exposure)	0/100	0/100	0/100
Methacrylonitrile (gavage)	0/50	0/50	0/50
Naphthalene (inhalation)	0/49	0/49	0/49
<i>o</i> -Nitrotoluene (feed)	0/60	0/60	0/60
<i>p</i> -Nitrotoluene (feed)	0/50	0/50	0/50
Riddelliine (gavage)	0/50	0/50	0/50
Sodium nitrite (drinking water)	0/50	0/50	0/50
Vanadium pentoxide (inhalation)	0/50	0/50	0/50
Overall Historical Incidence in Controls Given NTP-2000 Diet			
Total	0/659	0/659	0/659
Historical Incidence in Corn Oil Gavage Controls Given NIH-07 Diet at Southern Research Institute^b			
Salicylazosulfapyridine	0/50	0/50	0/50
Theophylline	0/50	0/50	0/50
Overall Historical Incidence in Corn Oil Gavage Controls Given NIH-07 Diet			
Total	2/401 (0.5%)	0/401	2/401 (0.5%)
Mean ± standard deviation	0.5% ± 0.9%		0.5% ± 0.9%
Range	0%-2%		0%-2%

^a Data as of January 17, 2001

^b Data as of December 21, 1999

TABLE B5
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths		1	1	6
Moribund	12	9	6	9
Natural deaths	1	1	2	4
Survivors				
Died last week of study		1		
Terminal sacrifice	37	38	41	31
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(48)	(47)	(50)	(48)
Inflammation, chronic				1 (2%)
Perforation				1 (2%)
Intestine large, cecum	(50)	(50)	(50)	(50)
Edema		1 (2%)		2 (4%)
Inflammation, chronic active		1 (2%)		
Liver	(50)	(50)	(50)	(50)
Angiectasis	1 (2%)	1 (2%)	2 (4%)	2 (4%)
Basophilic focus	47 (94%)	49 (98%)	48 (96%)	45 (90%)
Clear cell focus	7 (14%)	8 (16%)	11 (22%)	4 (8%)
Eosinophilic focus	9 (18%)	10 (20%)	6 (12%)	4 (8%)
Hepatodiaphragmatic nodule	9 (18%)	6 (12%)	6 (12%)	4 (8%)
Infiltration cellular, mixed cell	15 (30%)	10 (20%)	10 (20%)	17 (34%)
Mixed cell focus	6 (12%)	12 (24%)	6 (12%)	9 (18%)
Bile duct, hyperplasia	5 (10%)			1 (2%)
Hepatocyte, cytomegaly	2 (4%)			1 (2%)
Hepatocyte, necrosis	2 (4%)		1 (2%)	3 (6%)
Hepatocyte, vacuolization cytoplasmic	1 (2%)	3 (6%)		1 (2%)
Kupffer cell, pigmentation	2 (4%)	3 (6%)		1 (2%)
Mesentery	(13)	(16)	(19)	(15)
Fat, necrosis	13 (100%)	16 (100%)	18 (95%)	15 (100%)
Pancreas	(50)	(50)	(50)	(50)
Atrophy	7 (14%)	12 (24%)	8 (16%)	8 (16%)
Cyst	2 (4%)	4 (8%)	1 (2%)	3 (6%)
Acinus, cytoplasmic alteration	1 (2%)	2 (4%)		2 (4%)
Acinus, hyperplasia, focal	3 (6%)	2 (4%)	2 (4%)	1 (2%)
Salivary glands	(50)	(50)	(50)	(50)
Atrophy		2 (4%)	5 (10%)	4 (8%)
Necrosis		1 (2%)		
Stomach, forestomach	(50)	(49)	(50)	(50)
Edema	1 (2%)	1 (2%)		2 (4%)
Erosion	1 (2%)			
Inflammation, chronic active			1 (2%)	2 (4%)
Ulcer	1 (2%)			1 (2%)
Epithelium, cyst				1 (2%)
Epithelium, hyperplasia	2 (4%)	16 (33%)	37 (74%)	41 (82%)
Stomach, glandular	(50)	(49)	(50)	(50)
Edema	1 (2%)			
Erosion				1 (2%)
Ulcer			1 (2%)	1 (2%)
Tongue	(1)		(2)	(1)
Hyperplasia	1 (100%)			

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

TABLE B5
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	20 (40%)	20 (40%)	17 (34%)	13 (26%)
Myocardium, necrosis		1 (2%)		
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Accessory adrenal cortical nodule	13 (26%)	13 (26%)	13 (26%)	13 (26%)
Angiectasis	3 (6%)	2 (4%)	1 (2%)	2 (4%)
Degeneration, fatty	12 (24%)	11 (22%)	11 (22%)	11 (22%)
Hyperplasia, diffuse	2 (4%)		1 (2%)	1 (2%)
Hyperplasia, focal	3 (6%)	6 (12%)	5 (10%)	4 (8%)
Hypertrophy, focal	9 (18%)	9 (18%)	8 (16%)	5 (10%)
Necrosis				2 (4%)
Adrenal medulla	(50)	(49)	(50)	(50)
Hyperplasia	3 (6%)	1 (2%)	1 (2%)	1 (2%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Hyperplasia	1 (2%)	2 (4%)	1 (2%)	
Pituitary gland	(50)	(50)	(50)	(50)
Pars distalis, angiectasis	9 (18%)	3 (6%)	5 (10%)	2 (4%)
Pars distalis, cyst	22 (44%)	14 (28%)	12 (24%)	15 (30%)
Pars distalis, hyperplasia		3 (6%)	1 (2%)	
Pars distalis, hyperplasia, focal	13 (26%)	8 (16%)	7 (14%)	9 (18%)
Pars intermedia, angiectasis			1 (2%)	
Pars intermedia, cyst		1 (2%)		
Thyroid gland	(50)	(50)	(50)	(50)
Ultimobranchial cyst		2 (4%)		2 (4%)
C-cell, hyperplasia	24 (48%)	22 (44%)	26 (52%)	14 (28%)
Follicle, cyst		1 (2%)	1 (2%)	
Follicular cell, hyperplasia				1 (2%)
General Body System				
None				
Genital System				
Clitoral gland	(50)	(50)	(50)	(50)
Cyst	8 (16%)	6 (12%)	2 (4%)	2 (4%)
Hyperplasia	1 (2%)			1 (2%)
Inflammation, chronic	1 (2%)	6 (12%)	3 (6%)	5 (10%)
Ovary	(50)	(50)	(50)	(50)
Cyst	12 (24%)	7 (14%)	8 (16%)	9 (18%)
Uterus	(50)	(50)	(50)	(50)
Hydrometra	2 (4%)	7 (14%)	6 (12%)	2 (4%)
Hyperplasia, cystic	3 (6%)	4 (8%)	4 (8%)	5 (10%)
Inflammation, chronic			1 (2%)	2 (4%)

TABLE B5
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hyperplasia	2 (4%)	5 (10%)	5 (10%)	2 (4%)
Infiltration cellular, histiocyte		1 (2%)		
Lymph node	(29)	(30)	(30)	(38)
Deep cervical, hemorrhage	1 (3%)	1 (3%)	1 (3%)	1 (3%)
Deep cervical, hyperplasia, lymphoid				1 (3%)
Deep cervical, pigmentation	1 (3%)	1 (3%)	1 (3%)	1 (3%)
Mediastinal, hemorrhage		3 (10%)	4 (13%)	2 (5%)
Mediastinal, hyperplasia, lymphoid	5 (17%)	8 (27%)	10 (33%)	3 (8%)
Mediastinal, pigmentation	4 (14%)	8 (27%)	7 (23%)	3 (8%)
Pancreatic, hemorrhage	8 (28%)	8 (27%)	13 (43%)	17 (45%)
Pancreatic, hyperplasia	1 (3%)			
Pancreatic, hyperplasia, lymphoid	17 (59%)	24 (80%)	18 (60%)	28 (74%)
Pancreatic, pigmentation	24 (83%)	25 (83%)	24 (80%)	32 (84%)
Lymph node, mandibular	(6)	(2)	(5)	(1)
Ectasia	2 (33%)	1 (50%)	2 (40%)	
Hyperplasia, lymphoid	1 (17%)			
Pigmentation	3 (50%)	1 (50%)		
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Hemorrhage	10 (20%)	6 (12%)	3 (6%)	4 (8%)
Hyperplasia, lymphoid	9 (18%)	17 (34%)	15 (30%)	13 (26%)
Pigmentation	42 (84%)	42 (84%)	33 (66%)	34 (68%)
Spleen	(50)	(50)	(50)	(50)
Fibrosis				2 (4%)
Hematopoietic cell proliferation	34 (68%)	26 (52%)	29 (58%)	21 (42%)
Infiltration cellular, mixed cell				1 (2%)
Pigmentation	29 (58%)	34 (68%)	35 (70%)	36 (72%)
Lymphoid follicle, atrophy				4 (8%)
Lymphoid follicle, hyperplasia				1 (2%)
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Hyperplasia	43 (86%)	47 (94%)	48 (96%)	40 (80%)
Hyperplasia, lobular	2 (4%)			
Skin	(49)	(49)	(50)	(50)
Inflammation, chronic				1 (2%)
Subcutaneous tissue, edema				1 (2%)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Femur, osteopetrosis	2 (4%)			
Nervous System				
Brain	(50)	(50)	(50)	(50)
Compression	7 (14%)	12 (24%)	6 (12%)	8 (16%)
Hydrocephalus	3 (6%)	8 (16%)	2 (4%)	1 (2%)

TABLE B5
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	22.5 mg/kg	45 mg/kg	90 mg/kg
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Congestion	1 (2%)	2 (4%)	2 (4%)	7 (14%)
Edema		2 (4%)	3 (6%)	7 (14%)
Foreign body		1 (2%)	1 (2%)	
Hemorrhage			1 (2%)	4 (8%)
Infiltration cellular, histiocyte	41 (82%)	41 (82%)	41 (82%)	43 (86%)
Inflammation, granulomatous		1 (2%)	1 (2%)	
Metaplasia, osseous	1 (2%)	2 (4%)		
Alveolar epithelium, hyperplasia	2 (4%)	6 (12%)	7 (14%)	5 (10%)
Alveolar epithelium, hyperplasia, multifocal	1 (2%)			
Nose	(50)	(50)	(50)	(50)
Foreign body	2 (4%)		3 (6%)	2 (4%)
Inflammation, chronic	4 (8%)	3 (6%)	4 (8%)	4 (8%)
Respiratory epithelium, hyperplasia	3 (6%)	1 (2%)	3 (6%)	
Special Senses System				
Eye	(1)		(3)	(1)
Cataract	1 (100%)		3 (100%)	1 (100%)
Retina, degeneration	1 (100%)		3 (100%)	1 (100%)
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Cyst		1 (2%)		1 (2%)
Hydronephrosis				2 (4%)
Inflammation, chronic		2 (4%)		
Nephropathy	2 (4%)	6 (12%)	6 (12%)	6 (12%)
Renal tubule, cytoplasmic alteration	3 (6%)	4 (8%)	5 (10%)	4 (8%)
Renal tubule, dilatation			1 (2%)	
Renal tubule, infarct				1 (2%)
Renal tubule, necrosis		1 (2%)		2 (4%)
Renal tubule, pigmentation	4 (8%)	2 (4%)	3 (6%)	8 (16%)
Transitional epithelium, hyperplasia		1 (2%)	1 (2%)	1 (2%)
Urinary bladder	(50)	(50)	(50)	(50)
Inflammation, chronic		1 (2%)		
Transitional epithelium, hyperplasia		1 (2%)		

APPENDIX C
SUMMARY OF LESIONS IN MALE MICE
IN THE 2-YEAR GAVAGE STUDY
OF 2,4-HEXADIENAL

TABLE C1	Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal	144
TABLE C2	Individual Animal Tumor Pathology of Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal	148
TABLE C3	Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal	172
TABLE C4a	Historical Incidence of Forestomach Neoplasms in Control Male B6C3F₁ Mice	176
TABLE C4b	Historical Incidence of Oral Cavity (Tongue) Neoplasms in Control Male B6C3F₁ Mice	177
TABLE C5	Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal	178

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	3	3	3	8
Natural deaths	3	8	3	3
Survivors				
Died last week of study				1
Terminal sacrifice	44	39	44	38
Animals examined microscopically	50	50	50	50
Alimentary System				
Intestine small, duodenum	(47)	(47)	(48)	(49)
Carcinoma		1 (2%)		
Polyp adenomatous		1 (2%)		
Intestine small, jejunum	(47)	(45)	(49)	(48)
Carcinoma	1 (2%)		1 (2%)	3 (6%)
Intestine small, ileum	(47)	(45)	(47)	(48)
Carcinoma			1 (2%)	1 (2%)
Liver	(50)	(50)	(50)	(50)
Cholangiocarcinoma			1 (2%)	1 (2%)
Hemangiosarcoma	1 (2%)	2 (4%)	1 (2%)	2 (4%)
Hemangiosarcoma, multiple			1 (2%)	
Hepatoblastoma	2 (4%)	3 (6%)		2 (4%)
Hepatocellular carcinoma	5 (10%)	6 (12%)	6 (12%)	12 (24%)
Hepatocellular carcinoma, multiple	3 (6%)	5 (10%)	1 (2%)	2 (4%)
Hepatocellular adenoma	15 (30%)	18 (36%)	14 (28%)	12 (24%)
Hepatocellular adenoma, multiple	8 (16%)	12 (24%)	16 (32%)	13 (26%)
Histiocytic sarcoma			1 (2%)	
Mesentery	(25)	(28)	(33)	(32)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (3%)
Cholangiocarcinoma, metastatic, liver				1 (3%)
Fibrosarcoma	1 (4%)			
Hemangiosarcoma		2 (7%)		1 (3%)
Histiocytic sarcoma			1 (3%)	
Sarcoma, metastatic, tissue NOS				1 (3%)
Pancreas	(50)	(50)	(50)	(50)
Cholangiocarcinoma, metastatic, liver				1 (2%)
Salivary glands	(50)	(50)	(50)	(50)
Stomach, forestomach	(50)	(50)	(48)	(50)
Squamous cell carcinoma		1 (2%)		2 (4%)
Squamous cell papilloma	1 (2%)	3 (6%)	5 (10%)	6 (12%)
Squamous cell papilloma, multiple	1 (2%)	1 (2%)		2 (4%)
Tongue				(2)
Squamous cell carcinoma				2 (100%)
Tooth	(19)	(21)	(16)	(19)
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (2%)
Cholangiocarcinoma, metastatic, liver				1 (2%)
Hemangiosarcoma		1 (2%)		

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Bilateral, subcapsular, adenoma		1 (2%)	1 (2%)	
Subcapsular, adenoma	4 (8%)	4 (8%)	5 (10%)	4 (8%)
Subcapsular, adenoma, multiple		1 (2%)		
Adrenal medulla	(49)	(50)	(50)	(50)
Pheochromocytoma benign				2 (4%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Adenoma	2 (4%)		1 (2%)	
Pituitary gland	(48)	(47)	(48)	(49)
Pars distalis, adenoma		1 (2%)		
Thyroid gland	(50)	(50)	(50)	(50)
Follicular cell, adenoma	1 (2%)		1 (2%)	
General Body System				
Tissue NOS				(2)
Abdominal, sarcoma				1 (50%)
Genital System				
Coagulating gland	(1)	(2)	(1)	(1)
Epididymis	(50)	(50)	(50)	(50)
Hemangiosarcoma, metastatic, mesentery				1 (2%)
Preputial gland	(50)	(50)	(50)	(50)
Adenoma				1 (2%)
Prostate	(49)	(50)	(50)	(50)
Seminal vesicle	(50)	(50)	(50)	(50)
Testes	(50)	(50)	(50)	(50)
Hemangioma			1 (2%)	
Hemangiosarcoma, metastatic, mesentery				1 (2%)
Interstitial cell, adenoma	1 (2%)		1 (2%)	3 (6%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Histiocytic sarcoma			1 (2%)	
Lymph node	(1)	(4)	(2)	(7)
Bronchial, alveolar/bronchiolar carcinoma, metastatic, lung				1 (14%)
Bronchial, histiocytic sarcoma			1 (50%)	
Inguinal, histiocytic sarcoma			1 (50%)	
Lymph node, mandibular	(48)	(48)	(49)	(49)
Rhabdomyosarcoma, metastatic, skeletal muscle			1 (2%)	
Lymph node, mesenteric	(50)	(49)	(47)	(50)
Histiocytic sarcoma			2 (4%)	
Spleen	(49)	(50)	(50)	(49)
Hemangiosarcoma	2 (4%)	1 (2%)	3 (6%)	1 (2%)
Histiocytic sarcoma		1 (2%)	1 (2%)	
Thymus	(44)	(46)	(47)	(42)
Cholangiocarcinoma, metastatic, liver				1 (2%)

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Integumentary System				
Skin	(50)	(50)	(50)	(50)
Keratoacanthoma	1 (2%)			
Pinna, sarcoma		1 (2%)		
Prepuce, squamous cell papilloma	1 (2%)			
Sebaceous gland, adenoma		1 (2%)		
Subcutaneous tissue, hemangioma	1 (2%)			
Subcutaneous tissue, hemangiosarcoma	1 (2%)	1 (2%)		
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Osteosarcoma	1 (2%)			
Cranium, carcinoma, metastatic, harderian gland		1 (2%)		
Skeletal muscle	(1)		(2)	(3)
Cholangiocarcinoma, metastatic, liver				1 (33%)
Hemangiosarcoma				1 (33%)
Rhabdomyosarcoma			1 (50%)	
Nervous System				
None				
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	9 (18%)	8 (16%)	6 (12%)	6 (12%)
Alveolar/bronchiolar adenoma, multiple	2 (4%)	1 (2%)	2 (4%)	2 (4%)
Alveolar/bronchiolar carcinoma	9 (18%)	3 (6%)	5 (10%)	8 (16%)
Alveolar/bronchiolar carcinoma, multiple			2 (4%)	2 (4%)
Carcinoma, metastatic, harderian gland		1 (2%)		
Cholangiocarcinoma, metastatic, liver				1 (2%)
Hepatoblastoma, metastatic, liver		1 (2%)		1 (2%)
Hepatocellular carcinoma, metastatic, liver	4 (8%)		2 (4%)	4 (8%)
Histiocytic sarcoma			1 (2%)	
Lymphatic, mediastinum, alveolar/bronchiolar carcinoma, metastatic, lung				1 (2%)
Mediastinum, alveolar/bronchiolar carcinoma, metastatic, lung				2 (4%)
Mediastinum, cholangiocarcinoma, metastatic, liver				1 (2%)
Mediastinum, hepatocellular carcinoma, metastatic, liver	1 (2%)			
Nose	(50)	(50)	(48)	(50)
Carcinoma, metastatic, harderian gland		1 (2%)		
Pleura				(1)
Cholangiocarcinoma, metastatic, liver				1 (100%)
Special Senses System				
Harderian gland	(9)	(10)	(4)	(5)
Adenoma	7 (78%)	6 (60%)	3 (75%)	4 (80%)
Carcinoma	2 (22%)	4 (40%)	1 (25%)	1 (20%)
Zymbal's gland	(1)			
Adenoma	1 (100%)			

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Urinary System				
Kidney	(50)	(48)	(50)	(50)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (2%)
Cholangiocarcinoma, metastatic, liver				1 (2%)
Histiocytic sarcoma			1 (2%)	
Sarcoma, metastatic, tissue NOS				1 (2%)
Renal tubule, adenoma	1 (2%)	1 (2%)	1 (2%)	
Ureter			(1)	
Urethra	(1)		(1)	
Urinary bladder	(50)	(49)	(49)	(50)
Hemangioma			1 (2%)	
Systemic Lesions				
Multiple organs ^b	(50)	(50)	(50)	(50)
Histiocytic sarcoma		1 (2%)	2 (4%)	
Lymphoma Malignant	2 (4%)	4 (8%)	2 (4%)	
Neoplasm Summary				
Total animals with primary neoplasms ^c	45	44	43	47
Total primary neoplasms	86	95	86	97
Total animals with benign neoplasms	37	36	38	34
Total benign neoplasms	56	59	58	55
Total animals with malignant neoplasms	23	27	22	30
Total malignant neoplasms	30	36	28	42
Total animals with metastatic neoplasms	4	2	3	11
Total metastatic neoplasms	5	4	3	25

^a Number of animals examined microscopically at the site and the number of animals with neoplasm

^b Number of animals with any tissue examined microscopically

^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE C2
Individual Animal Tumor Pathology of Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal: Vehicle Control

Number of Days on Study	7 7	
	3 3	
	0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2	
Carcass ID Number	0 0	Total Tissues/Tumors
	4 4 0 0 0 0 1 1 2 2 2 2 2 2 3 3 4 4 1 1 2 2 2 3 4 4	
	3 5 1 3 5 6 1 4 0 2 3 5 8 2 4 1 8 2 6 1 4 7 9 4 9	
Special Senses System		
Ear		1
Eye		1
Harderian gland	+ + +	9
Adenoma	X X X	7
Carcinoma	X X	2
Zymbal's gland		1
Adenoma		1
Urinary System		
Kidney	+ +	50
Renal tubule, adenoma		1
Urethra		1
Urinary bladder	+ +	50
Systemic Lesions		
Multiple organs	+ +	50
Lymphoma malignant		2

TABLE C2
Individual Animal Tumor Pathology of Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal: 30 mg/kg

Number of Days on Study	7 7	
	3 3	
	0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 2 2 2 2	
Carcass ID Number	0 0	Total Tissues/Tumors
	7 7 8 8 8 8 9 9 9 9 5 5 5 6 6 6 7 7 8 8 9 5 6 8 9	
	8 9 0 2 4 5 0 2 6 7 3 8 9 3 6 7 1 6 3 7 3 6 8 8 9	
Special Senses System		
Ear		1
Eye		3
Harderian gland		10
Adenoma	+ + + +	6
Carcinoma	X X X X	4
Urinary System		
Kidney	+ +	48
Renal tubule, adenoma		1
Urinary bladder	+ +	49
Systemic Lesions		
Multiple organs	+ +	50
Histiocytic sarcoma		1
Lymphoma malignant	X X	4

TABLE C2
Individual Animal Tumor Pathology of Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal: 60 mg/kg

Number of Days on Study	7 7	
	3 3	
	0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2	
Carcass ID Number	1 1	Total Tissues/Tumors
	3 3 4 4 4 0 0 0 0 1 1 1 1 2 2 2 3 4 0 2 2 3 3 4 5	
	1 4 1 8 9 1 3 5 6 0 1 2 8 0 6 9 2 3 9 1 8 3 7 4 0	
Special Senses System		
Harderian gland		4
Adenoma	X	3
Carcinoma		1
Urinary System		
Kidney	+ +	50
Histiocytic sarcoma		1
Renal tubule, adenoma		1
Ureter		1
Urethra		1
Urinary bladder	+ +	49
Hemangioma		1
Systemic Lesions		
Multiple organs	+ +	50
Histiocytic sarcoma		2
Lymphoma malignant		2

TABLE C3
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Adrenal Cortex: Adenoma				
Overall rate ^a	4/50 (8%)	6/50 (12%)	6/50 (12%)	4/50 (8%)
Adjusted rate ^b	8.2%	13.4%	12.6%	8.8%
Terminal rate ^c	4/44 (9%)	6/39 (15%)	5/44 (11%)	4/39 (10%)
First incidence (days) ^d	729 (T)	729 (T)	704	729 (T)
Poly-3 test	P=0.538N	P=0.320	P=0.361	P=0.606
Harderian Gland: Adenoma				
Overall rate	7/50 (14%)	6/50 (12%)	3/50 (6%)	4/50 (8%)
Adjusted rate	14.4%	13.4%	6.3%	8.8%
Terminal rate	6/44 (14%)	6/39 (15%)	3/44 (7%)	4/39 (10%)
First incidence (days)	718	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.183N	P=0.563N	P=0.166N	P=0.301N
Harderian Gland: Carcinoma				
Overall rate	2/50 (4%)	4/50 (8%)	1/50 (2%)	1/50 (2%)
Adjusted rate	4.1%	8.9%	2.1%	2.2%
Terminal rate	1/44 (2%)	3/39 (8%)	1/44 (2%)	1/39 (3%)
First incidence (days)	670	643	729 (T)	729 (T)
Poly-3 test	P=0.251N	P=0.302	P=0.508N	P=0.524N
Harderian Gland: Adenoma or Carcinoma				
Overall rate	9/50 (18%)	10/50 (20%)	4/50 (8%)	5/50 (10%)
Adjusted rate	18.4%	22.2%	8.4%	11.0%
Terminal rate	7/44 (16%)	9/39 (23%)	4/44 (9%)	5/39 (13%)
First incidence (days)	670	643	729 (T)	729 (T)
Poly-3 test	P=0.097N	P=0.423	P=0.125N	P=0.235N
Small Intestine (Jejunum): Carcinoma				
Overall rate	1/50 (2%)	0/50 (0%)	1/50 (2%)	3/50 (6%)
Adjusted rate	2.1%	0.0%	2.1%	6.6%
Terminal rate	1/44 (2%)	0/39 (0%)	1/44 (2%)	3/39 (8%)
First incidence (days)	729 (T)	— ^e	729 (T)	729 (T)
Poly-3 test	P=0.094	P=0.516N	P=0.757	P=0.282
Small Intestine (Duodenum, Jejunum, or Ileum): Carcinoma				
Overall rate	1/50 (2%)	1/50 (2%)	2/50 (4%)	4/50 (8%)
Adjusted rate	2.1%	2.2%	4.2%	8.8%
Terminal rate	1/44 (2%)	0/39 (0%)	2/44 (5%)	4/39 (10%)
First incidence (days)	729 (T)	675	729 (T)	729 (T)
Poly-3 test	P=0.066	P=0.743	P=0.494	P=0.160
Small Intestine (Duodenum, Jejunum, or Ileum): Adenomatous Polyp or Carcinoma				
Overall rate	1/50 (2%)	2/50 (4%)	2/50 (4%)	4/50 (8%)
Adjusted rate	2.1%	4.5%	4.2%	8.8%
Terminal rate	1/44 (2%)	1/39 (3%)	2/44 (5%)	4/39 (10%)
First incidence (days)	729 (T)	675	729 (T)	729 (T)
Poly-3 test	P=0.103	P=0.473	P=0.494	P=0.160
Liver: Hepatocellular Adenoma				
Overall rate	23/50 (46%)	30/50 (60%)	30/50 (60%)	25/50 (50%)
Adjusted rate	46.8%	65.2%	62.0%	54.8%
Terminal rate	21/44 (48%)	27/39 (69%)	28/44 (64%)	24/39 (62%)
First incidence (days)	568	544	587	690
Poly-3 test	P=0.348	P=0.051	P=0.095	P=0.283

TABLE C3
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Liver: Hepatocellular Carcinoma				
Overall rate	8/50 (16%)	11/50 (22%)	7/50 (14%)	14/50 (28%)
Adjusted rate	16.3%	23.6%	14.6%	29.9%
Terminal rate	5/44 (11%)	7/39 (18%)	6/44 (14%)	10/39 (26%)
First incidence (days)	670	400	649	558
Poly-3 test	P=0.100	P=0.265	P=0.517N	P=0.090
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	31/50 (62%)	36/50 (72%)	35/50 (70%)	36/50 (72%)
Adjusted rate	62.5%	75.1%	71.9%	76.5%
Terminal rate	26/44 (59%)	29/39 (74%)	32/44 (73%)	31/39 (80%)
First incidence (days)	568	400	587	558
Poly-3 test	P=0.115	P=0.129	P=0.220	P=0.098
Liver: Hepatoblastoma				
Overall rate	2/50 (4%)	3/50 (6%)	0/50 (0%)	2/50 (4%)
Adjusted rate	4.1%	6.7%	0.0%	4.3%
Terminal rate	2/44 (5%)	3/39 (8%)	0/44 (0%)	1/39 (3%)
First incidence (days)	729 (T)	729 (T)	—	504
Poly-3 test	P=0.480N	P=0.463	P=0.241N	P=0.675
Liver: Hepatocellular Carcinoma or Hepatoblastoma				
Overall rate	10/50 (20%)	13/50 (26%)	7/50 (14%)	16/50 (32%)
Adjusted rate	20.4%	27.9%	14.6%	33.7%
Terminal rate	7/44 (16%)	9/39 (23%)	6/44 (14%)	11/39 (28%)
First incidence (days)	670	400	649	504
Poly-3 test	P=0.128	P=0.271	P=0.314N	P=0.107
Liver: Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma				
Overall rate	31/50 (62%)	36/50 (72%)	35/50 (70%)	37/50 (74%)
Adjusted rate	62.5%	75.1%	71.9%	77.6%
Terminal rate	26/44 (59%)	29/39 (74%)	32/44 (73%)	31/39 (80%)
First incidence (days)	568	400	587	504
Poly-3 test	P=0.092	P=0.129	P=0.220	P=0.078
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	11/50 (22%)	9/50 (18%)	8/50 (16%)	8/50 (16%)
Adjusted rate	22.6%	20.1%	16.7%	17.6%
Terminal rate	10/44 (23%)	9/39 (23%)	7/44 (16%)	7/39 (18%)
First incidence (days)	701	729 (T)	637	713
Poly-3 test	P=0.295N	P=0.484N	P=0.316N	P=0.364N
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	9/50 (18%)	3/50 (6%)	7/50 (14%)	10/50 (20%)
Adjusted rate	18.5%	6.7%	14.7%	21.2%
Terminal rate	7/44 (16%)	3/39 (8%)	7/44 (16%)	6/39 (15%)
First incidence (days)	698	729 (T)	729 (T)	504
Poly-3 test	P=0.243	P=0.081N	P=0.410N	P=0.470

TABLE C3
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	19/50 (38%)	12/50 (24%)	15/50 (30%)	17/50 (34%)
Adjusted rate	38.9%	26.8%	31.2%	36.0%
Terminal rate	16/44 (36%)	12/39 (31%)	14/44 (32%)	12/39 (31%)
First incidence (days)	698	729 (T)	637	504
Poly-3 test	P=0.527N	P=0.153N	P=0.281N	P=0.464N
Spleen: Hemangiosarcoma				
Overall rate	2/49 (4%)	1/50 (2%)	3/50 (6%)	1/49 (2%)
Adjusted rate	4.2%	2.2%	6.3%	2.3%
Terminal rate	2/44 (5%)	1/39 (3%)	2/44 (5%)	1/38 (3%)
First incidence (days)	729 (T)	729 (T)	680	729 (T)
Poly-3 test	P=0.494N	P=0.523N	P=0.501	P=0.525N
Stomach (Forestomach): Squamous Cell Papilloma				
Overall rate	2/50 (4%)	4/50 (8%)	5/50 (10%)	8/50 (16%)
Adjusted rate	4.1%	8.9%	10.5%	17.6%
Terminal rate	2/44 (5%)	4/39 (10%)	5/44 (11%)	8/39 (21%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.022	P=0.301	P=0.210	P=0.035
Stomach (Forestomach): Squamous Cell Papilloma or Squamous Cell Carcinoma				
Overall rate	2/50 (4%)	4/50 (8%)	5/50 (10%)	10/50 (20%)
Adjusted rate	4.1%	8.9%	10.5%	22.0%
Terminal rate	2/44 (5%)	4/39 (10%)	5/44 (11%)	10/39 (26%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.004	P=0.301	P=0.210	P=0.009
Testes: Adenoma				
Overall rate	1/50 (2%)	0/50 (0%)	1/50 (2%)	3/50 (6%)
Adjusted rate	2.1%	0.0%	2.1%	6.6%
Terminal rate	1/44 (2%)	0/39 (0%)	1/44 (2%)	3/39 (8%)
First incidence (days)	729 (T)	—	729 (T)	729 (T)
Poly-3 test	P=0.094	P=0.516N	P=0.757	P=0.282
All Organs: Hemangiosarcoma				
Overall rate	4/50 (8%)	6/50 (12%)	4/50 (8%)	4/50 (8%)
Adjusted rate	8.2%	13.2%	8.4%	8.8%
Terminal rate	4/44 (9%)	5/39 (13%)	3/44 (7%)	3/39 (8%)
First incidence (days)	729 (T)	400	680	713
Poly-3 test	P=0.499N	P=0.331	P=0.636	P=0.607
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	5/50 (10%)	6/50 (12%)	6/50 (12%)	4/50 (8%)
Adjusted rate	10.3%	13.2%	12.5%	8.8%
Terminal rate	5/44 (11%)	5/39 (13%)	5/44 (11%)	3/39 (8%)
First incidence (days)	729 (T)	400	680	713
Poly-3 test	P=0.429N	P=0.456	P=0.491	P=0.540N

TABLE C3
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
All Organs: Malignant Lymphoma				
Overall rate	2/50 (4%)	4/50 (8%)	2/50 (4%)	0/50 (0%)
Adjusted rate	4.1%	8.8%	4.2%	0.0%
Terminal rate	2/44 (5%)	3/39 (8%)	2/44 (5%)	0/39 (0%)
First incidence (days)	729 (T)	504	729 (T)	—
Poly-3 test	P=0.125N	P=0.307	P=0.687	P=0.252N
All Organs: Benign Neoplasms				
Overall rate	37/50 (74%)	36/50 (72%)	38/50 (76%)	34/50 (68%)
Adjusted rate	75.2%	78.3%	77.4%	74.3%
Terminal rate	34/44 (77%)	33/39 (85%)	33/44 (75%)	31/39 (80%)
First incidence (days)	568	544	587	690
Poly-3 test	P=0.475N	P=0.452	P=0.492	P=0.558N
All Organs: Malignant Neoplasms				
Overall rate	23/50 (46%)	27/50 (54%)	22/50 (44%)	30/50 (60%)
Adjusted rate	46.9%	56.6%	45.1%	61.4%
Terminal rate	19/44 (43%)	21/39 (54%)	18/44 (41%)	21/39 (54%)
First incidence (days)	670	400	587	504
Poly-3 test	P=0.138	P=0.225	P=0.510N	P=0.108
All Organs: Benign or Malignant Neoplasms				
Overall rate	45/50 (90%)	44/50 (88%)	43/50 (86%)	47/50 (94%)
Adjusted rate	90.8%	90.5%	87.6%	95.9%
Terminal rate	40/44 (91%)	36/39 (92%)	38/44 (86%)	37/39 (95%)
First incidence (days)	568	400	587	504
Poly-3 test	P=0.231	P=0.622N	P=0.423N	P=0.270

(T)Terminal sacrifice

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, liver, lung, spleen, and testis; for other tissues, denominator is number of animals necropsied.

^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the vehicle control incidence are the P values 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. A negative trend or a lower incidence in a dosed group is indicated by N.

^e Not applicable; no neoplasms in animal group

TABLE C4a
Historical Incidence of Forestomach Neoplasms in Control Male B6C3F₁ Mice

Study	Incidence in Controls		
	Squamous Cell Papilloma	Squamous Cell Carcinoma	Squamous Cell Papilloma or Squamous Cell Carcinoma
Historical Incidence in Controls Given NTP-2000 Diet^a			
Acrylonitrile (gavage)	3/50	0/50	3/50
Citral (feed)	0/100	0/100	0/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	0/50	0/50	0/50
Indium phosphide (inhalation)	1/50	1/50	2/50
60-Hz Magnetic fields (whole body exposure)	0/100	0/100	0/100
Methacrylonitrile (gavage)	1/49	0/49	1/49
<i>o</i> -Nitrotoluene (feed)	0/60	0/60	0/60
<i>p</i> -Nitrotoluene (feed)	1/50	0/50	1/50
Riddelliine (gavage)	1/50	0/50	1/50
Sodium nitrite (drinking water)	1/50	0/50	1/50
Vanadium pentoxide (inhalation)	2/50	0/50	2/50
Overall Historical Incidence in Controls Given NTP-2000 Diet			
Total	10/659 (1.5%)	1/659 (0.2%)	11/659 (1.7%)
Mean ± standard deviation	1.8% ± 1.9%	0.2% ± 0.6%	2.0% ± 2.0%
Range	0%-6%	0%-2%	0%-6%
Historical Incidence in Corn Oil Gavage Controls Given NIH-07 Diet at Southern Research Institute^b			
<i>p</i> -Nitroaniline	3/50	1/50	4/50
Salicylazosulfapyridine	3/50	0/50	3/50
Theophylline	1/50	0/50	1/50
Overall Historical Incidence in Corn Oil Gavage Controls Given NIH-07 Diet			
Total	19/464 (4.1%)	3/464 (0.6%)	22/464 (4.7%)
Mean ± standard deviation	4.1% ± 1.7%	0.7% ± 1.0%	4.7% ± 2.0%
Range	2%-6%	0%-2%	2%-8%

^a Data as of December 20, 2000

^b Data as of December 23, 1999

TABLE C4b
Historical Incidence of Oral Cavity (Tongue) Neoplasms in Control Male B6C3F₁ Mice

Study	Incidence in Controls		
	Squamous Cell Papilloma	Squamous Cell Carcinoma	Squamous Cell Papilloma or Squamous Cell Carcinoma
Historical Incidence in Controls Given NTP-2000 Diet^a			
Acrylonitrile (gavage)	0/50	0/50	0/50
Citral (feed)	0/100	0/100	0/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	0/50	0/50	0/50
Indium phosphide (inhalation)	0/50	0/50	0/50
60-Hz Magnetic fields (whole body exposure)	0/100	0/100	0/100
Methacrylonitrile (gavage)	0/49	0/49	0/49
<i>o</i> -Nitrotoluene (feed)	0/60	0/60	0/60
<i>p</i> -Nitrotoluene (feed)	0/50	0/50	0/50
Riddelliine (gavage)	0/50	0/50	0/50
Sodium nitrite (drinking water)	0/50	0/50	0/50
Vanadium pentoxide (inhalation)	1/50	0/50	1/50
Overall Historical Incidence in Controls Given NTP-2000 Diet			
Total	1/659 (0.2%)	0/659	1/659 (0.2%)
Mean ± standard deviation	0.2% ± 0.6%		0.2% ± 0.6%
Range	0%-2%		0%-2%
Historical Incidence in Corn Oil Gavage Controls Given NIH-07 Diet at Southern Research Institute^b			
<i>p</i> -Nitroaniline	0/50	0/50	0/50
Salicylazosulfapyridine	0/50	0/50	0/50
Theophylline	0/50	0/50	0/50
Overall Historical Incidence in Corn Oil Gavage Controls Given NIH-07 Diet			
Total	0/464	0/464	0/464

^a Data as of December 20, 2000

^b Data as of December 23, 1999

TABLE C5
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	3	3	3	8
Natural deaths	3	8	3	3
Survivors				
Died last week of study				1
Terminal sacrifice	44	39	44	38
Animals examined microscopically	50	50	50	50
Alimentary System				
Gallbladder	(48)	(47)	(45)	(46)
Cyst	2 (4%)		2 (4%)	1 (2%)
Cyst, multiple	1 (2%)		1 (2%)	
Infiltration cellular, polymorphonuclear Epithelium, degeneration, hyaline		1 (2%)	2 (4%)	
Epithelium, hyperplasia, adenomatous		1 (2%)	1 (2%)	
Intestine large, cecum	(47)	(45)	(48)	(48)
Serosa, fibrosis, focal	1 (2%)			
Intestine small, duodenum	(47)	(47)	(48)	(49)
Epithelium, cyst	1 (2%)			
Intestine small, jejunum	(47)	(45)	(49)	(48)
Inflammation, chronic active				1 (2%)
Peyer's patch, hyperplasia, histiocytic		1 (2%)		
Peyer's patch, hyperplasia, lymphoid			1 (2%)	
Intestine small, ileum	(47)	(45)	(47)	(48)
Infiltration cellular, mixed cell			1 (2%)	
Serosa, fibrosis, focal	1 (2%)			
Liver	(50)	(50)	(50)	(50)
Angiectasis	2 (4%)			
Angiectasis, focal		1 (2%)		
Clear cell focus				1 (2%)
Congestion	1 (2%)			
Congestion, focal	1 (2%)	3 (6%)	1 (2%)	3 (6%)
Eosinophilic focus				1 (2%)
Eosinophilic focus, multiple		1 (2%)		
Erythrophagocytosis			1 (2%)	
Hematopoietic cell proliferation				1 (2%)
Hemorrhage, focal		2 (4%)	1 (2%)	2 (4%)
Hyperplasia, focal, histiocytic			1 (2%)	
Hyperplasia, focal, lymphoid			1 (2%)	
Hyperplasia, histiocytic		1 (2%)		
Hyperplasia, lymphoid		1 (2%)		
Infarct	2 (4%)			
Infiltration cellular, lymphocyte			1 (2%)	
Infiltration cellular, mixed cell	31 (62%)	27 (54%)	30 (60%)	31 (62%)
Inflammation, chronic			1 (2%)	1 (2%)
Mixed cell focus	1 (2%)	1 (2%)		3 (6%)
Necrosis, focal		2 (4%)	1 (2%)	
Pigmentation, focal			1 (2%)	

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

TABLE C5
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Alimentary System (continued)				
Liver (continued)	(50)	(50)	(50)	(50)
Bile duct, hyperplasia				1 (2%)
Hepatocyte, basophilic focus		2 (4%)	1 (2%)	1 (2%)
Hepatocyte, cytomegaly	2 (4%)	2 (4%)		2 (4%)
Hepatocyte, eosinophilic focus	3 (6%)	5 (10%)	2 (4%)	4 (8%)
Hepatocyte, eosinophilic focus, multiple			1 (2%)	
Hepatocyte, karyomegaly	2 (4%)			2 (4%)
Hepatocyte, mixed cell focus	1 (2%)	6 (12%)	9 (18%)	5 (10%)
Hepatocyte, mixed cell focus, multiple	1 (2%)	3 (6%)		3 (6%)
Hepatocyte, necrosis, focal	1 (2%)	1 (2%)	2 (4%)	2 (4%)
Hepatocyte, vacuolization cytoplasmic, diffuse	6 (12%)	1 (2%)	1 (2%)	4 (8%)
Hepatocyte, vacuolization cytoplasmic, focal	13 (26%)	15 (30%)	16 (32%)	12 (24%)
Hepatocyte, periportal, vacuolization cytoplasmic	3 (6%)	3 (6%)	2 (4%)	2 (4%)
Hepatocyte, centrilobular, depletion glycogen		1 (2%)		
Hepatocyte, centrilobular, necrosis		1 (2%)		
Hepatocyte, midzonal, vacuolization cytoplasmic	17 (34%)	9 (18%)	20 (40%)	10 (20%)
Oval cell, hyperplasia			1 (2%)	1 (2%)
Mesentery	(25)	(28)	(33)	(32)
Fibrosis, focal	1 (4%)		1 (3%)	
Hemorrhage		1 (4%)	1 (3%)	1 (3%)
Hemorrhage, focal	1 (4%)			
Inflammation, chronic	1 (4%)	4 (14%)		2 (6%)
Fat, necrosis	24 (96%)	27 (96%)	32 (97%)	31 (97%)
Pancreas	(50)	(50)	(50)	(50)
Necrosis, focal		1 (2%)		1 (2%)
Acinus, atrophy, diffuse				1 (2%)
Acinus, atrophy, focal	1 (2%)	1 (2%)		1 (2%)
Acinus, cytoplasmic alteration		1 (2%)		
Duct, cyst			1 (2%)	1 (2%)
Duct, inflammation, chronic, focal				1 (2%)
Stomach, forestomach	(50)	(50)	(48)	(50)
Inflammation, focal	3 (6%)	1 (2%)	3 (6%)	6 (12%)
Ulcer	2 (4%)		3 (6%)	10 (20%)
Ulcer, focal				1 (2%)
Epithelium, cyst				1 (2%)
Epithelium, hyperplasia, squamous	14 (28%)	7 (14%)	9 (19%)	26 (52%)
Stomach, glandular	(50)	(48)	(48)	(50)
Erosion				1 (2%)
Hyperplasia				1 (2%)
Mineralization	1 (2%)			
Glands, degeneration, cystic, focal				3 (6%)
Tooth	(19)	(21)	(16)	(19)
Malformation	11 (58%)	13 (62%)	10 (63%)	12 (63%)
Peridontal tissue, inflammation, chronic	10 (53%)	9 (43%)	8 (50%)	10 (53%)
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Infiltration cellular, mixed cell				2 (4%)
Inflammation, chronic, focal	1 (2%)			2 (4%)
Mineralization, focal		1 (2%)		
Valve, hemorrhage, focal				1 (2%)
Valve, inflammation, chronic, focal				1 (2%)

TABLE C5
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Accessory adrenal cortical nodule	1 (2%)		1 (2%)	5 (10%)
Cytoplasmic alteration, focal	7 (14%)	2 (4%)	3 (6%)	5 (10%)
Hypertrophy, focal	1 (2%)		1 (2%)	
Bilateral, accessory adrenal cortical nodule				1 (2%)
Subcapsular, hyperplasia, focal	11 (22%)	7 (14%)	8 (16%)	7 (14%)
Adrenal medulla	(49)	(50)	(50)	(50)
Angiectasis		1 (2%)		
Hyperplasia	1 (2%)		1 (2%)	1 (2%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Hyperplasia		1 (2%)	1 (2%)	2 (4%)
Parathyroid gland	(47)	(48)	(49)	(46)
Cyst	1 (2%)		2 (4%)	1 (2%)
Pituitary gland	(48)	(47)	(48)	(49)
Pars distalis, cyst	3 (6%)	2 (4%)	3 (6%)	3 (6%)
Pars distalis, cyst, multiple			1 (2%)	
Pars distalis, cytoplasmic alteration, focal				1 (2%)
Pars distalis, degeneration, cystic, focal			1 (2%)	
Pars distalis, hyperplasia, focal		2 (4%)	1 (2%)	
Thyroid gland	(50)	(50)	(50)	(50)
Degeneration, cystic, focal	7 (14%)	5 (10%)	5 (10%)	6 (12%)
Follicle, cyst	1 (2%)	1 (2%)		1 (2%)
General Body System				
Peritoneum		(1)		
Inflammation, focal		1 (100%)		
Genital System				
Coagulating gland	(1)	(2)	(1)	(1)
Inflammation, chronic				1 (100%)
Epididymis	(50)	(50)	(50)	(50)
Hemorrhage				1 (2%)
Inflammation, chronic	1 (2%)	1 (2%)		
Bilateral, hemorrhage	1 (2%)			
Preputial gland	(50)	(50)	(50)	(50)
Degeneration, cystic	16 (32%)	17 (34%)	9 (18%)	21 (42%)
Inflammation, chronic	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Metaplasia, squamous				1 (2%)
Prostate	(49)	(50)	(50)	(50)
Inflammation, chronic	1 (2%)			1 (2%)
Epithelium, hyperplasia, focal				1 (2%)
Seminal vesicle	(50)	(50)	(50)	(50)
Dilatation	1 (2%)		2 (4%)	
Inflammation, chronic		1 (2%)	2 (4%)	1 (2%)
Bilateral, dilatation			1 (2%)	
Testes	(50)	(50)	(50)	(50)
Hemorrhage	1 (2%)			
Mineralization				1 (2%)
Interstitial cell, hyperplasia	1 (2%)			

TABLE C5
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Fibrosis, focal		1 (2%)		
Thrombosis, chronic		1 (2%)		
Myeloid cell, hyperplasia	1 (2%)	1 (2%)	2 (4%)	3 (6%)
Lymph node	(1)	(4)	(2)	(7)
Inguinal, hyperplasia, lymphoid		1 (25%)		
Inguinal, hyperplasia, plasma cell				1 (14%)
Mediastinal, congestion		1 (25%)		
Mediastinal, hyperplasia, lymphoid	1 (100%)			
Mediastinal, hyperplasia, plasma cell			1 (50%)	3 (43%)
Mediastinal, infiltration cellular, mixed cell				1 (14%)
Lymph node, mandibular	(48)	(48)	(49)	(49)
Hyperplasia, histiocytic	1 (2%)			
Hyperplasia, lymphoid	1 (2%)	3 (6%)	1 (2%)	1 (2%)
Hyperplasia, plasma cell	2 (4%)	1 (2%)	1 (2%)	3 (6%)
Pigmentation			1 (2%)	
Lymph node, mesenteric	(50)	(49)	(47)	(50)
Congestion				1 (2%)
Hemorrhage	1 (2%)		2 (4%)	
Hyperplasia, histiocytic		1 (2%)		
Hyperplasia, lymphoid		1 (2%)		
Hyperplasia, plasma cell	6 (12%)		2 (4%)	2 (4%)
Infiltration cellular, polymorphonuclear	1 (2%)			
Spleen	(49)	(50)	(50)	(49)
Depletion cellular		1 (2%)		1 (2%)
Hematopoietic cell proliferation	14 (29%)	18 (36%)	18 (36%)	23 (47%)
Hyperplasia, histiocytic		1 (2%)		
Hyperplasia, lymphoid	3 (6%)	3 (6%)	2 (4%)	
Thymus	(44)	(46)	(47)	(42)
Angiectasis	1 (2%)	1 (2%)		
Atrophy	1 (2%)	1 (2%)	1 (2%)	
Cyst	10 (23%)	9 (20%)	5 (11%)	9 (21%)
Cyst, multiple			2 (4%)	
Hyperplasia, lymphoid		1 (2%)	1 (2%)	
Integumentary System				
Skin	(50)	(50)	(50)	(50)
Hyperkeratosis, focal				1 (2%)
Inflammation, chronic, focal			1 (2%)	1 (2%)
Ulcer			2 (4%)	
Epidermis, hyperplasia, focal				1 (2%)
Subcutaneous tissue, congestion, focal	1 (2%)			
Subcutaneous tissue, necrosis, fatty, focal			1 (2%)	1 (2%)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Cranium, hyperostosis	1 (2%)			
Skeletal muscle	(1)		(2)	(3)
Infiltration cellular, focal, lipocyte	1 (100%)		1 (50%)	

TABLE C5
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Nervous System				
Brain	(50)	(50)	(50)	(50)
Compression, focal		1 (2%)		
Hemorrhage, focal			1 (2%)	
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Congestion	1 (2%)		3 (6%)	1 (2%)
Hemorrhage		1 (2%)	2 (4%)	1 (2%)
Hyperplasia, histiocytic	5 (10%)	3 (6%)	6 (12%)	6 (12%)
Hyperplasia, lymphoid		2 (4%)	2 (4%)	1 (2%)
Infiltration cellular, eosinophil, focal				1 (2%)
Infiltration cellular, focal, mixed cell		1 (2%)		
Infiltration cellular, mixed cell		2 (4%)	2 (4%)	1 (2%)
Inflammation, chronic, focal		1 (2%)		
Metaplasia, focal, osseous		1 (2%)		
Pigmentation, focal		1 (2%)		
Alveolar epithelium, hyperplasia	5 (10%)	6 (12%)	4 (8%)	
Alveolar epithelium, hyperplasia, focal				1 (2%)
Mediastinum, necrosis, fatty, focal		1 (2%)		
Nose	(50)	(50)	(48)	(50)
Foreign body		1 (2%)		
Inflammation, suppurative	7 (14%)	11 (22%)	13 (27%)	13 (26%)
Mineralization, focal				1 (2%)
Polyp, inflammatory			2 (4%)	
Glands, cytoplasmic alteration	1 (2%)			
Mucosa, glands, dilatation, focal	4 (8%)	2 (4%)		6 (12%)
Nasolacrimal duct, inflammation	2 (4%)	2 (4%)		1 (2%)
Olfactory epithelium, cytoplasmic alteration	24 (48%)	19 (38%)	17 (35%)	19 (38%)
Respiratory epithelium, cytoplasmic alteration	48 (96%)	47 (94%)	45 (94%)	45 (90%)
Respiratory epithelium, metaplasia, squamous			1 (2%)	
Sinus, glands, fibrosis, focal			1 (2%)	
Vomeronasal organ, cyst		1 (2%)		
Special Senses System				
Eye	(1)	(3)		(3)
Atrophy		1 (33%)		
Cornea, hyperplasia, focal, squamous		1 (33%)		3 (100%)
Cornea, inflammation, chronic		2 (67%)		
Cornea, inflammation, focal		1 (33%)		3 (100%)
Cornea, necrosis, focal				2 (67%)
Iris, synechia		1 (33%)		

TABLE C5
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Urinary System				
Kidney	(50)	(48)	(50)	(50)
Accumulation, hyaline droplet			1 (2%)	
Atrophy			1 (2%)	
Atrophy, focal	1 (2%)	2 (4%)	1 (2%)	
Congestion	2 (4%)	1 (2%)	1 (2%)	
Cyst	7 (14%)	11 (23%)	4 (8%)	5 (10%)
Cyst, multiple		1 (2%)	1 (2%)	1 (2%)
Hydronephrosis			1 (2%)	
Hyperplasia, lymphoid	1 (2%)		1 (2%)	
Infiltration cellular, focal, mixed cell				1 (2%)
Inflammation, suppurative	1 (2%)			1 (2%)
Metaplasia, focal, osseous	3 (6%)	1 (2%)	1 (2%)	
Nephropathy	47 (94%)	44 (92%)	48 (96%)	46 (92%)
Papilla, necrosis		1 (2%)		
Renal tubule, accumulation, hyaline droplet				1 (2%)
Renal tubule, dilatation, focal	1 (2%)			
Renal tubule, hyperplasia, focal	1 (2%)	2 (4%)	2 (4%)	4 (8%)
Renal tubule, pigmentation	1 (2%)	1 (2%)		
Urethra	(1)		(1)	
Inflammation	1 (100%)			
Necrosis, focal	1 (100%)			
Urinary bladder	(50)	(49)	(49)	(50)
Inflammation, suppurative	1 (2%)			
Transitional epithelium, hyperplasia				1 (2%)

APPENDIX D
SUMMARY OF LESIONS IN FEMALE MICE
IN THE 2-YEAR GAVAGE STUDY
OF 2,4-HEXADIENAL

TABLE D1	Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal	186
TABLE D2	Individual Animal Tumor Pathology of Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal	190
TABLE D3	Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal	214
TABLE D4	Historical Incidence of Forestomach Neoplasms in Control Female B6C3F₁ Mice	218
TABLE D5	Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal	219

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death				1
Moribund	2	8	8	4
Natural deaths	6	4	5	6
Survivors				
Died last week of study				2
Terminal sacrifice	42	37	37	37
Missing		1		
Animals examined microscopically	50	49	50	50
Alimentary System				
Esophagus	(49)	(49)	(50)	(50)
Squamous cell carcinoma, metastatic, stomach, forestomach				1 (2%)
Gallbladder	(44)	(45)	(47)	(41)
Sarcoma, metastatic, skeletal muscle	1 (2%)			
Intestine large, colon	(46)	(46)	(49)	(49)
Histiocytic sarcoma		1 (2%)		
Intestine large, cecum	(45)	(45)	(47)	(44)
Intestine small, duodenum	(45)	(44)	(47)	(43)
Polyp adenomatous	1 (2%)			
Intestine small, ileum	(43)	(46)	(48)	(44)
Histiocytic sarcoma		1 (2%)		
Liver	(50)	(49)	(50)	(50)
Cholangiocarcinoma		1 (2%)		
Hepatocellular carcinoma	3 (6%)	3 (6%)	3 (6%)	4 (8%)
Hepatocellular carcinoma, multiple		1 (2%)		
Hepatocellular adenoma	10 (20%)	3 (6%)	10 (20%)	5 (10%)
Hepatocellular adenoma, multiple	1 (2%)		1 (2%)	1 (2%)
Histiocytic sarcoma	1 (2%)	1 (2%)		1 (2%)
Ito cell tumor benign			1 (2%)	1 (2%)
Ito cell tumor malignant			1 (2%)	
Serosa, sarcoma, metastatic, skeletal muscle	1 (2%)			
Mesentery	(31)	(30)	(37)	(25)
Histiocytic sarcoma	1 (3%)	1 (3%)		1 (4%)
Ito cell tumor malignant, metastatic, liver			1 (3%)	
Sarcoma, metastatic, skeletal muscle	1 (3%)			
Squamous cell carcinoma, metastatic, stomach, forestomach				3 (12%)
Pancreas	(47)	(46)	(50)	(47)
Histiocytic sarcoma				1 (2%)
Squamous cell carcinoma, metastatic, stomach, forestomach				2 (4%)
Salivary glands	(49)	(48)	(48)	(50)
Stomach, forestomach	(48)	(48)	(50)	(48)
Squamous cell carcinoma				7 (15%)
Squamous cell papilloma	2 (4%)	2 (4%)	9 (18%)	11 (23%)
Squamous cell papilloma, multiple			2 (4%)	2 (4%)
Stomach, glandular	(45)	(45)	(48)	(46)
Squamous cell carcinoma, metastatic, stomach, forestomach				3 (7%)
Serosa, sarcoma, metastatic, skeletal muscle	1 (2%)			
Tooth	(1)			(2)
Peridontal tissue, sarcoma	1 (100%)			

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Cardiovascular System				
Heart	(49)	(49)	(50)	(50)
Histiocytic sarcoma	1 (2%)	1 (2%)		
Endocrine System				
Adrenal cortex	(50)	(48)	(50)	(50)
Hepatocellular carcinoma, metastatic, liver			1 (2%)	
Histiocytic sarcoma	1 (2%)			
Adrenal medulla	(50)	(48)	(49)	(49)
Pheochromocytoma malignant			1 (2%)	
Pheochromocytoma benign	1 (2%)			
Islets, pancreatic	(47)	(46)	(49)	(49)
Adenoma	1 (2%)			
Carcinoma				1 (2%)
Pituitary gland	(46)	(48)	(47)	(50)
Pars distalis, adenoma	2 (4%)	5 (10%)	7 (15%)	3 (6%)
Pars distalis, carcinoma			1 (2%)	
Pars intermedia, adenoma	2 (4%)		1 (2%)	1 (2%)
Thyroid gland	(49)	(46)	(49)	(50)
Bilateral, follicular cell, adenoma	1 (2%)			
Follicular cell, carcinoma			2 (4%)	
General Body System				
Peritoneum	(1)			
Sarcoma, metastatic, skeletal muscle	1 (100%)			
Tissue NOS	(1)	(1)	(2)	(1)
Fibrosarcoma			1 (50%)	
Abdominal, sarcoma				1 (100%)
Thoracic, sarcoma, multiple	1 (100%)			
Genital System				
Ovary	(49)	(47)	(48)	(48)
Cystadenoma	1 (2%)	1 (2%)	3 (6%)	1 (2%)
Hemangioma				1 (2%)
Hemangiosarcoma			2 (4%)	
Histiocytic sarcoma	1 (2%)			
Luteoma	1 (2%)			
Squamous cell carcinoma, metastatic, stomach, forestomach				1 (2%)
Uterus	(50)	(49)	(50)	(50)
Hemangiosarcoma				2 (4%)
Histiocytic sarcoma	1 (2%)	1 (2%)		
Leiomyoma				1 (2%)
Polyp stromal			1 (2%)	
Sarcoma	1 (2%)			
Endometrium, adenoma				1 (2%)
Endometrium, polyp stromal		1 (2%)	2 (4%)	1 (2%)
Vagina		(1)	(1)	(1)
Hemangioma			1 (100%)	

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Hematopoietic System				
Bone marrow	(50)	(47)	(50)	(48)
Histiocytic sarcoma	1 (2%)	1 (2%)		1 (2%)
Lymph node	(8)	(9)	(9)	(7)
Bronchial, histiocytic sarcoma		1 (11%)		
Iliac, histiocytic sarcoma		1 (11%)		
Inguinal, histiocytic sarcoma		1 (11%)		
Mediastinal, histiocytic sarcoma	1 (13%)			1 (14%)
Mediastinal, squamous cell carcinoma, metastatic, stomach, forestomach				2 (29%)
Renal, histiocytic sarcoma		1 (11%)		
Lymph node, mandibular	(47)	(47)	(48)	(49)
Histiocytic sarcoma	1 (2%)	1 (2%)		1 (2%)
Lymph node, mesenteric	(47)	(46)	(49)	(49)
Histiocytic sarcoma	1 (2%)	2 (4%)		1 (2%)
Sarcoma	1 (2%)			
Sarcoma, metastatic, skeletal muscle	1 (2%)			
Squamous cell carcinoma, metastatic, stomach, forestomach				1 (2%)
Spleen	(48)	(47)	(50)	(49)
Hemangiosarcoma		1 (2%)		
Histiocytic sarcoma		1 (2%)		1 (2%)
Sarcoma, metastatic, skeletal muscle	1 (2%)			
Thymus	(44)	(46)	(48)	(46)
Histiocytic sarcoma		1 (2%)		
Integumentary System				
Mammary gland	(48)	(49)	(50)	(50)
Adenoacanthoma			1 (2%)	
Carcinoma	1 (2%)	3 (6%)	1 (2%)	
Skin	(50)	(49)	(50)	(50)
Pinna, fibrous histiocytoma			1 (2%)	
Sebaceous gland, pinna, adenoma	1 (2%)			
Subcutaneous tissue, fibrosarcoma		1 (2%)	3 (6%)	
Subcutaneous tissue, fibrosarcoma, multiple		2 (4%)	1 (2%)	
Subcutaneous tissue, hemangiosarcoma	1 (2%)			
Subcutaneous tissue, histiocytic sarcoma	1 (2%)			
Subcutaneous tissue, schwannoma malignant	1 (2%)			
Musculoskeletal System				
Bone	(43)	(49)	(49)	(39)
Osteoma	1 (2%)			
Skeletal muscle	(6)	(4)	(5)	(2)
Histiocytic sarcoma		1 (25%)		
Sarcoma	5 (83%)	1 (25%)	2 (40%)	
Squamous cell carcinoma, metastatic, stomach, forestomach				2 (100%)
Nervous System				
Brain	(50)	(48)	(50)	(50)

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Respiratory System				
Lung	(49)	(49)	(50)	(50)
Alveolar/bronchiolar adenoma	2 (4%)	3 (6%)		1 (2%)
Alveolar/bronchiolar carcinoma			1 (2%)	
Alveolar/bronchiolar carcinoma, multiple				1 (2%)
Carcinoma, metastatic, harderian gland			1 (2%)	
Carcinoma, metastatic, mammary gland		1 (2%)		
Fibrosarcoma, metastatic, skin			1 (2%)	
Hepatocellular carcinoma, metastatic, liver		1 (2%)		1 (2%)
Histiocytic sarcoma	1 (2%)	1 (2%)		1 (2%)
Sarcoma	1 (2%)			
Sarcoma, metastatic, skeletal muscle	1 (2%)			
Mediastinum, histiocytic sarcoma		1 (2%)		
Mediastinum, sarcoma, metastatic, skeletal muscle	1 (2%)			
Nose	(50)	(48)	(50)	(50)
Trachea	(49)	(49)	(50)	(50)
Peritracheal tissue, histiocytic sarcoma		1 (2%)		
Special Senses System				
Eye	(2)	(3)	(2)	(2)
Retrolbulbar, carcinoma, metastatic, harderian gland			1 (50%)	
Harderian gland	(8)	(6)	(4)	(1)
Adenoma	7 (88%)	6 (100%)	2 (50%)	1 (100%)
Carcinoma			2 (50%)	
Urinary System				
Kidney	(50)	(47)	(49)	(48)
Histiocytic sarcoma	1 (2%)	1 (2%)		1 (2%)
Urinary bladder	(48)	(47)	(48)	(48)
Systemic Lesions				
Multiple organs ^b	(50)	(49)	(50)	(50)
Histiocytic sarcoma	1 (2%)	2 (4%)		1 (2%)
Lymphoma malignant	4 (8%)	4 (8%)	6 (12%)	3 (6%)
Neoplasm Summary				
Total animals with primary neoplasms ^c	39	33	38	29
Total primary neoplasms	55	40	69	51
Total animals with benign neoplasms	27	18	27	21
Total benign neoplasms	34	21	40	31
Total animals with malignant neoplasms	17	18	22	15
Total malignant neoplasms	21	19	29	20
Total animals with metastatic neoplasms	1	2	5	4
Total metastatic neoplasms	9	2	5	16

^a Number of animals examined microscopically at the site and the number of animals with neoplasm

^b Number of animals with any tissue examined microscopically

^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE D2
Individual Animal Tumor Pathology of Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal: Vehicle Control

Number of Days on Study	7 7	
	3 3	
	3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5	
Carcass ID Number	2 2	Total Tissues/Tumors
	3 3 4 4 4 0 0 0 0 1 2 2 2 2 3 3 3 3 3 3 4 1 1 1 1	
	3 4 3 4 5 6 7 8 9 0 6 7 8 9 0 5 6 7 8 9 0 2 3 4 5	
General Body System		
Peritoneum		1
Sarcoma, metastatic, skeletal muscle		1
Tissue NOS		1
Thoracic, sarcoma, multiple		1
Genital System		
Clitoral gland	+ + + + + + + + + + M + + + + + + + + + + + + + + +	47
Ovary	+ +	49
Cystadenoma		1
Histiocytic sarcoma		1
Luteoma		1
Oviduct		1
Uterus	+ +	50
Histiocytic sarcoma		1
Sarcoma		1
Hematopoietic System		
Bone marrow	+ +	50
Histiocytic sarcoma		1
Lymph node		8
Mediastinal, histiocytic sarcoma		1
Lymph node, mandibular	+ +	47
Histiocytic sarcoma		1
Lymph node, mesenteric	+ +	47
Histiocytic sarcoma		1
Sarcoma		1
Sarcoma, metastatic, skeletal muscle		1
Spleen	+ +	48
Sarcoma, metastatic, skeletal muscle		1
Thymus	+ + + + + + + + + + + + + + + + + + M + + + + + + + + + +	44
Integumentary System		
Mammary gland	+ +	48
Carcinoma		1
Skin	+ +	50
Sebaceous gland, pinna, adenoma		1
Subcutaneous tissue, hemangiosarcoma		1
Subcutaneous tissue, histiocytic sarcoma		1
Subcutaneous tissue, schwannoma malignant		1
Musculoskeletal System		
Bone	+ +	43
Osteoma		1
Skeletal muscle		6
Sarcoma		5

TABLE D2
Individual Animal Tumor Pathology of Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal: Vehicle Control

Number of Days on Study	7 7	
	3 3	
	3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5	
Carcass ID Number	2 2	Total Tissues/Tumors
	3 3 4 4 4 0 0 0 0 1 2 2 2 2 2 3 3 3 3 3 3 4 1 1 1 1	
	3 4 3 4 5 6 7 8 9 0 6 7 8 9 0 5 6 7 8 9 0 2 3 4 5	
Nervous System		
Brain	+ +	50
Respiratory System		
Lung	+ +	49
Alveolar/bronchiolar adenoma	X	2
Histiocytic sarcoma		1
Sarcoma		1
Sarcoma, metastatic, skeletal muscle		1
Mediastinum, sarcoma, metastatic, skeletal muscle		1
Nose	+ +	50
Trachea	+ +	49
Special Senses System		
Eye		2
Harderian gland	+ + +	8
Adenoma	X X X	7
Urinary System		
Kidney	+ +	50
Histiocytic sarcoma		1
Urinary bladder	+ +	48
Systemic Lesions		
Multiple organs	+ +	50
Histiocytic sarcoma		1
Lymphoma malignant		4

TABLE D2
Individual Animal Tumor Pathology of Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal: 120 mg/kg

Number of Days on Study	7 7	
	3 3	
	4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5 5	
Carcass ID Number	3 3 3 3 3 3 3 3 3 4 3 3 3 3 3 3 3 3 3 3 3 3 3	Total Tissues/Tumors
	9 9 9 9 9 9 9 9 9 0 5 5 5 6 6 6 6 7 7 8 8 8 8 8	
	1 2 3 4 5 6 7 8 9 0 1 2 4 1 2 3 4 6 9 0 1 2 3 4 5	
Respiratory System		
Lung	+ +	50
Alveolar/bronchiolar adenoma		1
Alveolar/bronchiolar carcinoma, multiple		1
Hepatocellular carcinoma, metastatic, liver	X	1
Histiocytic sarcoma		1
Nose	+ +	50
Trachea	+ +	50
Special Senses System		
Eye		2
Harderian gland	+	1
Adenoma	X	1
Lacrimal gland		1
Urinary System		
Kidney	+ +	48
Histiocytic sarcoma		1
Urinary bladder	+ +	48
Systemic Lesions		
Multiple organs	+ +	50
Histiocytic sarcoma		1
Lymphoma malignant	X	3

TABLE D3
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Harderian Gland: Adenoma				
Overall rate ^a	7/50 (14%)	6/49 (12%)	2/50 (4%)	1/50 (2%)
Adjusted rate ^b	14.7%	13.1%	4.3%	2.2%
Terminal rate ^c	7/42 (17%)	6/37 (16%)	2/37 (5%)	1/39 (3%)
First incidence (days) ^d	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.011N	P=0.531N	P=0.084N	P=0.034N
Harderian Gland: Adenoma or Carcinoma				
Overall rate	7/50 (14%)	6/49 (12%)	4/50 (8%)	1/50 (2%)
Adjusted rate	14.7%	13.1%	8.5%	2.2%
Terminal rate	7/42 (17%)	6/37 (16%)	2/37 (5%)	1/39 (3%)
First incidence (days)	729 (T)	729 (T)	649	729 (T)
Poly-3 test	P=0.019N	P=0.531N	P=0.270N	P=0.034N
Liver: Hepatocellular Adenoma				
Overall rate	11/50 (22%)	3/49 (6%)	11/50 (22%)	6/50 (12%)
Adjusted rate	23.1%	6.6%	23.1%	13.0%
Terminal rate	10/42 (24%)	2/37 (5%)	6/37 (16%)	6/39 (15%)
First incidence (days)	698	713	627	729 (T)
Poly-3 test	P=0.302N	P=0.024N	P=0.595	P=0.160N
Liver: Hepatocellular Carcinoma				
Overall rate	3/50 (6%)	4/49 (8%)	3/50 (6%)	4/50 (8%)
Adjusted rate	6.3%	8.7%	6.4%	8.6%
Terminal rate	2/42 (5%)	2/37 (5%)	3/37 (8%)	3/39 (8%)
First incidence (days)	609	616	729 (T)	657
Poly-3 test	P=0.446	P=0.480	P=0.648	P=0.481
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	13/50 (26%)	7/49 (14%)	14/50 (28%)	10/50 (20%)
Adjusted rate	27.0%	15.1%	29.4%	21.6%
Terminal rate	11/42 (26%)	4/37 (11%)	9/37 (24%)	9/39 (23%)
First incidence (days)	609	616	627	657
Poly-3 test	P=0.485N	P=0.122N	P=0.488	P=0.354N
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	2/49 (4%)	3/49 (6%)	0/50 (0%)	1/50 (2%)
Adjusted rate	4.3%	6.6%	0.0%	2.2%
Terminal rate	2/41 (5%)	3/37 (8%)	0/37 (0%)	1/39 (3%)
First incidence (days)	729 (T)	729 (T)	— ^e	729 (T)
Poly-3 test	P=0.238N	P=0.491	P=0.237N	P=0.504N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	2/49 (4%)	3/49 (6%)	1/50 (2%)	2/50 (4%)
Adjusted rate	4.3%	6.6%	2.2%	4.3%
Terminal rate	2/41 (5%)	3/37 (8%)	1/37 (3%)	2/39 (5%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.498N	P=0.491	P=0.500N	P=0.690

TABLE D3
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Mammary Gland: Carcinoma				
Overall rate	1/50 (2%)	3/49 (6%)	1/50 (2%)	0/50 (0%)
Adjusted rate	2.1%	6.6%	2.2%	0.0%
Terminal rate	1/42 (2%)	2/37 (5%)	1/37 (3%)	0/39 (0%)
First incidence (days)	729 (T)	710	729 (T)	—
Poly-3 test	P=0.206N	P=0.291	P=0.756	P=0.506N
Ovary: Cystadenoma				
Overall rate	1/49 (2%)	1/47 (2%)	3/48 (6%)	1/48 (2%)
Adjusted rate	2.2%	2.3%	6.7%	2.3%
Terminal rate	1/41 (2%)	1/36 (3%)	3/36 (8%)	1/37 (3%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.531	P=0.747	P=0.290	P=0.748
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	2/46 (4%)	5/48 (10%)	7/47 (15%)	3/50 (6%)
Adjusted rate	4.6%	11.1%	15.9%	6.5%
Terminal rate	2/40 (5%)	3/37 (8%)	6/36 (17%)	3/39 (8%)
First incidence (days)	729 (T)	649	725	729 (T)
Poly-3 test	P=0.494	P=0.228	P=0.079	P=0.521
Pituitary Gland (Pars Distalis): Adenoma or Carcinoma				
Overall rate	2/46 (4%)	5/48 (10%)	8/47 (17%)	3/50 (6%)
Adjusted rate	4.6%	11.1%	18.0%	6.5%
Terminal rate	2/40 (5%)	3/37 (8%)	6/36 (17%)	3/39 (8%)
First incidence (days)	729 (T)	649	643	729 (T)
Poly-3 test	P=0.477	P=0.228	P=0.046	P=0.521
Skeletal Muscle: Sarcoma				
Overall rate	5/50 (10%)	1/49 (2%)	2/50 (4%)	0/50 (0%)
Adjusted rate	10.5%	2.2%	4.3%	0.0%
Terminal rate	3/42 (7%)	1/37 (3%)	1/37 (3%)	0/39 (0%)
First incidence (days)	698	729 (T)	587	—
Poly-3 test	P=0.025N	P=0.112N	P=0.223N	P=0.034N
Skin (Subcutaneous Tissue): Fibrosarcoma				
Overall rate	0/50 (0%)	3/49 (6%)	4/50 (8%)	0/50 (0%)
Adjusted rate	0.0%	6.6%	8.4%	0.0%
Terminal rate	0/42 (0%)	2/37 (5%)	2/37 (5%)	0/39 (0%)
First incidence (days)	—	713	533	— ^f
Poly-3 test	P=0.495N	P=0.112	P=0.060	—
Skin: Fibrous Histiocytoma or Fibrosarcoma				
Overall rate	0/50 (0%)	3/49 (6%)	5/50 (10%)	0/50 (0%)
Adjusted rate	0.0%	6.6%	10.5%	0.0%
Terminal rate	0/42 (0%)	2/37 (5%)	2/37 (5%)	0/39 (0%)
First incidence (days)	—	713	533	—
Poly-3 test	P=0.524N	P=0.112	P=0.031	—

TABLE D3
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Stomach (Forestomach): Squamous Cell Papilloma				
Overall rate	2/50 (4%)	2/49 (4%)	11/50 (22%)	13/50 (26%)
Adjusted rate	4.2%	4.4%	23.5%	28.2%
Terminal rate	2/42 (5%)	2/37 (5%)	10/37 (27%)	13/39 (33%)
First incidence (days)	729 (T)	729 (T)	672	729 (T)
Poly-3 test	P<0.001	P=0.679	P=0.006	P<0.001
Stomach (Forestomach): Squamous Cell Carcinoma				
Overall rate	0/50 (0%)	0/49 (0%)	0/50 (0%)	7/50 (14%)
Adjusted rate	0.0%	0.0%	0.0%	15.0%
Terminal rate	0/42 (0%)	0/37 (0%)	0/37 (0%)	5/39 (13%)
First incidence (days)	—	—	—	614
Poly-3 test	P<0.001	—	—	P=0.007
Stomach (Forestomach): Squamous Cell Papilloma or Squamous Cell Carcinoma				
Overall rate	2/50 (4%)	2/49 (4%)	11/50 (22%)	18/50 (36%)
Adjusted rate	4.2%	4.4%	23.5%	38.6%
Terminal rate	2/42 (5%)	2/37 (5%)	10/37 (27%)	16/39 (41%)
First incidence (days)	729 (T)	729 (T)	672	614
Poly-3 test	P<0.001	P=0.679	P=0.006	P<0.001
Uterus: Stromal Polyp				
Overall rate	0/50 (0%)	1/49 (2%)	3/50 (6%)	1/50 (2%)
Adjusted rate	0.0%	2.2%	6.4%	2.2%
Terminal rate	0/42 (0%)	0/37 (0%)	2/37 (5%)	1/39 (3%)
First incidence (days)	—	349	725	729 (T)
Poly-3 test	P=0.331	P=0.496	P=0.115	P=0.494
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	1/50 (2%)	1/49 (2%)	3/50 (6%)	3/50 (6%)
Adjusted rate	2.1%	2.2%	6.3%	6.5%
Terminal rate	1/42 (2%)	0/37 (0%)	0/37 (0%)	3/39 (8%)
First incidence (days)	729 (T)	643	533	729 (T)
Poly-3 test	P=0.157	P=0.753	P=0.304	P=0.294
All Organs: Malignant Lymphoma				
Overall rate	4/50 (8%)	4/49 (8%)	6/50 (12%)	3/50 (6%)
Adjusted rate	8.3%	8.6%	12.8%	6.4%
Terminal rate	2/42 (5%)	2/37 (5%)	4/37 (11%)	1/39 (3%)
First incidence (days)	635	587	661	543
Poly-3 test	P=0.467N	P=0.621	P=0.354	P=0.518N
All Organs: Benign Neoplasms				
Overall rate	27/50 (54%)	18/49 (37%)	27/50 (54%)	21/50 (42%)
Adjusted rate	56.6%	38.4%	56.7%	45.6%
Terminal rate	26/42 (62%)	15/37 (41%)	21/37 (57%)	21/39 (54%)
First incidence (days)	698	349	627	729 (T)
Poly-3 test	P=0.341N	P=0.055N	P=0.581	P=0.192N

TABLE D3
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
All Organs: Malignant Neoplasms				
Overall rate	17/50 (34%)	18/49 (37%)	22/50 (44%)	15/50 (30%)
Adjusted rate	34.4%	37.8%	44.9%	31.3%
Terminal rate	10/42 (24%)	10/37 (27%)	13/37 (35%)	9/39 (23%)
First incidence (days)	538	587	533	543
Poly-3 test	P=0.415N	P=0.445	P=0.197	P=0.454N
All Organs: Benign or Malignant Neoplasms				
Overall rate	39/50 (78%)	33/49 (67%)	38/50 (76%)	29/50 (58%)
Adjusted rate	78.9%	67.6%	76.5%	60.4%
Terminal rate	32/42 (76%)	22/37 (60%)	26/37 (70%)	23/39 (59%)
First incidence (days)	538	349	533	543
Poly-3 test	P=0.051N	P=0.147N	P=0.481N	P=0.035N

(T)Terminal sacrifice

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for liver, lung, ovary, and pituitary gland; for other tissues, denominator is number of animals necropsied.

^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the vehicle control incidence are the P values 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. A negative trend or a lower incidence in a dosed group is indicated by N.

^e Not applicable; no neoplasms in animal group

^f Value of statistic cannot be computed.

TABLE D4
Historical Incidence of Forestomach Neoplasms in Control Female B6C3F₁ Mice

Study	Incidence in Controls		
	Squamous Cell Papilloma	Squamous Cell Carcinoma	Squamous Cell Papilloma or Squamous Cell Carcinoma
Historical Incidence in Controls Given NTP-2000 Diet^a			
Acrylonitrile (gavage)	3/50	0/50	3/50
Citral (feed)	1/99	0/99	1/99
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	0/50	0/50	0/50
Indium phosphide (inhalation)	0/50	1/50	1/50
60-Hz Magnetic fields (whole body exposure)	1/100	0/100	1/100
Methacrylonitrile (gavage)	0/50	0/50	0/50
<i>o</i> -Nitrotoluene (feed)	1/60	0/60	1/60
<i>p</i> -Nitrotoluene (feed)	0/50	0/50	0/50
Riddelliine (gavage)	0/50	0/50	0/50
Sodium nitrite (drinking water)	1/50	0/50	1/50
Vanadium pentoxide (inhalation)	2/50	0/50	2/50
Overall Historical Incidence in Controls Given NTP-2000 Diet			
Total	9/659 (1.4%)	1/659 (0.2%)	10/659 (1.5%)
Mean ± standard deviation	1.4% ± 2.0%	0.2% ± 0.6%	1.6% ± 1.9%
Range	0%-6%	0%-2%	0%-6%
Historical Incidence in Corn Oil Gavage Controls Given NIH-07 Diet at Southern Research Institute^b			
<i>p</i> -Nitroaniline	3/50	0/50	3/50
Salicylazosulfapyridine	5/50	0/50	5/50
Theophylline	4/50	0/50	4/50
Overall Historical Incidence in Corn Oil Gavage Controls Given NIH-07 Diet			
Total	19/463 (4.1%)	0/463	19/463 (4.1%)
Mean ± standard deviation	4.1% ± 3.5%		4.1% ± 3.5%
Range	0%-10%		0%-10%

^a Data as of December 20, 2000

^b Data as of December 23, 1999

TABLE D5
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death				1
Moribund	2	8	8	4
Natural deaths	6	4	5	6
Survivors				
Died last week of study				2
Terminal sacrifice	42	37	37	37
Missing		1		
Animals examined microscopically	50	49	50	50
Alimentary System				
Esophagus	(49)	(49)	(50)	(50)
Perforation				1 (2%)
Epithelium, hyperplasia, squamous	1 (2%)			
Periesophageal tissue, foreign body				1 (2%)
Gallbladder	(44)	(45)	(47)	(41)
Cyst		3 (7%)	1 (2%)	2 (5%)
Hyperplasia, lymphoid		1 (2%)		
Liver	(50)	(49)	(50)	(50)
Angiectasis		1 (2%)	2 (4%)	
Basophilic focus		2 (4%)		
Congestion, focal		1 (2%)	1 (2%)	1 (2%)
Fibrosis, focal	1 (2%)			
Hemorrhage, focal			1 (2%)	1 (2%)
Hyperplasia, focal, lymphoid	1 (2%)	1 (2%)		
Hyperplasia, lymphoid	2 (4%)		1 (2%)	
Infarct, chronic			1 (2%)	
Infiltration cellular, mixed cell	41 (82%)	35 (71%)	36 (72%)	31 (62%)
Mineralization, focal			1 (2%)	
Mixed cell focus			2 (4%)	
Mixed cell focus, multiple	1 (2%)			
Tension lipidosis			1 (2%)	
Bile duct, cyst	1 (2%)			1 (2%)
Bile duct, hyperplasia	1 (2%)			1 (2%)
Bile duct, hyperplasia, cystic	1 (2%)			
Hepatocyte, basophilic focus	1 (2%)	2 (4%)		1 (2%)
Hepatocyte, cytomegaly		1 (2%)		
Hepatocyte, depletion glycogen, diffuse	2 (4%)	1 (2%)	2 (4%)	1 (2%)
Hepatocyte, eosinophilic focus		1 (2%)		1 (2%)
Hepatocyte, karyomegaly		1 (2%)		
Hepatocyte, mixed cell focus		1 (2%)	1 (2%)	1 (2%)
Hepatocyte, necrosis, focal	1 (2%)	3 (6%)		2 (4%)
Hepatocyte, vacuolization cytoplasmic, diffuse	5 (10%)	2 (4%)	2 (4%)	5 (10%)
Hepatocyte, vacuolization cytoplasmic, focal	2 (4%)	1 (2%)		
Hepatocyte, periportal, depletion glycogen			1 (2%)	
Hepatocyte, periportal, vacuolization cytoplasmic	8 (16%)	5 (10%)	3 (6%)	1 (2%)
Hepatocyte, centrilobular, necrosis	1 (2%)			2 (4%)
Hepatocyte, centrilobular, vacuolization cytoplasmic			2 (4%)	1 (2%)
Hepatocyte, midzonal, vacuolization cytoplasmic	12 (24%)	7 (14%)	3 (6%)	6 (12%)
Oval cell, hyperplasia	1 (2%)			
Portal, infiltration cellular, focal, lymphocyte	1 (2%)			
Serosa, hyperplasia, focal, lymphoid		1 (2%)		

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

TABLE D5
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Alimentary System (continued)				
Mesentery	(31)	(30)	(37)	(25)
Angiectasis	1 (3%)	2 (7%)		
Angiectasis, focal	1 (3%)			1 (4%)
Congestion, focal		1 (3%)		
Fibrosis, focal		1 (3%)		
Hemorrhage		1 (3%)	1 (3%)	
Inflammation, chronic			1 (3%)	
Artery, mineralization	2 (6%)			
Fat, necrosis	29 (94%)	28 (93%)	34 (92%)	22 (88%)
Lymphatic, angiectasis		1 (3%)	3 (8%)	
Pancreas	(47)	(46)	(50)	(47)
Infiltration cellular, focal, mixed cell	1 (2%)			
Necrosis, focal			1 (2%)	
Acinus, atrophy, focal		1 (2%)		1 (2%)
Duct, cyst	1 (2%)		1 (2%)	
Salivary glands	(49)	(48)	(48)	(50)
Hyperplasia, lymphoid			2 (4%)	
Submandibular gland, vacuolization cytoplasmic		1 (2%)		
Stomach, forestomach	(48)	(48)	(50)	(48)
Diverticulum				1 (2%)
Erosion		1 (2%)	1 (2%)	
Erosion, focal			1 (2%)	
Inflammation, focal		2 (4%)		2 (4%)
Ulcer	1 (2%)	1 (2%)	2 (4%)	
Epithelium, hyperplasia, squamous	4 (8%)	8 (17%)	12 (24%)	31 (65%)
Epithelium, ulcer, focal				1 (2%)
Stomach, glandular	(45)	(45)	(48)	(46)
Glands, degeneration, cystic, focal	1 (2%)			1 (2%)
Muscularis, mineralization	1 (2%)			
Tooth	(1)			(2)
Peridental tissue, inflammation, chronic				2 (100%)
Cardiovascular System				
Blood vessel	(2)		(1)	
Aorta, mineralization	2 (100%)			
Pulmonary artery, mineralization	2 (100%)			
Pulmonary vein, mineralization	2 (100%)			
Heart	(49)	(49)	(50)	(50)
Infiltration cellular, mixed cell		1 (2%)		
Mineralization, focal	2 (4%)	1 (2%)	1 (2%)	
Endocrine System				
Adrenal cortex	(50)	(48)	(50)	(50)
Accessory adrenal cortical nodule		2 (4%)	2 (4%)	3 (6%)
Angiectasis	1 (2%)	1 (2%)		
Cyst	2 (4%)			
Cytoplasmic alteration, focal	1 (2%)			
Degeneration, cystic, focal		1 (2%)		
Infiltration cellular, mixed cell				1 (2%)
Subcapsular, hyperplasia, focal			1 (2%)	

TABLE D5
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Endocrine System (continued)				
Adrenal medulla	(50)	(48)	(49)	(49)
Hyperplasia		1 (2%)		
Parathyroid gland	(45)	(46)	(49)	(47)
Cyst	1 (2%)	1 (2%)		2 (4%)
Cyst, multiple			1 (2%)	
Hyperplasia, cystic		1 (2%)		
Pituitary gland	(46)	(48)	(47)	(50)
Angiectasis	3 (7%)	1 (2%)		1 (2%)
Pars distalis, angiectasis				1 (2%)
Pars distalis, cyst, multiple	1 (2%)			
Pars distalis, cytoplasmic alteration, focal	6 (13%)	3 (6%)	2 (4%)	4 (8%)
Pars distalis, degeneration, cystic, focal	2 (4%)	2 (4%)	4 (9%)	1 (2%)
Pars distalis, hemorrhage, focal		3 (6%)		1 (2%)
Pars distalis, hyperplasia			1 (2%)	
Pars distalis, hyperplasia, focal	2 (4%)	5 (10%)	6 (13%)	2 (4%)
Rathke's cleft, hyperplasia, cystic				1 (2%)
Thyroid gland	(49)	(46)	(49)	(50)
Atrophy, focal			1 (2%)	
Degeneration, cystic, focal	7 (14%)	14 (30%)	9 (18%)	11 (22%)
Ectopic thymus			2 (4%)	
Inflammation, chronic			1 (2%)	
Inflammation, chronic, focal			1 (2%)	
Follicle, cyst	1 (2%)	1 (2%)	1 (2%)	
General Body System				
Tissue NOS	(1)	(1)	(2)	(1)
Abdominal, abscess		1 (100%)		
Genital System				
Clitoral gland	(47)	(45)	(45)	(50)
Angiectasis	1 (2%)			
Degeneration, cystic		2 (4%)	2 (4%)	1 (2%)
Inflammation, chronic			1 (2%)	
Ovary	(49)	(47)	(48)	(48)
Angiectasis		1 (2%)	1 (2%)	1 (2%)
Cyst	13 (27%)	11 (23%)	11 (23%)	10 (21%)
Cyst, multiple	1 (2%)		1 (2%)	
Hemorrhage		1 (2%)		
Hyperplasia, cystic	1 (2%)			
Hyperplasia, tubular	1 (2%)			
Inflammation, suppurative				1 (2%)
Thrombosis		1 (2%)	1 (2%)	
Bilateral, cyst	1 (2%)			1 (2%)
Bilateral, follicle, cyst				1 (2%)
Bilateral, follicle, hemorrhage				1 (2%)
Periovarian tissue, mineralization				1 (2%)

TABLE D5
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Genital System (continued)				
Uterus	(50)	(49)	(50)	(50)
Angiectasis	1 (2%)		1 (2%)	2 (4%)
Congestion		1 (2%)		
Cyst	1 (2%)	2 (4%)	1 (2%)	
Cyst, multiple		1 (2%)		
Edema	1 (2%)			
Hemorrhage		1 (2%)		
Hydrometra	33 (66%)	35 (71%)	40 (80%)	39 (78%)
Inflammation, chronic				1 (2%)
Endometrium, hyperplasia, cystic	39 (78%)	39 (80%)	42 (84%)	40 (80%)
Hematopoietic System				
Bone marrow	(50)	(47)	(50)	(48)
Hyperplasia, megakaryocyte			1 (2%)	
Myeloid cell, hyperplasia	3 (6%)	5 (11%)	4 (8%)	5 (10%)
Lymph node	(8)	(9)	(9)	(7)
Iliac, hyperplasia, plasma cell	1 (13%)	1 (11%)		1 (14%)
Iliac, pigmentation	1 (13%)			
Inguinal, hyperplasia, lymphoid	1 (13%)		2 (22%)	
Inguinal, hyperplasia, plasma cell	1 (13%)	1 (11%)		
Inguinal, infiltration cellular, mixed cell				1 (14%)
Mediastinal, hemorrhage				1 (14%)
Mediastinal, hyperplasia, lymphoid	1 (13%)	2 (22%)	2 (22%)	
Mediastinal, hyperplasia, plasma cell	1 (13%)			1 (14%)
Renal, hyperplasia, plasma cell				1 (14%)
Renal, infiltration cellular, mixed cell				1 (14%)
Lymph node, mandibular	(47)	(47)	(48)	(49)
Congestion			1 (2%)	
Hyperplasia, lymphoid	1 (2%)			
Hyperplasia, plasma cell	1 (2%)			
Lymph node, mesenteric	(47)	(46)	(49)	(49)
Angiectasis		2 (4%)		
Atypia cellular		1 (2%)		
Hyperplasia, cystic				1 (2%)
Hyperplasia, histiocytic		1 (2%)	2 (4%)	
Hyperplasia, lymphoid		1 (2%)	2 (4%)	
Hyperplasia, plasma cell	3 (6%)	2 (4%)		
Spleen	(48)	(47)	(50)	(49)
Accessory spleen		1 (2%)		
Angiectasis		1 (2%)		1 (2%)
Congestion	1 (2%)	3 (6%)		2 (4%)
Depletion cellular			2 (4%)	
Hematopoietic cell proliferation	20 (42%)	29 (62%)	29 (58%)	17 (35%)
Hemorrhage	1 (2%)	1 (2%)		1 (2%)
Hyperplasia, histiocytic	1 (2%)			
Hyperplasia, lymphoid	8 (17%)	9 (19%)	7 (14%)	7 (14%)
Necrosis, focal	1 (2%)	2 (4%)		
Pigmentation, focal			1 (2%)	
Thrombosis				1 (2%)
Thymus	(44)	(46)	(48)	(46)
Atrophy			1 (2%)	
Cyst			1 (2%)	3 (7%)
Hemorrhage	1 (2%)			
Hyperplasia, lymphoid		1 (2%)	1 (2%)	2 (4%)

TABLE D5
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Integumentary System				
Mammary gland	(48)	(49)	(50)	(50)
Ectasia	2 (4%)	1 (2%)	2 (4%)	1 (2%)
Hyperplasia	2 (4%)		3 (6%)	
Metaplasia, focal, squamous		1 (2%)		
Skin	(50)	(49)	(50)	(50)
Ulcer	1 (2%)			
Hair follicle, atrophy, focal	1 (2%)			
Subcutaneous tissue, fibrosis, focal	1 (2%)			
Subcutaneous tissue, hemorrhage, focal		1 (2%)		1 (2%)
Subcutaneous tissue, infiltration cellular, focal, mixed cell		1 (2%)		
Subcutaneous tissue, inflammation, chronic			1 (2%)	
Musculoskeletal System				
Skeletal muscle	(6)	(4)	(5)	(2)
Fibrosis			1 (20%)	
Inflammation, chronic	1 (17%)			
Nervous System				
Brain	(50)	(48)	(50)	(50)
Compression, focal	2 (4%)	3 (6%)	2 (4%)	1 (2%)
Hemorrhage, focal			1 (2%)	1 (2%)
Ventricle, hydrocephalus	1 (2%)		1 (2%)	
Respiratory System				
Lung	(49)	(49)	(50)	(50)
Congestion	1 (2%)	2 (4%)	2 (4%)	2 (4%)
Fibrosis, focal	1 (2%)			
Hemorrhage	1 (2%)	2 (4%)	2 (4%)	
Hemorrhage, focal				1 (2%)
Hyperplasia, histiocytic	2 (4%)		1 (2%)	
Hyperplasia, lymphoid	1 (2%)	3 (6%)	2 (4%)	
Infiltration cellular, focal, mixed cell				1 (2%)
Infiltration cellular, mixed cell		1 (2%)		
Inflammation, suppurative				1 (2%)
Thrombosis	1 (2%)			
Alveolar epithelium, hyperplasia				1 (2%)
Artery, mineralization	1 (2%)			
Vein, mineralization	1 (2%)			
Nose	(50)	(48)	(50)	(50)
Hemorrhage				1 (2%)
Inflammation, suppurative	18 (36%)	22 (46%)	23 (46%)	16 (32%)
Mucosa, glands, dilatation, focal	5 (10%)		2 (4%)	6 (12%)
Nasolacrimal duct, cyst			1 (2%)	
Nasolacrimal duct, inflammation	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Nasolacrimal duct, mineralization	1 (2%)			
Olfactory epithelium, cytoplasmic alteration	28 (56%)	23 (48%)	34 (68%)	28 (56%)
Respiratory epithelium, cytoplasmic alteration	50 (100%)	47 (98%)	49 (98%)	48 (96%)
Respiratory epithelium, necrosis		1 (2%)		1 (2%)

TABLE D5
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of 2,4-Hexadienal

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
Special Senses System				
Eye	(2)	(3)	(2)	(2)
Atrophy		2 (67%)	2 (100%)	
Cornea, hyperplasia, focal, squamous	1 (50%)	1 (33%)		
Cornea, inflammation, focal	1 (50%)	1 (33%)		
Harderian gland	(8)	(6)	(4)	(1)
Hyperplasia, focal	1 (13%)			
Urinary System				
Kidney	(50)	(47)	(49)	(48)
Atrophy				1 (2%)
Congestion		1 (2%)	1 (2%)	
Cyst	3 (6%)	4 (9%)	1 (2%)	1 (2%)
Cyst, multiple			1 (2%)	
Hydronephrosis				1 (2%)
Hyperplasia, lymphoid	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Infarct	3 (6%)	1 (2%)	5 (10%)	4 (8%)
Infarct, focal	1 (2%)			
Inflammation, chronic, focal		1 (2%)		
Metaplasia, focal, osseous	2 (4%)	1 (2%)		1 (2%)
Nephropathy	19 (38%)	15 (32%)	17 (35%)	22 (46%)
Artery, mineralization	2 (4%)			
Papilla, mineralization, focal			1 (2%)	
Papilla, necrosis			4 (8%)	1 (2%)
Renal tubule, accumulation, hyaline droplet	3 (6%)		2 (4%)	1 (2%)
Renal tubule, accumulation, hyaline droplet, focal				1 (2%)
Renal tubule, necrosis			1 (2%)	
Renal tubule, pigmentation		1 (2%)		2 (4%)
Urinary bladder	(48)	(47)	(48)	(48)
Transitional epithelium, hyperplasia	1 (2%)			

APPENDIX E

GENETIC TOXICOLOGY

<i>SALMONELLA TYPHIMURIUM</i> MUTAGENICITY TEST PROTOCOL	226
RAT AND MOUSE BONE MARROW MICRONUCLEUS TEST PROTOCOL	226
MOUSE PERIPHERAL BLOOD MICRONUCLEUS TEST PROTOCOL	227
EVALUATION PROTOCOL	227
RESULTS	227
TABLE E1 Mutagenicity of 2,4-Hexadienal in <i>Salmonella typhimurium</i>	228
TABLE E2 Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Rats Treated with 2,4-Hexadienal by a Single Intraperitoneal Injection	231
TABLE E3 Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Mice Treated with 2,4-Hexadienal by Intraperitoneal Injection	231
TABLE E4 Frequency of Micronuclei in Peripheral Blood Normochromatic Erythrocytes of Mice Following Treatment with 2,4-Hexadienal by Gavage for 14 Weeks	232

GENETIC TOXICOLOGY

SALMONELLA TYPHIMURIUM MUTAGENICITY TEST PROTOCOL

Testing was performed as reported by Zeiger *et al.* (1992). 2,4-Hexadienal was sent to the laboratories as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains TA98, TA100, and TA1535 either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and at least five doses of 2,4-hexadienal. The high dose was limited by toxicity. All positive trials were repeated under the conditions that elicited the positive response or with a higher S9 fraction.

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

RAT AND MOUSE BONE MARROW MICRONUCLEUS TEST PROTOCOL

Preliminary range-finding studies were performed. Factors affecting dose selection included chemical solubility and toxicity and the extent of cell cycle delay induced by 2,4-hexadienal exposure. The standard three-exposure protocol (three injections at 24-hour intervals) used in the mouse study is described in detail by Shelby *et al.* (1993); the protocol used in the rat study is similar except only one injection was administered. Male F344/N rats and B6C3F₁ mice were injected intraperitoneally with 2,4-hexadienal dissolved in corn oil. Vehicle control animals were injected with corn oil only. The positive control animals received injections of cyclophosphamide (25 mg/kg). The animals were killed 24 hours after the final injection, and blood smears were prepared from bone marrow cells obtained from the femurs. Air-dried smears were fixed and stained; 2,000 polychromatic erythrocytes (PCEs) were scored for the frequency of micronucleated cells in up to five animals per dose group.

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

MOUSE PERIPHERAL BLOOD MICRONUCLEUS TEST PROTOCOL

Detailed discussions of this assay are presented by MacGregor *et al.* (1990) and Witt *et al.* (2000). At the end of the 14-week toxicity study, peripheral blood samples were obtained from male and female mice. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange (Tice *et al.*, 1990) and coded. Slides were scanned to determine the frequency of micronuclei in 1,000 normochromatic erythrocytes (NCEs) in up to 10 animals per dose group.

The results were tabulated as described for polychromatic erythrocytes in the bone marrow micronucleus test. Results of the 14-week studies were accepted without repeat tests, because additional test data could not be obtained.

EVALUATION PROTOCOL

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

RESULTS

2,4-Hexadienal was tested at two laboratories for induction of mutations in three strains of *S. typhimurium* (Table E1). Neither laboratory detected mutagenic activity in strains TA98 or TA1535, with or without Aroclor 1254-induced rat or hamster liver S9 enzymes. At one laboratory, significant responses were seen in strain TA100 without S9 and in the presence of 5%, 10%, or 30% S9 from rat and hamster liver. At the second laboratory, results in TA100 in the absence of S9 were negative; a positive response was noted with 30% hamster and 30% rat liver S9. Additional concentrations of S9 were not tested in this second laboratory study. Both laboratories tested similar concentrations of 2,4-hexadienal. Strain TA100 mutates via base pair substitution. Results of acute tests with 2,4-hexadienal for induction of micronuclei in bone marrow PCEs of male rats (Table E2) and male mice (Table E3) were judged to be inconclusive. Each of the initial trials, one in rats and one in mice, gave an indication of an effect. In the mouse study, trend analysis of the response over the dose range of 40 to 160 mg/kg 2,4-hexadienal yielded a P value of 0.024, which is significant. However, no individual groups were significantly elevated over the concurrent vehicle control group. In the rat study, the trend test P value was 0.017, which is also significant. As with the study in male mice, none of the mean values for the individual groups of treated rats differed significantly from the concurrent control group value. Because no repeat testing was performed to confirm the response in either rats or mice, the results in both bone marrow micronucleus tests were judged to be inconclusive. No increases in the frequencies of micronucleated normochromatic (mature) erythrocytes were seen in peripheral blood samples obtained from male or female mice after 14 weeks exposure to 2,4-hexadienal (7.5 to 120 mg/kg) by gavage (Table E4).

In summary, 2,4-hexadienal induced gene mutations in *S. typhimurium*, but it did not conclusively affect erythrocyte micronucleus frequencies, an indirect indicator of numerical or chromosomal damage, in rats or mice after acute or subchronic administration.

TABLE E1
Mutagenicity of 2,4-Hexadienal in *Salmonella typhimurium*^a

Strain	Dose ($\mu\text{g}/\text{plate}$)	Revertants/Plate ^b					
		-S9					
		Trial 1	Trial 2	Trial 3			
Study performed at SRI International							
TA100	0	108 \pm 2.0	120 \pm 2.6	99 \pm 4.9			
	3	119 \pm 1.5					
	10	128 \pm 3.3	119 \pm 4.7	114 \pm 0.9			
	33	121 \pm 1.9	131 \pm 0.3	139 \pm 6.2			
	66		150 \pm 3.6	143 \pm 5.7			
	100	171 \pm 8.0	155 \pm 4.3	162 \pm 3.3			
	166		210 \pm 10.0	248 \pm 10.0			
	333	82 \pm 5.8 ^c		Toxic			
Trial summary		Weakly Positive	Positive				
Positive control ^d	910 \pm 17.3	946 \pm 11.3	1,166 \pm 24.6				
				+hamster S9			
		5%	5%	10%	30%	30%	30%
TA100 (continued)	0	92 \pm 6.9	129 \pm 13.8	128 \pm 1.2	143 \pm 3.6	129 \pm 5.2	102 \pm 5.7
	3		126 \pm 8.5				
	10	119 \pm 7.5	156 \pm 22.6		140 \pm 2.8		
	33	125 \pm 2.4	185 \pm 9.5		141 \pm 7.8		
	66	172 \pm 1.5	248 \pm 6.2	118 \pm 19.3		129 \pm 1.2	113 \pm 8.2
	100	251 \pm 11.8	281 \pm 13.2	157 \pm 18.8	140 \pm 5.5	142 \pm 7.0	132 \pm 2.5
	166	195 \pm 16.0	228 \pm 13.5 ^c	240 \pm 22.5		151 \pm 1.5	161 \pm 11.0
	333	Toxic		290 \pm 14.1	143 \pm 3.8	187 \pm 6.2	256 \pm 23.0
	666			Toxic		140 \pm 1.3	163 \pm 7.0
	1,000				167 \pm 2.3		
Trial summary	Positive	Positive	Positive	Negative	Equivocal	Positive	
Positive control	758 \pm 21.1	585 \pm 8.1	742 \pm 4.7	477 \pm 7.6	525 \pm 13.3	627 \pm 17.9	
				+rat S9			
		5%	5%	10%	30%	30%	30%
TA100 (continued)	0	96 \pm 2.6	121 \pm 5.4	94 \pm 4.5	145 \pm 5.0	127 \pm 5.2	135 \pm 9.5
	10	110 \pm 1.9			148 \pm 5.8		
	33	113 \pm 12.9	157 \pm 5.8		145 \pm 5.7		
	66	181 \pm 3.8	181 \pm 3.1	153 \pm 2.7		136 \pm 6.6	162 \pm 15.0
	100	314 \pm 13.2	282 \pm 12.7	209 \pm 9.3	163 \pm 5.0	144 \pm 10.0	167 \pm 12.4
	166	384 \pm 13.4	327 \pm 11.3	246 \pm 10.4		163 \pm 10.7	215 \pm 9.2
	333	Toxic	123 \pm 32.9 ^c	364 \pm 8.3	175 \pm 4.8	205 \pm 10.9	253 \pm 13.8
	666			229 \pm 31.0 ^c		159 \pm 13.3	136 \pm 5.2
	1,000				84 \pm 9.8 ^c		
Trial summary	Positive	Positive	Positive	Negative	Weakly Positive	Weakly Positive	
Positive control	757 \pm 18.9	552 \pm 23.3	630 \pm 8.5	423 \pm 16.8	473 \pm 8.2	544 \pm 17.8	

TABLE E1
Mutagenicity of 2,4-Hexadienal in *Salmonella typhimurium*

Strain	Dose ($\mu\text{g}/\text{plate}$)	Revertants/Plate					
		-S9	+30% hamster S9	+30% rat S9			
Study performed at SRI International (continued)							
TA1535	0	10 \pm 0.9	11 \pm 0.9	10 \pm 1.2			
	10	12 \pm 0.0					
	33	10 \pm 1.2					
	66	11 \pm 2.0	11 \pm 0.9	10 \pm 0.9			
	100	11 \pm 0.7	9 \pm 0.6	10 \pm 0.9			
	166	11 \pm 0.9	9 \pm 0.3	11 \pm 1.5			
	333		10 \pm 1.2	12 \pm 2.2			
	666		9 \pm 0.6	12 \pm 3.7			
Trial summary	Negative	Negative	Negative				
Positive control	825 \pm 18.3	202 \pm 12.1	152 \pm 14.5				
TA98	0	15 \pm 1.8	17 \pm 1.2	24 \pm 1.5			
	3	20 \pm 4.2					
	10	16 \pm 1.2	19 \pm 3.5	25 \pm 1.2			
	33	21 \pm 1.7	20 \pm 5.4	29 \pm 3.8			
	100	23 \pm 1.5	15 \pm 1.5	29 \pm 2.9			
	166						
	333	5 ^e	18 \pm 0.3	30 \pm 2.3			
	1,000		11 \pm 0.9	19 \pm 5.5			
Trial summary	Negative	Negative	Negative				
Positive control	456 \pm 17.3	356 \pm 8.0	312 \pm 8.1				
Study performed at Environmental Health Research and Testing, Inc.							
		-S9		+30% hamster S9		+30% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
TA100	0	141 \pm 3.5	103 \pm 2.4	139 \pm 3.2	104 \pm 2.0	144 \pm 3.5	116 \pm 3.2
	50	135 \pm 3.2	105 \pm 3.2				
	75	138 \pm 1.8	109 \pm 1.2				
	100	135 \pm 2.6	120 \pm 1.2				
	125	142 \pm 2.8	117 \pm 2.1				
	150	139 \pm 2.4	119 \pm 2.3				
	500			164 \pm 2.2	167 \pm 1.7	317 \pm 3.5	321 \pm 2.3
	750			156 \pm 3.6	161 \pm 3.2	355 \pm 5.0	305 \pm 4.2
	1,000			152 \pm 3.5	171 \pm 3.2	514 \pm 5.0	464 \pm 2.6
	1,250			240 \pm 3.7	260 \pm 2.3	723 \pm 4.6	656 \pm 3.5
	1,500			233 \pm 4.3	263 \pm 4.3	225 \pm 5.7	206 \pm 2.4
	Trial summary	Negative	Negative	Weakly Positive	Positive	Positive	Positive
Positive control	591 \pm 5.8	458 \pm 17.5	859 \pm 32.9	532 \pm 10.1	457 \pm 9.0	414 \pm 13.2	

TABLE E1
Mutagenicity of 2,4-Hexadienal in *Salmonella typhimurium*

Strain	Dose ($\mu\text{g}/\text{plate}$)	Revertants/Plate		
		-S9	+30% hamster S9	+30% rat S9
Study performed at Environmental Health Research and Testing, Inc. (continued)				
TA1535	0	20 \pm 1.9	18 \pm 1.5	17 \pm 1.9
	50	17 \pm 1.7		
	75	17 \pm 1.0		
	100	21 \pm 1.9		
	125	17 \pm 1.2		
	150	13 \pm 1.5		
	500		17 \pm 2.0	19 \pm 1.5
	750		20 \pm 1.9	19 \pm 2.5
	1,000		18 \pm 1.5	20 \pm 2.3
	1,250		19 \pm 2.6	21 \pm 1.2
	1,500		23 \pm 2.3	19 \pm 2.0
	Trial summary	Negative	Negative	Negative
	Positive control	267 \pm 8.1	253 \pm 8.4	243 \pm 3.2
	TA98	0	24 \pm 2.1	33 \pm 2.3
50		21 \pm 2.0		
75		18 \pm 2.0		
100		20 \pm 1.8		
125		20 \pm 1.5		
150		18 \pm 1.2		
500			37 \pm 2.4	29 \pm 0.9
750			37 \pm 3.3	35 \pm 1.5
1,000			40 \pm 2.0	37 \pm 3.0
1,250			35 \pm 2.6	22 \pm 3.5
1,500			29 \pm 1.5	34 \pm 2.4
Trial summary		Negative	Negative	Negative
Positive control		264 \pm 7.8	487 \pm 21.8	425 \pm 8.4

^a The detailed protocol is presented by Zeiger *et al.* (1992). 0 $\mu\text{g}/\text{plate}$ was the solvent control.

^b Revertants are presented as mean \pm standard error from three plates.

^c Slight toxicity

^d The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535) and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

^e Precipitate observed with slight toxicity

TABLE E2
Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Rats Treated with 2,4-Hexadienal by a Single Intraperitoneal Injection^a

	Dose (mg/kg)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs ^b	P Value ^c
Corn oil ^d		5	0.30 ± 0.20	
2,4-Hexadienal	50	5	0.80 ± 0.44	0.0658
	100	5	1.00 ± 0.35	0.0261
	150	5	1.10 ± 0.48	0.0162
	200	3	1.17 ± 0.17	0.0169
			P=0.017 ^e	
Cyclophosphamide ^f	25	5	7.50 ± 2.77	0.0000

^a Study was performed at ILS, Inc. The detailed protocol is presented by Shelby *et al.* (1993). PCE=polychromatic erythrocyte

^b Mean ± standard error

^c Pairwise comparison with the vehicle control. Dosed group values are significant at P≤0.006; positive control values are significant at P≤0.05 (ILS, 1990)

^d Vehicle control

^e Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test; significant at P≤0.025 (ILS, 1990)

^f Positive control

TABLE E3
Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Mice Treated with 2,4-Hexadienal by Intraperitoneal Injection^a

	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs ^b	P Value ^c
Corn oil ^d		5	1.80 ± 0.49	
2,4-Hexadienal	40	5	1.40 ± 0.33	0.7604
	80	5	1.90 ± 0.37	0.4346
	120	3	1.67 ± 0.17	0.5774
	160	4	3.13 ± 1.16	0.0352
			P=0.024 ^e	
Cyclophosphamide ^f	25	4	11.25 ± 2.17	0.0000

^a Study was performed at ILS, Inc. The detailed protocol is presented by Shelby *et al.* (1993). PCE=polychromatic erythrocyte

^b Mean ± standard error

^c Pairwise comparison with the vehicle control. Dosed group values are significant at P≤0.006; positive control values are significant at P≤0.05 (ILS, 1990)

^d Vehicle control

^e Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test; significant at P≤0.025 (ILS, 1990)

^f Positive control

TABLE E4
Frequency of Micronuclei in Peripheral Blood Normochromatic Erythrocytes
of Mice Following Treatment with 2,4-Hexadienal by Gavage for 14 Weeks^a

	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs ^b	P Value ^c
Male				
Corn oil ^d		8	2.25 ± 0.41	
2,4-Hexadienal	7.5	10	1.60 ± 0.16	0.8409
	15	10	2.20 ± 0.42	0.5282
	30	10	1.80 ± 0.39	0.7490
	60	9	2.33 ± 0.44	0.4549
	120	10	2.10 ± 0.38	0.5851
			P=0.360 ^e	
Female				
Corn oil		10	1.20 ± 0.25	
2,4-Hexadienal	7.5	10	1.40 ± 0.34	0.3473
	15	10	1.60 ± 0.31	0.2247
	30	10	1.70 ± 0.21	0.1764
	60	10	1.30 ± 0.21	0.4207
	120	10	1.80 ± 0.36	0.1365
			P=0.215	

^a Study was performed at ILS, Inc. The detailed protocol is presented by MacGregor *et al.* (1990). NCE=normochromatic erythrocyte

^b Mean ± standard error

^c Pairwise comparison with the vehicle control; significant at P≤0.005 (ILS, 1990)

^d Vehicle control

^e Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test; significant at P≤0.025 (ILS, 1990)

APPENDIX F

CLINICAL PATHOLOGY RESULTS

TABLE F1	Hematology and Clinical Chemistry Data for Rats in the 14-Week Gavage Study of 2,4-Hexadienal	234
TABLE F2	Hematology Data for Mice in the 14-Week Gavage Study of 2,4-Hexadienal	239

TABLE F1
Hematology and Chemical Chemistry Data for Rats in the 14-Week Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Male						
n						
Day 4	10	10	10	10	10	10
Day 19	10	10	10	10	10	9
Week 14	10	10	10	10	10	10
Hematology						
Hematocrit (%)						
Day 4	42.7 ± 0.6	43.0 ± 0.8	42.8 ± 0.7	42.4 ± 0.6	42.0 ± 0.6	43.9 ± 0.6
Day 19	45.7 ± 0.6	44.9 ± 0.7	43.5 ± 1.2	43.6 ± 0.7	43.2 ± 1.0	44.4 ± 0.8
Week 14	46.1 ± 0.4	44.5 ± 0.3*	45.7 ± 0.4	45.7 ± 0.4	45.2 ± 0.3	47.3 ± 0.3
Hemoglobin (g/dL)						
Day 4	13.7 ± 0.2	13.8 ± 0.4	13.7 ± 0.2	13.4 ± 0.2	13.4 ± 0.3	13.9 ± 0.2
Day 19	15.5 ± 0.2	15.1 ± 0.3	14.7 ± 0.4	14.7 ± 0.2	14.6 ± 0.4	14.8 ± 0.2
Week 14	15.4 ± 0.1	14.8 ± 0.1**	15.0 ± 0.1*	15.4 ± 0.1	15.1 ± 0.1	15.4 ± 0.1
Erythrocytes (10⁶/μL)						
Day 4	7.29 ± 0.13	7.39 ± 0.17	7.29 ± 0.10	7.22 ± 0.11	7.18 ± 0.12	7.54 ± 0.10
Day 19	7.77 ± 0.14	7.67 ± 0.16	7.36 ± 0.18	7.29 ± 0.14	7.34 ± 0.18	7.56 ± 0.15
Week 14	9.05 ± 0.08	8.76 ± 0.05*	8.93 ± 0.08	9.01 ± 0.09	8.83 ± 0.06	9.10 ± 0.06
Reticulocytes (10⁶/μL)						
Day 4	5.49 ± 0.37	5.14 ± 0.36	5.89 ± 0.36	5.59 ± 0.22	4.94 ± 0.19	5.74 ± 0.29
Day 19	3.40 ± 0.14	3.46 ± 0.12	3.47 ± 0.12	3.68 ± 0.17	3.51 ± 0.14	3.49 ± 0.15
Week 14	2.53 ± 0.06	2.62 ± 0.15	2.53 ± 0.05	2.45 ± 0.05	2.44 ± 0.04	2.52 ± 0.10
Mean cell volume (fL)						
Day 4	58.7 ± 0.5	58.2 ± 0.4	58.7 ± 0.5	58.8 ± 0.3	58.5 ± 0.2	58.3 ± 0.2
Day 19	58.9 ± 0.7	58.6 ± 0.6	59.1 ± 0.5	59.9 ± 0.2	58.9 ± 0.5	58.7 ± 0.3
Week 14	51.0 ± 0.2	50.8 ± 0.2	51.1 ± 0.1	50.7 ± 0.2	51.2 ± 0.2	51.9 ± 0.2*
Mean cell hemoglobin (pg)						
Day 4	18.8 ± 0.1	18.7 ± 0.1	18.8 ± 0.2	18.6 ± 0.2	18.6 ± 0.2	18.4 ± 0.1
Day 19	20.0 ± 0.2	19.7 ± 0.1	20.0 ± 0.2	20.2 ± 0.1	19.8 ± 0.1	19.5 ± 0.2
Week 14	17.0 ± 0.1	16.9 ± 0.0	16.8 ± 0.1*	17.0 ± 0.1	17.1 ± 0.1	16.9 ± 0.1
Mean cell hemoglobin concentration (g/dL)						
Day 4	32.0 ± 0.3	32.2 ± 0.4	32.1 ± 0.4	31.6 ± 0.3	31.8 ± 0.3	31.6 ± 0.2
Day 19	33.9 ± 0.3	33.6 ± 0.3	33.8 ± 0.3	33.7 ± 0.1	33.6 ± 0.2	33.3 ± 0.2
Week 14	33.4 ± 0.1	33.3 ± 0.2	32.8 ± 0.1*	33.6 ± 0.1	33.4 ± 0.1	32.5 ± 0.1**
Platelets (10³/μL)						
Day 4	937.3 ± 16.4	981.2 ± 35.0	961.5 ± 21.9	972.0 ± 31.1	916.8 ± 18.5	982.1 ± 32.7
Day 19	890.0 ± 16.3	945.0 ± 50.5	926.9 ± 30.3	931.0 ± 21.3	954.0 ± 21.0	991.4 ± 22.7*
Week 14	705.2 ± 15.8	700.3 ± 11.5	689.8 ± 21.0	700.1 ± 10.3	719.7 ± 9.1	688.6 ± 11.0
Leukocytes (10³/μL)						
Day 4	8.64 ± 0.42	8.51 ± 0.55	8.52 ± 0.37	8.10 ± 0.51	9.36 ± 0.46	6.80 ± 0.42
Day 19	8.98 ± 0.31	9.09 ± 0.34	9.82 ± 0.56	9.24 ± 0.30	9.67 ± 0.44	8.60 ± 0.36
Week 14	7.96 ± 0.30	7.54 ± 0.31	8.23 ± 0.37	7.43 ± 0.17	7.17 ± 0.31	6.61 ± 0.32**
Segmented neutrophils (10³/μL)						
Day 4	1.16 ± 0.07	1.02 ± 0.06	1.10 ± 0.07	1.12 ± 0.06	1.33 ± 0.09	1.48 ± 0.11*
Day 19	0.83 ± 0.04	0.88 ± 0.04	0.86 ± 0.05	0.98 ± 0.07	0.90 ± 0.03	1.49 ± 0.11**
Week 14	1.26 ± 0.04	1.27 ± 0.09	1.31 ± 0.11	1.20 ± 0.05	1.19 ± 0.03	1.69 ± 0.17
Lymphocytes (10³/μL)						
Day 4	7.13 ± 0.35	7.19 ± 0.47	7.12 ± 0.33	6.69 ± 0.44	7.66 ± 0.41	5.06 ± 0.37*
Day 19	7.85 ± 0.28	7.96 ± 0.31	8.66 ± 0.51	7.99 ± 0.25	8.47 ± 0.41	6.78 ± 0.36
Week 14	6.27 ± 0.25	5.87 ± 0.34	6.51 ± 0.28	5.89 ± 0.20	5.63 ± 0.30	4.61 ± 0.23**

TABLE F1
Hematology and Chemical Chemistry Data for Rats in the 14-Week Gavage Study of 2,4-Hexadienal

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Male (continued)						
n						
Day 4	10	10	10	10	10	10
Day 19	10	10	10	10	10	9
Week 14	10	10	10	10	10	10
Hematology (continued)						
Activated lymphocytes (10 ³ /μL)						
Day 4	0.13 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.12 ± 0.02	0.13 ± 0.01	0.16 ± 0.01
Day 19	0.14 ± 0.01	0.11 ± 0.01	0.14 ± 0.02	0.12 ± 0.01	0.14 ± 0.01	0.15 ± 0.03
Week 14	0.17 ± 0.02	0.17 ± 0.01	0.17 ± 0.02	0.13 ± 0.01	0.14 ± 0.01	0.12 ± 0.01
Monocytes (10 ³ /μL)						
Day 4	0.08 ± 0.01	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.01*	0.09 ± 0.02	0.06 ± 0.01
Day 19	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.00	0.06 ± 0.01
Week 14	0.11 ± 0.02	0.08 ± 0.00	0.11 ± 0.02	0.10 ± 0.01	0.08 ± 0.00	0.10 ± 0.01
Basophils (10 ³ /μL)						
Day 4	0.064 ± 0.007	0.059 ± 0.009	0.064 ± 0.009	0.058 ± 0.011	0.069 ± 0.011	0.038 ± 0.005
Day 19	0.053 ± 0.006	0.039 ± 0.003	0.064 ± 0.009	0.056 ± 0.005	0.057 ± 0.007	0.057 ± 0.016
Week 14	0.059 ± 0.010	0.063 ± 0.008	0.048 ± 0.007	0.034 ± 0.004	0.039 ± 0.007	0.030 ± 0.005
Eosinophils (10 ³ /μL)						
Day 4	0.06 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.07 ± 0.01	0.03 ± 0.00**
Day 19	0.05 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
Week 14	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.00	0.07 ± 0.01
Clinical Chemistry						
Urea nitrogen (mg/dL)						
Day 4	7.9 ± 0.4	8.4 ± 0.5	8.2 ± 0.2	8.2 ± 0.2	8.1 ± 0.5	8.3 ± 0.3
Day 19	11.9 ± 0.4	13.5 ± 0.5	13.2 ± 0.5	12.6 ± 0.4	12.4 ± 0.4	11.4 ± 0.6
Week 14	13.6 ± 0.2	11.4 ± 0.5**	12.1 ± 0.4*	12.3 ± 0.5	12.6 ± 0.3	12.7 ± 0.5
Creatinine (mg/dL)						
Day 4	0.57 ± 0.03	0.58 ± 0.01	0.61 ± 0.01	0.57 ± 0.01	0.57 ± 0.01	0.55 ± 0.02
Day 19	0.67 ± 0.01	0.66 ± 0.02	0.69 ± 0.02	0.69 ± 0.01	0.69 ± 0.02	0.64 ± 0.02
Week 14	0.67 ± 0.01	0.66 ± 0.02	0.68 ± 0.02	0.63 ± 0.02	0.65 ± 0.02	0.64 ± 0.02
Total protein (g/dL)						
Day 4	5.6 ± 0.1	5.7 ± 0.1	5.6 ± 0.1	5.4 ± 0.1	5.5 ± 0.1	5.2 ± 0.1**
Day 19	6.2 ± 0.0	6.2 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.3 ± 0.1
Week 14	6.4 ± 0.1	6.2 ± 0.1	6.4 ± 0.1	6.0 ± 0.1*	6.2 ± 0.1	6.4 ± 0.1
Albumin (g/dL)						
Day 4	4.0 ± 0.1	4.1 ± 0.1	3.9 ± 0.1	4.0 ± 0.0	3.9 ± 0.1	3.7 ± 0.1**
Day 19	4.1 ± 0.0	4.2 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.2 ± 0.1
Week 14	4.7 ± 0.0	4.7 ± 0.1	4.6 ± 0.1	4.3 ± 0.1	4.7 ± 0.1	4.7 ± 0.1
Alanine aminotransferase (IU/L)						
Day 4	61 ± 3	58 ± 3	63 ± 3	63 ± 3	60 ± 2	54 ± 1
Day 19	46 ± 2	49 ± 2	45 ± 1	46 ± 2	45 ± 2	48 ± 2
Week 14	129 ± 9	103 ± 9	128 ± 18	123 ± 14	104 ± 13*	86 ± 5**
Alkaline phosphatase (IU/L)						
Day 4	845 ± 37	848 ± 39	907 ± 20	905 ± 28	881 ± 30	718 ± 18
Day 19	636 ± 18	639 ± 23	655 ± 10	664 ± 11	635 ± 13	571 ± 15
Week 14	278 ± 7	258 ± 6	279 ± 8	268 ± 7	263 ± 11	253 ± 6

TABLE F1
Hematology and Chemical Chemistry Data for Rats in the 14-Week Gavage Study of 2,4-Hexadienal

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Male (continued)						
n						
Day 4	10	10	10	10	10	10
Day 19	10	10	10	10	10	9
Week 14	10	10	10	10	10	10
Clinical Chemistry (continued)						
Creatine kinase (IU/L)						
Day 4	737 ± 183	341 ± 27 ^b	659 ± 117	452 ± 75	628 ± 109	374 ± 39
Day 19	352 ± 53	250 ± 26	268 ± 41	227 ± 20	256 ± 21	216 ± 14
Week 14	201 ± 14	175 ± 30	180 ± 17	191 ± 22	235 ± 43	254 ± 26
Sorbitol dehydrogenase (IU/L)						
Day 4	7 ± 1	9 ± 1	8 ± 0	8 ± 1	8 ± 0	6 ± 1
Day 19	10 ± 0	11 ± 1	12 ± 1	10 ± 1	12 ± 1	10 ± 1
Week 14	34 ± 2	31 ± 3	35 ± 4	38 ± 5	28 ± 3	21 ± 2**
Bile acids (µmol/L)						
Day 4	26.8 ± 4.6	38.8 ± 7.3	33.7 ± 5.8	33.0 ± 3.6	37.4 ± 4.9	27.6 ± 3.9
Day 19	17.8 ± 2.6	22.4 ± 2.0	20.5 ± 1.9	20.3 ± 2.1	25.0 ± 1.9**	33.3 ± 3.1**
Week 14	18.4 ± 2.2	14.2 ± 2.2	15.8 ± 2.2	15.4 ± 1.8	18.3 ± 2.6	18.5 ± 1.9
Female						
n						
Day 4	10	10	10	10	10	10
Day 19	9	10	10	10	9	10
Week 14	10	10	10	10	10	10
Hematology						
Hematocrit (%)						
Day 4	44.6 ± 0.7	43.7 ± 0.4	44.0 ± 0.8	44.4 ± 0.9	43.0 ± 0.7	45.8 ± 0.4
Day 19	45.5 ± 0.5	45.5 ± 0.7	45.3 ± 0.5	44.1 ± 0.7	45.5 ± 0.7	45.9 ± 0.5
Week 14	43.3 ± 0.4	44.4 ± 0.3*	43.3 ± 0.3	44.1 ± 0.3	44.7 ± 0.3**	44.9 ± 0.3**
Hemoglobin (g/dL)						
Day 4	14.5 ± 0.3	14.6 ± 0.1	14.4 ± 0.2	14.5 ± 0.2	14.3 ± 0.3	14.9 ± 0.2
Day 19	15.0 ± 0.1	15.2 ± 0.2	15.0 ± 0.1	14.8 ± 0.2	15.1 ± 0.1	15.2 ± 0.2
Week 14	14.7 ± 0.1	15.3 ± 0.1**	14.9 ± 0.1	15.1 ± 0.1	15.1 ± 0.1	15.2 ± 0.1
Erythrocytes (10 ⁶ /µL)						
Day 4	7.65 ± 0.15	7.52 ± 0.09	7.51 ± 0.13	7.64 ± 0.12	7.40 ± 0.15	7.89 ± 0.11
Day 19	7.62 ± 0.07	7.66 ± 0.07	7.61 ± 0.10	7.49 ± 0.08	7.59 ± 0.13	7.71 ± 0.09
Week 14	8.04 ± 0.07	8.28 ± 0.09	8.09 ± 0.06	8.24 ± 0.06	8.28 ± 0.06	8.29 ± 0.06*
Reticulocytes (10 ⁶ /µL)						
Day 4	5.01 ± 0.33	4.68 ± 0.32	4.95 ± 0.25	4.83 ± 0.28	4.65 ± 0.21	4.78 ± 0.28
Day 19	2.41 ± 0.09	2.37 ± 0.09	2.30 ± 0.10	2.29 ± 0.11	2.34 ± 0.06	2.71 ± 0.09
Week 14	2.22 ± 0.10	2.39 ± 0.06	2.33 ± 0.09	2.28 ± 0.06	2.20 ± 0.05	2.40 ± 0.08
Mean cell volume (fL)						
Day 4	58.4 ± 0.4	58.2 ± 0.6	58.7 ± 0.5	58.1 ± 0.5	58.1 ± 0.4	58.1 ± 0.5
Day 19	59.7 ± 0.5	59.4 ± 0.5	59.6 ± 0.5	58.9 ± 0.6	60.0 ± 0.4	59.6 ± 0.4
Week 14	53.9 ± 0.2	53.7 ± 0.2	53.5 ± 0.2	53.5 ± 0.2	54.0 ± 0.1	54.1 ± 0.2

TABLE F1
Hematology and Chemical Chemistry Data for Rats in the 14-Week Gavage Study of 2,4-Hexadienal

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Female (continued)						
n						
Day 4	10	10	10	10	10	10
Day 19	9	10	10	10	9	10
Week 14	10	10	10	10	10	10
Hematology (continued)						
Mean cell hemoglobin (pg)						
Day 4	18.9 ± 0.1	19.4 ± 0.1	19.2 ± 0.1	19.0 ± 0.1	19.4 ± 0.1	18.9 ± 0.1
Day 19	19.7 ± 0.1	19.8 ± 0.1	19.8 ± 0.2	19.8 ± 0.1	19.9 ± 0.2	19.7 ± 0.1
Week 14	18.3 ± 0.1	18.5 ± 0.1	18.4 ± 0.1	18.3 ± 0.1	18.3 ± 0.0	18.3 ± 0.1
Mean cell hemoglobin concentration (g/dL)						
Day 4	32.4 ± 0.2	33.4 ± 0.2	32.7 ± 0.3	32.7 ± 0.3	33.3 ± 0.2	32.5 ± 0.3
Day 19	33.0 ± 0.2	33.4 ± 0.2	33.2 ± 0.3	33.7 ± 0.3	33.2 ± 0.2	33.0 ± 0.3
Week 14	34.0 ± 0.1	34.5 ± 0.1	34.5 ± 0.2	34.2 ± 0.1	33.8 ± 0.1	33.9 ± 0.1
Platelets (10 ³ /μL)						
Day 4	894.3 ± 28.3	946.1 ± 37.8	888.2 ± 30.8	892.9 ± 29.8	917.0 ± 36.3	930.7 ± 25.9
Day 19	913.4 ± 22.6	913.7 ± 24.9	908.2 ± 29.7	919.8 ± 25.7	939.6 ± 30.9	974.5 ± 34.0
Week 14	740.5 ± 40.1	773.7 ± 34.6	771.0 ± 18.5	797.0 ± 27.1	776.2 ± 16.0	752.6 ± 14.7
Leukocytes (10 ³ /μL)						
Day 4	11.00 ± 0.49	10.33 ± 0.43	10.54 ± 0.38	9.83 ± 0.38	10.06 ± 0.34	6.35 ± 0.22**
Day 19	7.91 ± 0.44	8.15 ± 0.41	8.31 ± 0.35	7.81 ± 0.34	8.16 ± 0.28	7.50 ± 0.24
Week 14	8.00 ± 0.43	7.14 ± 0.25	6.98 ± 0.28	6.96 ± 0.56	7.22 ± 0.39	7.33 ± 0.36
Segmented neutrophils (10 ³ /μL)						
Day 4	1.16 ± 0.04	1.21 ± 0.08	1.11 ± 0.04	1.09 ± 0.09	1.16 ± 0.06	1.25 ± 0.11
Day 19	1.10 ± 0.08	0.95 ± 0.06	1.02 ± 0.07	0.77 ± 0.04**	0.86 ± 0.04	1.25 ± 0.07
Week 14	1.18 ± 0.11	1.19 ± 0.09	1.24 ± 0.07	1.16 ± 0.11	1.39 ± 0.11	1.39 ± 0.13
Lymphocytes (10 ³ /μL)						
Day 4	9.31 ± 0.47	8.68 ± 0.34	8.95 ± 0.35	8.32 ± 0.31	8.42 ± 0.30	4.70 ± 0.18**
Day 19	6.51 ± 0.34	6.94 ± 0.39	7.00 ± 0.31	6.77 ± 0.32	7.02 ± 0.28	5.97 ± 0.24
Week 14	6.38 ± 0.32	5.59 ± 0.21	5.42 ± 0.23	5.47 ± 0.42	5.45 ± 0.28	5.58 ± 0.30
Activated lymphocytes (10 ³ /μL)						
Day 4	0.23 ± 0.02	0.18 ± 0.02	0.20 ± 0.02	0.18 ± 0.01	0.22 ± 0.02	0.24 ± 0.02
Day 19	0.13 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.12 ± 0.02	0.11 ± 0.01
Week 14	0.20 ± 0.03	0.16 ± 0.03	0.14 ± 0.02	0.15 ± 0.03	0.16 ± 0.02	0.16 ± 0.01
Monocytes (10 ³ /μL)						
Day 4	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
Day 19	0.05 ± 0.00	0.05 ± 0.00	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Week 14	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
Basophils (10 ³ /μL)						
Day 4	0.134 ± 0.012	0.100 ± 0.015	0.121 ± 0.016	0.108 ± 0.010	0.129 ± 0.011	0.057 ± 0.006**
Day 19	0.053 ± 0.009	0.044 ± 0.005	0.048 ± 0.004	0.050 ± 0.006	0.051 ± 0.007	0.051 ± 0.004
Week 14	0.071 ± 0.013	0.040 ± 0.006	0.038 ± 0.005	0.052 ± 0.011	0.051 ± 0.015	0.050 ± 0.007
Eosinophils (10 ³ /μL)						
Day 4	0.06 ± 0.00	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.00	0.06 ± 0.00	0.03 ± 0.00**
Day 19	0.07 ± 0.01	0.06 ± 0.00	0.06 ± 0.00	0.07 ± 0.01	0.06 ± 0.00	0.06 ± 0.01
Week 14	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.06 ± 0.01

TABLE F1
Hematology and Chemical Chemistry Data for Rats in the 14-Week Gavage Study of 2,4-Hexadienal

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Female (continued)						
n						
Day 4	10	10	10	10	10	10
Day 19	9	10	10	10	9	10
Week 14	10	10	10	10	10	10
Clinical Chemistry						
Urea nitrogen (mg/dL)						
Day 4	8.3 ± 0.7	7.9 ± 0.5	7.6 ± 0.5	7.6 ± 0.3	8.3 ± 0.6	7.7 ± 0.3
Day 19	12.9 ± 0.4	14.5 ± 0.3*	13.2 ± 0.3	13.5 ± 0.4	13.5 ± 0.5	14.2 ± 0.5
Week 14	12.1 ± 0.2	13.2 ± 0.3	13.0 ± 0.3	11.8 ± 0.3	11.3 ± 0.2	11.4 ± 0.3
Creatinine (mg/dL)						
Day 4	0.61 ± 0.02	0.60 ± 0.02	0.58 ± 0.02	0.60 ± 0.01	0.62 ± 0.01	0.58 ± 0.01
Day 19	0.67 ± 0.02	0.61 ± 0.02	0.65 ± 0.02	0.62 ± 0.02	0.66 ± 0.02	0.64 ± 0.02
Week 14	0.71 ± 0.04	0.70 ± 0.05	0.62 ± 0.05	0.61 ± 0.04	0.60 ± 0.05	0.60 ± 0.04
Total protein (g/dL)						
Day 4	5.6 ± 0.1	5.7 ± 0.1	5.6 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	5.1 ± 0.1**
Day 19	6.1 ± 0.1	6.1 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	6.3 ± 0.1	6.1 ± 0.1
Week 14	6.3 ± 0.1	6.5 ± 0.1	6.4 ± 0.1	6.3 ± 0.1	6.4 ± 0.1	6.2 ± 0.0
Albumin (g/dL)						
Day 4	4.0 ± 0.1	4.1 ± 0.1	4.0 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	3.6 ± 0.1*
Day 19	4.2 ± 0.0	4.3 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	4.3 ± 0.1	4.2 ± 0.1
Week 14	4.7 ± 0.1	5.0 ± 0.1	5.0 ± 0.1*	4.9 ± 0.1	4.9 ± 0.1	4.8 ± 0.1
Alanine aminotransferase (IU/L)						
Day 4	54 ± 3	55 ± 2	56 ± 2	53 ± 3	52 ± 3	46 ± 2*
Day 19	44 ± 1	43 ± 1	40 ± 1	40 ± 2	39 ± 2	44 ± 2
Week 14	53 ± 3	75 ± 7*	70 ± 7	68 ± 6	56 ± 5	57 ± 2
Alkaline phosphatase (IU/L)						
Day 4	762 ± 38	757 ± 34	781 ± 25	760 ± 39	723 ± 31	480 ± 14**
Day 19	523 ± 13	543 ± 14	538 ± 13	527 ± 9	508 ± 15	466 ± 10*
Week 14	230 ± 8	295 ± 10**	278 ± 8*	297 ± 11**	274 ± 8*	247 ± 8
Creatine kinase (IU/L)						
Day 4	669 ± 153	754 ± 129	844 ± 167	455 ± 46	767 ± 199	799 ± 144
Day 19	303 ± 31	324 ± 42	305 ± 33	369 ± 64	447 ± 53	372 ± 53
Week 14	195 ± 34	209 ± 40	161 ± 21	190 ± 28	198 ± 35	210 ± 32
Sorbitol dehydrogenase (IU/L)						
Day 4	9 ± 1	10 ± 1	10 ± 1	11 ± 1	10 ± 1	8 ± 1
Day 19	7 ± 1	7 ± 1	5 ± 1	6 ± 0	6 ± 1	6 ± 1
Week 14	8 ± 1	12 ± 2	14 ± 2**	12 ± 2	10 ± 2	10 ± 1
Bile acids (µmol/L)						
Day 4	22.1 ± 2.3	26.7 ± 3.2	22.0 ± 3.5	28.2 ± 3.4	22.6 ± 2.9	19.7 ± 2.1
Day 19	13.9 ± 1.7	18.4 ± 1.9	17.5 ± 1.7	18.8 ± 1.8*	19.6 ± 2.2*	24.5 ± 1.9**
Week 14	21.3 ± 2.4	21.7 ± 3.4	17.4 ± 2.4	17.2 ± 2.2	18.5 ± 1.9	26.4 ± 3.8

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test

** $P \leq 0.01$

^a Data are given as mean ± standard error. Statistical tests were performed on unrounded data.

^b n=9

TABLE F2
Hematology Data for Mice in the 14-Week Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Male						
n	8	10	10	10	9	10
Hematocrit (%)	47.9 ± 0.7	46.7 ± 0.7	48.0 ± 1.1	47.0 ± 0.5	47.0 ± 0.3	47.9 ± 0.3
Hemoglobin (g/dL)	15.8 ± 0.3	15.8 ± 0.3	16.2 ± 0.3	15.8 ± 0.2	15.7 ± 0.1	15.7 ± 0.1
Erythrocytes (10 ⁶ /μL)	10.60 ± 0.17	10.29 ± 0.17	10.54 ± 0.23	10.32 ± 0.13	10.35 ± 0.09	10.39 ± 0.09
Reticulocytes (10 ⁶ /μL)	3.91 ± 0.19	3.78 ± 0.12	3.95 ± 0.16	3.67 ± 0.16	3.74 ± 0.11	3.63 ± 0.13
Mean cell volume (fL)	45.2 ± 0.1	45.4 ± 0.2	45.5 ± 0.2	45.6 ± 0.2	45.5 ± 0.2	46.1 ± 0.2**
Mean cell hemoglobin (pg)	14.9 ± 0.1	15.3 ± 0.1**	15.4 ± 0.1**	15.4 ± 0.1**	15.1 ± 0.1	15.1 ± 0.1
Mean cell hemoglobin concentration (g/dL)	32.9 ± 0.1	33.8 ± 0.2*	33.9 ± 0.3*	33.6 ± 0.2	33.3 ± 0.2	32.8 ± 0.2
Platelets (10 ³ /μL)	1,171.4 ± 43.7	1,261.8 ± 60.4	1,235.0 ± 37.4	1,218.7 ± 22.3	1,176.3 ± 26.4	1,175.7 ± 26.8
Leukocytes (10 ³ /μL)	3.17 ± 0.17	2.40 ± 0.19	2.65 ± 0.20	2.53 ± 0.23	2.99 ± 0.21	2.79 ± 0.20
Segmented neutrophils (10 ³ /μL)	0.37 ± 0.02	0.51 ± 0.07	0.57 ± 0.09	0.48 ± 0.06	0.55 ± 0.09	0.55 ± 0.06
Lymphocytes (10 ³ /μL)	2.66 ± 0.15	1.78 ± 0.15**	1.95 ± 0.14*	1.92 ± 0.18*	2.33 ± 0.18	2.11 ± 0.14
Activated lymphocytes (10 ³ /μL)	0.02 ± 0.00	0.02 ± 0.00 ^b	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.01 ^c	0.02 ± 0.00
Monocytes (10 ³ /μL)	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00
Basophils (10 ³ /μL)	0.008 ± 0.002	0.006 ± 0.002	0.006 ± 0.002	0.005 ± 0.002	0.003 ± 0.002	0.006 ± 0.002
Eosinophils (10 ³ /μL)	0.09 ± 0.02	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.03	0.08 ± 0.02	0.08 ± 0.01
Female						
n	10	10	10	10	10	10
Hematocrit (%)	47.8 ± 0.9	48.0 ± 1.1	47.6 ± 0.7	47.3 ± 0.5	48.4 ± 0.7	47.2 ± 0.6
Hemoglobin (g/dL)	16.6 ± 0.3	16.8 ± 0.2	16.6 ± 0.2	16.5 ± 0.1	16.6 ± 0.2	16.2 ± 0.2
Erythrocytes (10 ⁶ /μL)	10.38 ± 0.18	10.50 ± 0.18	10.41 ± 0.14	10.29 ± 0.09	10.43 ± 0.12	10.27 ± 0.12
Reticulocytes (10 ⁶ /μL)	3.56 ± 0.10	4.14 ± 0.43	3.36 ± 0.12	3.39 ± 0.17	3.31 ± 0.10	3.43 ± 0.16
Mean cell volume (fL)	46.0 ± 0.1	45.7 ± 0.3	45.7 ± 0.2	46.0 ± 0.2	46.5 ± 0.2	46.0 ± 0.3
Mean cell hemoglobin (pg)	16.0 ± 0.1	16.0 ± 0.2	15.9 ± 0.2	16.1 ± 0.1	16.0 ± 0.2	15.7 ± 0.1
Mean cell hemoglobin concentration (g/dL)	34.7 ± 0.2	35.1 ± 0.6	34.9 ± 0.4	34.9 ± 0.3	34.3 ± 0.2	34.2 ± 0.1
Platelets (10 ³ /μL)	931.4 ± 49.2	1,029.3 ± 50.1	878.3 ± 42.9	970.4 ± 26.3	922.1 ± 45.7	971.4 ± 57.7 ^b
Leukocytes (10 ³ /μL)	3.61 ± 0.26	4.39 ± 0.21	3.48 ± 0.34	4.41 ± 0.38	3.34 ± 0.28	4.12 ± 0.83
Segmented neutrophils (10 ³ /μL)	0.66 ± 0.17	0.68 ± 0.08	0.42 ± 0.06	0.69 ± 0.09	0.58 ± 0.07	0.61 ± 0.10
Lymphocytes (10 ³ /μL)	2.82 ± 0.18	3.53 ± 0.16	2.83 ± 0.27	3.52 ± 0.30	2.63 ± 0.23	3.37 ± 0.71
Activated lymphocytes (10 ³ /μL)	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01 ^d	0.03 ± 0.00	0.02 ± 0.00 ^b	0.03 ± 0.01
Monocytes (10 ³ /μL)	0.04 ± 0.00	0.05 ± 0.00	0.03 ± 0.01	0.06 ± 0.00	0.04 ± 0.01	0.04 ± 0.01
Basophils (10 ³ /μL)	0.012 ± 0.002	0.012 ± 0.001	0.010 ± 0.004	0.015 ± 0.002	0.014 ± 0.002	0.015 ± 0.003
Eosinophils (10 ³ /μL)	0.06 ± 0.01	0.09 ± 0.01	0.07 ± 0.02	0.11 ± 0.02	0.05 ± 0.01	0.07 ± 0.02

* Significantly different (P ≤ 0.05) from the vehicle control group by Dunn's or Shirley's test

** P ≤ 0.01

^a Data are given as mean ± standard error. Statistical tests were performed on unrounded data.

^b n=9

^c n=5

^d n=8

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

TABLE G1	Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 16-Day Gavage Study of 2,4-Hexadienal	242
TABLE G2	Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 14-Week Gavage Study of 2,4-Hexadienal	243
TABLE G3	Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 16-Day Gavage Study of 2,4-Hexadienal	245
TABLE G4	Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 14-Week Gavage Study of 2,4-Hexadienal	246

TABLE G1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 16-Day Gavage Study
of 2,4-Hexadienal^a

	Vehicle Control	3 mg/kg	9 mg/kg	27 mg/kg	80 mg/kg	240 mg/kg
n	5	5	5	5	5	2
Male						
Necropsy body wt	175 ± 5	181 ± 4	178 ± 7	177 ± 6	176 ± 1	145 ± 12*
R. Kidney						
Absolute	0.720 ± 0.028	0.724 ± 0.032	0.724 ± 0.031	0.733 ± 0.029	0.750 ± 0.007	0.651 ± 0.017
Relative	4.121 ± 0.055	4.005 ± 0.118	4.068 ± 0.046	4.154 ± 0.057	4.264 ± 0.055	4.514 ± 0.249*
Liver						
Absolute	8.897 ± 0.375	9.034 ± 0.224	8.816 ± 0.429	8.854 ± 0.299	8.949 ± 0.133	7.610 ± 0.397
Relative	50.908 ± 1.024	50.027 ± 0.281	49.494 ± 0.642	50.208 ± 0.733	50.881 ± 1.049	52.691 ± 1.502
Female						
Necropsy body wt	131 ± 5	134 ± 4	133 ± 6	130 ± 2	131 ± 2	120 ± 15
R. Kidney						
Absolute	0.537 ± 0.020	0.559 ± 0.015	0.569 ± 0.027	0.548 ± 0.008	0.569 ± 0.007	0.573 ± 0.068
Relative	4.113 ± 0.069	4.174 ± 0.160	4.263 ± 0.080	4.232 ± 0.114	4.342 ± 0.078	4.779 ± 0.031**
Liver						
Absolute	5.871 ± 0.190	6.196 ± 0.069	5.925 ± 0.274	5.997 ± 0.087	5.949 ± 0.062	6.869 ± 0.514*
Relative	44.990 ± 0.587	46.279 ± 1.500	44.369 ± 0.378	46.278 ± 0.842	45.410 ± 0.665	57.647 ± 2.877**

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunnett's test

** $P \leq 0.01$

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE G2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 14-Week Gavage Study
of 2,4-Hexadienal^a

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Male						
n	10	10	10	10	10	10
Necropsy body wt	338 ± 8	329 ± 8	345 ± 3	318 ± 5*	318 ± 5*	287 ± 9**
Heart						
Absolute	0.919 ± 0.024	0.864 ± 0.024	0.908 ± 0.017	0.877 ± 0.017	0.878 ± 0.025	0.824 ± 0.018*
Relative	2.716 ± 0.022	2.628 ± 0.037	2.631 ± 0.032	2.754 ± 0.025	2.755 ± 0.042	2.888 ± 0.065**
R. Kidney						
Absolute	0.971 ± 0.028	0.949 ± 0.023	1.006 ± 0.026	0.947 ± 0.019	0.918 ± 0.033	0.865 ± 0.030**
Relative	2.868 ± 0.032	2.889 ± 0.041	2.913 ± 0.057	2.974 ± 0.028	2.877 ± 0.069	3.019 ± 0.048
Liver						
Absolute	10.733 ± 0.296	9.851 ± 0.374	11.014 ± 0.295	10.197 ± 0.247	9.909 ± 0.245	8.998 ± 0.314**
Relative	31.710 ± 0.315	29.917 ± 0.579	31.895 ± 0.649	31.998 ± 0.354	31.107 ± 0.427	31.418 ± 0.597
Lung						
Absolute	1.342 ± 0.047	1.279 ± 0.043	1.368 ± 0.035	1.214 ± 0.018	1.287 ± 0.039	1.259 ± 0.056
Relative	3.970 ± 0.113	3.889 ± 0.077	3.970 ± 0.116	3.819 ± 0.061	4.037 ± 0.077	4.389 ± 0.124**
Spleen						
Absolute	0.627 ± 0.012	0.607 ± 0.016	0.655 ± 0.014	0.602 ± 0.013	0.607 ± 0.015	0.529 ± 0.019**
Relative	1.856 ± 0.017	1.848 ± 0.028	1.900 ± 0.041	1.891 ± 0.027	1.908 ± 0.036	1.846 ± 0.030
R. Testis						
Absolute	1.437 ± 0.015	1.420 ± 0.030	1.431 ± 0.014	1.423 ± 0.026	1.431 ± 0.023	1.346 ± 0.035
Relative	4.265 ± 0.095	4.331 ± 0.092	4.149 ± 0.041	4.470 ± 0.058	4.499 ± 0.036*	4.714 ± 0.102**
Thymus						
Absolute	0.277 ± 0.009	0.261 ± 0.011	0.281 ± 0.024	0.281 ± 0.014	0.263 ± 0.011	0.223 ± 0.015
Relative	0.822 ± 0.035	0.794 ± 0.029	0.812 ± 0.065	0.879 ± 0.036	0.827 ± 0.036	0.776 ± 0.041

TABLE G2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 14-Week Gavage Study of 2,4-Hexadienal

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Female						
n	10	10	10	10	10	10
Necropsy body wt	191 ± 4	195 ± 3	190 ± 3	186 ± 2	192 ± 2	182 ± 3
Heart						
Absolute	0.560 ± 0.008	0.582 ± 0.005	0.586 ± 0.011	0.586 ± 0.025	0.581 ± 0.011	0.555 ± 0.010
Relative	2.942 ± 0.053	2.987 ± 0.035	3.081 ± 0.050	3.145 ± 0.101	3.035 ± 0.052	3.045 ± 0.051
R. Kidney						
Absolute	0.612 ± 0.015	0.629 ± 0.011	0.620 ± 0.015	0.612 ± 0.020	0.619 ± 0.014	0.601 ± 0.016
Relative	3.207 ± 0.041	3.224 ± 0.025	3.254 ± 0.036	3.287 ± 0.071	3.232 ± 0.060	3.294 ± 0.067
Liver						
Absolute	5.437 ± 0.155	5.695 ± 0.140	5.750 ± 0.161	5.400 ± 0.124	5.650 ± 0.067	5.388 ± 0.115
Relative	28.459 ± 0.337	29.162 ± 0.386	30.169 ± 0.513*	29.045 ± 0.516	29.521 ± 0.336	29.536 ± 0.440
Lung						
Absolute	0.913 ± 0.021	0.961 ± 0.027	0.934 ± 0.017	0.942 ± 0.029	0.951 ± 0.021	0.887 ± 0.013
Relative	4.795 ± 0.117	4.931 ± 0.139	4.911 ± 0.091	5.063 ± 0.122	4.967 ± 0.100	4.867 ± 0.064
Spleen						
Absolute	0.401 ± 0.012	0.425 ± 0.011	0.417 ± 0.010	0.398 ± 0.007	0.422 ± 0.008	0.421 ± 0.013
Relative	2.102 ± 0.047	2.178 ± 0.040	2.191 ± 0.042	2.141 ± 0.028	2.206 ± 0.048	2.311 ± 0.073*
Thymus						
Absolute	0.203 ± 0.008	0.224 ± 0.009	0.205 ± 0.008	0.200 ± 0.007	0.201 ± 0.006	0.198 ± 0.007
Relative	1.062 ± 0.032	1.144 ± 0.035	1.072 ± 0.030	1.072 ± 0.026	1.049 ± 0.026	1.089 ± 0.044

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE G3
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 16-Day Gavage Study
of 2,4-Hexadienal^a

	Vehicle Control	3 mg/kg	9 mg/kg	27 mg/kg	80 mg/kg	240 mg/kg
Male						
n	5	5	5	5	5	4
Necropsy body wt	25.2 ± 0.3	25.1 ± 0.7	25.7 ± 0.4	25.9 ± 0.5	24.6 ± 0.3	24.5 ± 0.3
R. Kidney						
Absolute	0.231 ± 0.008	0.234 ± 0.009	0.227 ± 0.009	0.236 ± 0.012	0.218 ± 0.011	0.238 ± 0.006
Relative	9.166 ± 0.214	9.335 ± 0.364	8.819 ± 0.264	9.112 ± 0.329	8.859 ± 0.357	9.703 ± 0.327
Liver						
Absolute	1.461 ± 0.053	1.440 ± 0.040	1.441 ± 0.034	1.492 ± 0.063	1.378 ± 0.031	1.464 ± 0.025
Relative	58.028 ± 1.476	57.351 ± 0.708	56.121 ± 1.590	57.651 ± 2.266	56.014 ± 1.120	59.693 ± 0.915
Female						
n	5	4	5	5	5	4
Necropsy body wt	19.9 ± 0.7	19.8 ± 0.5	20.1 ± 0.6	21.2 ± 0.4	20.2 ± 0.6	17.9 ± 0.9
R. Kidney						
Absolute	0.144 ± 0.005	0.151 ± 0.003	0.152 ± 0.004	0.156 ± 0.005	0.145 ± 0.005	0.145 ± 0.008
Relative	7.238 ± 0.146	7.611 ± 0.150	7.604 ± 0.091	7.347 ± 0.166	7.173 ± 0.111	8.107 ± 0.322**
Liver						
Absolute	1.016 ± 0.058	1.012 ± 0.025	1.054 ± 0.057	1.159 ± 0.035	1.012 ± 0.018	1.109 ± 0.054
Relative	50.820 ± 1.279	51.045 ± 0.493	52.379 ± 1.481	54.656 ± 0.994	50.111 ± 1.073	62.602 ± 4.395**

** Significantly different ($P \leq 0.01$) from the vehicle control group by Dunnett's test

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE G4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 14-Week Gavage Study
of 2,4-Hexadienal^a

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Male						
n	8	10	10	10	9	10
Necropsy body wt	38.2 ± 0.8	37.8 ± 1.1	40.7 ± 0.8	39.0 ± 1.3	38.5 ± 1.2	37.4 ± 0.7
Heart						
Absolute	0.151 ± 0.004	0.151 ± 0.003	0.143 ± 0.003	0.147 ± 0.004	0.151 ± 0.004	0.146 ± 0.002
Relative	3.970 ± 0.139	4.038 ± 0.191	3.519 ± 0.079	3.802 ± 0.152	3.935 ± 0.114	3.921 ± 0.094
R. Kidney						
Absolute	0.268 ± 0.007	0.287 ± 0.006	0.287 ± 0.007	0.283 ± 0.011	0.296 ± 0.010*	0.296 ± 0.004*
Relative	7.000 ± 0.128	7.649 ± 0.254	7.054 ± 0.101	7.284 ± 0.276	7.681 ± 0.212*	7.946 ± 0.178**
Liver						
Absolute	1.233 ± 0.043	1.317 ± 0.036	1.361 ± 0.028	1.320 ± 0.040	1.422 ± 0.066*	1.269 ± 0.024
Relative	32.216 ± 0.805	35.176 ± 1.587	33.487 ± 0.610	34.012 ± 1.065	36.857 ± 1.051*	33.999 ± 0.524
Lung						
Absolute	0.204 ± 0.011	0.189 ± 0.010	0.184 ± 0.007	0.180 ± 0.006	0.190 ± 0.011	0.170 ± 0.011
Relative	5.361 ± 0.335	5.074 ± 0.374	4.542 ± 0.196	4.653 ± 0.193	4.926 ± 0.210	4.578 ± 0.332
Spleen						
Absolute	0.073 ± 0.003	0.071 ± 0.003	0.072 ± 0.003	0.068 ± 0.003	0.072 ± 0.004	0.073 ± 0.004
Relative	1.902 ± 0.088	1.908 ± 0.136	1.770 ± 0.065	1.758 ± 0.091	1.880 ± 0.094	1.957 ± 0.122
R. Testis						
Absolute	0.113 ± 0.004	0.106 ± 0.003	0.113 ± 0.003	0.118 ± 0.003	0.104 ± 0.010	0.115 ± 0.003
Relative	2.966 ± 0.109	2.828 ± 0.110	2.787 ± 0.094	3.036 ± 0.105	2.747 ± 0.279	3.089 ± 0.097
Thymus						
Absolute	0.031 ± 0.003	0.033 ± 0.001	0.031 ± 0.001	0.033 ± 0.002	0.032 ± 0.002	0.031 ± 0.001
Relative	0.808 ± 0.065	0.881 ± 0.047	0.754 ± 0.027	0.835 ± 0.042	0.842 ± 0.045	0.831 ± 0.048

TABLE G4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 14-Week Gavage Study of 2,4-Hexadienal

	Vehicle Control	7.5 mg/kg	15 mg/kg	30 mg/kg	60 mg/kg	120 mg/kg
Female						
n	10	10	10	10	10	10
Necropsy body wt	33.0 ± 0.6	33.6 ± 1.1	33.5 ± 0.8	32.5 ± 1.7	33.1 ± 0.8	32.6 ± 1.2
Heart						
Absolute	0.123 ± 0.005	0.124 ± 0.004	0.122 ± 0.004	0.120 ± 0.005	0.124 ± 0.003	0.118 ± 0.006
Relative	3.741 ± 0.175	3.715 ± 0.109	3.662 ± 0.130	3.758 ± 0.173	3.759 ± 0.111	3.631 ± 0.161
R. Kidney						
Absolute	0.172 ± 0.006	0.171 ± 0.005	0.182 ± 0.005	0.173 ± 0.008	0.178 ± 0.005	0.167 ± 0.004
Relative	5.224 ± 0.215	5.116 ± 0.123	5.449 ± 0.124	5.399 ± 0.240	5.402 ± 0.181	5.150 ± 0.139
Liver						
Absolute	0.996 ± 0.026	1.096 ± 0.035	1.100 ± 0.018	1.096 ± 0.043	1.160 ± 0.022**	1.067 ± 0.028
Relative	30.187 ± 0.666	32.733 ± 0.572*	33.009 ± 0.741*	34.107 ± 1.071**	35.183 ± 0.911**	32.916 ± 0.944**
Lung						
Absolute	0.224 ± 0.017	0.200 ± 0.009	0.236 ± 0.012	0.193 ± 0.011	0.212 ± 0.015	0.207 ± 0.022
Relative	6.845 ± 0.590	5.990 ± 0.237	7.065 ± 0.338	6.045 ± 0.379	6.433 ± 0.469	6.294 ± 0.531
Spleen						
Absolute	0.096 ± 0.005	0.095 ± 0.003	0.096 ± 0.004	0.088 ± 0.005	0.096 ± 0.004	0.087 ± 0.004
Relative	2.913 ± 0.166	2.850 ± 0.094	2.876 ± 0.125	2.770 ± 0.203	2.896 ± 0.093	2.699 ± 0.179
Thymus						
Absolute	0.044 ± 0.002	0.043 ± 0.004	0.042 ± 0.001	0.043 ± 0.002	0.041 ± 0.002	0.037 ± 0.002
Relative	1.322 ± 0.055	1.265 ± 0.110	1.260 ± 0.052	1.370 ± 0.113	1.251 ± 0.062	1.136 ± 0.078

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test

** $P \leq 0.01$

^a Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

APPENDIX H

REPRODUCTIVE TISSUE EVALUATIONS AND ESTROUS CYCLE CHARACTERIZATION

TABLE H1	Summary of Reproductive Tissue Evaluations for Male Rats in the 14-Week Gavage Study of 2,4-Hexadienal	250
TABLE H2	Estrous Cycle Characterization for Female Rats in the 14-Week Gavage Study of 2,4-Hexadienal	250
TABLE H3	Summary of Reproductive Tissue Evaluations for Male Mice in the 14-Week Gavage Study of 2,4-Hexadienal	251
TABLE H4	Estrous Cycle Characterization for Female Mice in the 14-Week Gavage Study of 2,4-Hexadienal	251

TABLE H1
Summary of Reproductive Tissue Evaluations for Male Rats in the 14-Week Gavage Study
of 2,4-Hexadienal^a

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
n	10	10	10	10
Weights (g)				
Necropsy body wt	338 ± 8	318 ± 5	318 ± 5	286 ± 9**
L. Cauda epididymis	0.1616 ± 0.0024	0.1587 ± 0.0045	0.1584 ± 0.0048	0.1485 ± 0.0087
L. Epididymis	0.4297 ± 0.0057	0.4131 ± 0.0060	0.4212 ± 0.0105	0.4025 ± 0.0151
L. Testis	1.5212 ± 0.0276	1.4737 ± 0.0249	1.5128 ± 0.0219	1.4401 ± 0.0334
Spermatid and sperm measurements				
Spermatid heads (10 ⁷ /g testis)	153.63 ± 8.37	161.88 ± 12.93	143.67 ± 6.92	164.91 ± 8.16
Spermatid heads (10 ⁷ /testis)	192.63 ± 9.56	193.63 ± 11.34	189.13 ± 8.71	209.50 ± 9.39
Sperm heads (10 ⁷ /g cauda epididymis)	132.12 ± 5.35	117.26 ± 7.44	110.36 ± 9.12	111.87 ± 9.07
Sperm heads (10 ⁷ /cauda epididymis)	816.77 ± 28.09	736.11 ± 34.96	697.63 ± 52.81	752.21 ± 37.25
Epididymal sperm motility (%)	86.15 ± 0.69	84.52 ± 0.59	85.45 ± 0.51	84.13 ± 0.43

** Significantly different ($P \leq 0.01$) from the vehicle control group by William's test

^a Data are given as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (tissue weights) or Dunn's test (spermatid and sperm measurements and epididymal sperm motility)

TABLE H2
Estrous Cycle Characterization for Female Rats in the 14-Week Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
n	10	10	10	10
Necropsy body wt (g)	191 ± 4	186 ± 2	191 ± 2	182 ± 3
Estrous cycle length (days)	4.833 ± 0.118 ^b	5.100 ± 0.145	5.100 ± 0.125	5.000 ± 0.129
Estrous stages (% of cycle)				
Diestrus	39.2	44.2	43.3	44.2
Proestrus	14.2	9.2	14.2	17.5
Estrus	28.3	27.5	26.7	24.2
Metestrus	18.3	19.2	15.8	14.2

^a Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body weight) or Dunn's test (estrous cycle length). By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages.

^b Estrous cycle was longer than 12 days or unclear in 1 of 10 animals.

TABLE H3
Summary of Reproductive Tissue Evaluations for Male Mice in the 14-Week Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
n	8	10	8	10
Weights (g)				
Necropsy body wt	38.2 ± 0.8	38.0 ± 0.8	37.6 ± 0.7	37.4 ± 0.7
L. Cauda epididymis	0.0177 ± 0.0006	0.0184 ± 0.0016	0.0168 ± 0.0009	0.0182 ± 0.0006
L. Epididymis	0.0432 ± 0.0010	0.0440 ± 0.0022	0.0425 ± 0.0020	0.0439 ± 0.0010
L. Testis	0.1098 ± 0.0033	0.1140 ± 0.0033	0.1100 ± 0.0037	0.1115 ± 0.0029
Spermatid and sperm measurements				
Spermatid heads (10 ⁷ /g testis)	230.22 ± 8.81 ^b	224.01 ± 6.84	231.49 ± 18.72	208.14 ± 11.10
Spermatid heads (10 ⁷ /testis)	21.73 ± 1.19 ^b	22.10 ± 1.25	21.64 ± 1.80	20.56 ± 1.35
Sperm heads (10 ⁷ /g cauda epididymis)	26.26 ± 2.14	26.50 ± 2.57	24.40 ± 2.14	24.88 ± 1.81
Sperm heads (10 ⁷ /cauda epididymis)	1,472.83 ± 81.46	1,423.61 ± 138.41	1,459.14 ± 118.90	1,373.66 ± 96.00
Epididymal sperm motility (%)	83.94 ± 0.53	82.17 ± 0.39	82.33 ± 0.59	82.63 ± 0.65

^a Data are given as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body and tissue weights) or by Dunn's test (spermatid and sperm measurements and epididymal sperm motility)

^b n=6

TABLE H4
Estrous Cycle Characterization for Female Mice in the 14-Week Gavage Study of 2,4-Hexadienal^a

	Vehicle Control	30 mg/kg	60 mg/kg	120 mg/kg
n	10	10	10	10
Necropsy body wt (g)	33.0 ± 0.6	32.5 ± 1.7	33.1 ± 0.8	32.6 ± 1.2
Estrous cycle length (days)	4.200 ± 0.226	4.590 ± 0.414	4.167 ± 0.207 ^b	4.120 ± 0.088
Estrous stages (% of cycle)				
Diestrus	26.7	32.5	39.2	35.0
Proestrus	2.5	1.7	0.8	0.8
Estrus	49.2	42.5	37.5	41.7
Metestrus	21.7	23.3	22.5	22.5

^a Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body weight) or Dunn's test (estrous cycle length). By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages.

^b Estrous cycle was longer than 12 days or unclear in 1 of 10 animals.

APPENDIX I

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF 2,4-HEXADIENAL	254
PREPARATION AND ANALYSIS OF DOSE FORMULATIONS	255
FIGURE I1 Infrared Absorption Spectrum of 2,4-Hexadienal	256
FIGURE I2 Nuclear Magnetic Resonance Spectrum of 2,4-Hexadienal	257
TABLE I1 Gas Chromatography Systems Used in the Gavage Studies of 2,4-Hexadienal	258
TABLE I2 High-Performance Liquid Chromatography Systems Used in the Gavage Studies of 2,4-Hexadienal	259
TABLE I3 Preparation and Storage of Dose Formulations in the Gavage Studies of 2,4-Hexadienal	259
TABLE I4 Results of Analyses of Dose Formulations Administered to Rats and Mice in the 16-Day Gavage Studies of 2,4-Hexadienal	260
TABLE I5 Results of Analyses of Dose Formulations Administered to Rats and Mice in the 14-Week Gavage Studies of 2,4-Hexadienal	261
TABLE I6 Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Gavage Studies of 2,4-Hexadienal	263

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF 2,4-HEXADIENAL

2,4-Hexadienal was obtained from Lancaster Synthesis, Inc. (Windham, NH), in two lots (90000345 and P09653). Lot 90000345 was used in the 16-day studies, and lot P09653 was used during the 14-week and 2-year studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Research Triangle Institute (Research Triangle Park, NC) and the study laboratories. Reports on analyses performed in support of the 2,4-hexadienal studies are on file at the National Institute of Environmental Health Sciences.

Both lots of the chemical, a pale to dark yellow liquid, were identified as 2,4-hexadienal by the analytical chemistry laboratory using infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy. Lot P09653 was also identified using gas chromatography (GC) with IR spectroscopy (GC/IR) by system A (Table I1) and GC with mass spectrometry (GC/MS) by system B. The study laboratories confirmed the identity of the chemical using infrared (both lots) and NMR (lot P09653) spectroscopy. The infrared and NMR spectra were consistent with the literature spectra (Aldrich, 1981, 1983) and/or with the structure of 2,4-hexadienal; the infrared and NMR spectra are presented in Figures I1 and I2. GC and GC/MS indicated that the major component of the bulk chemical was the *trans,trans* isomer of 2,4-hexadienal.

Purity was determined by the analytical chemistry laboratory using high-performance liquid chromatography (HPLC) by system 1 (Table I2). GC/IR and GC/MS analyses (systems A and B) were performed by the analytical chemistry laboratory to identify impurities in the bulk chemical. Purity was confirmed by the study laboratory using GC by systems C (lot 90000345) and D (lot P09653).

For lot 90000345, HPLC by system 1 indicated one major peak and one major impurity peak with an area of 11.5% of the total integrated area. GC by system C conducted by the study laboratory indicated a purity of 95.2%; one major peak, one minor impurity peak, and four major impurity peaks were observed.

For lot P09653, HPLC by system 1 indicated a purity of approximately 89%; one minor impurity peak with an area of approximately 11% of the total integrated area was detected. GC by systems A and B indicated that the impurity was *cis,trans*-2,4-hexadienal. GC by system D conducted by the study laboratory indicated a purity of 98.4%.

Inconsistencies in the purity results of the chemical were resolved during the 2-year study. A close examination of the gas chromatography method revealed that incomplete resolution of the impurity and main peak caused underestimation of the impurity content. When an optimized HPLC method was used, analyses indicated that 2,4-hexadienal contained approximately 89% of the *trans,trans* isomer and approximately 11% of the *cis,trans* isomer.

To ensure stability, the bulk chemical was stored refrigerated and protected from light in sealed containers under a nitrogen headspace. Stability was monitored relative to a frozen reference sample by the study laboratories using GC by systems C (lot 90000345) and D (lot P09653). Gas chromatography was used to allow comparison of data to previous bulk chemical analyses. No degradation of the bulk chemical was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared once for the 16-day studies and every 4 weeks for the 14-week and 2-year studies by mixing 2,4-hexadienal with corn oil to give the required concentrations (Table I3). The dose formulations were stored refrigerated and protected from light under nitrogen in amber glass containers.

Stability studies were performed on 0.290, 0.292, and 0.298 mg/mL dose formulations by the analytical chemistry laboratory using HPLC by system 2. Samples were extracted by shaking on a horizontal shaker for 30 minutes and then filtered prior to analysis. Stability was confirmed for samples stored protected from air, refrigerated or at room temperature, for up to 35 days and for samples stored at room temperature, open to air and light, for 3 hours; samples exposed to air for 3 days showed a total loss of 2,4-hexadienal. Homogeneity studies of the 0.75 and 24 mg/mL dose formulations for the 14-week studies and stability studies of a 0.77 mg/mL dose formulation were performed by the study laboratory with HPLC by system 3. Homogeneity was confirmed; stability was confirmed for dose formulations stored under a nitrogen headspace at up to room temperature for 24 hours and for dose formulations stored frozen under a nitrogen headspace and then thawed, open to air, for up to 3 hours.

Periodic analyses of the dose formulations of 2,4-hexadienal were conducted by the analytical chemistry laboratory (16-day studies) and the study laboratory (14-week and 2-year studies) using HPLC by systems 1 (16-day studies) and 2. During the 16-day studies, the dose formulations were analyzed once; four of five dose formulations for rats and mice were within 10% of the target concentrations; the dose formulation that was 84% of the target concentration was used and not remixed (Table I4). Animal room samples of these dose formulations were also analyzed; four of five animal room samples for rats and five of five for mice were within 10% of the target concentrations. During the 14-week studies, the dose formulations were analyzed at the beginning, midpoint, and end of the studies; animal room samples of these dose formulations were also analyzed (Table I5). All dose formulations analyzed were within 10% of the target concentrations, with no value greater than 109% of the target concentration; 14 of 16 animal room samples for rats and 13 of 17 for mice were within 10% of the target concentrations. During the 2-year studies, the dose formulations were analyzed every 8 to 12 weeks; animal room samples were also analyzed periodically (Table I6). All dose formulations analyzed for rats and 32 of 33 for mice were within 10% of the target concentrations, with no value greater than 113% of the target concentration; the dose formulation for mice that was not within the acceptable range was remixed and was found to be within 10% of the target concentration. Of the animal room samples analyzed, 10 of 12 for rats and all for mice were within 10% of the target concentrations. Periodic analyses of the corn oil vehicle by the study laboratories demonstrated that peroxide concentrations were within the acceptable limit of 3.0 mEq/kg.

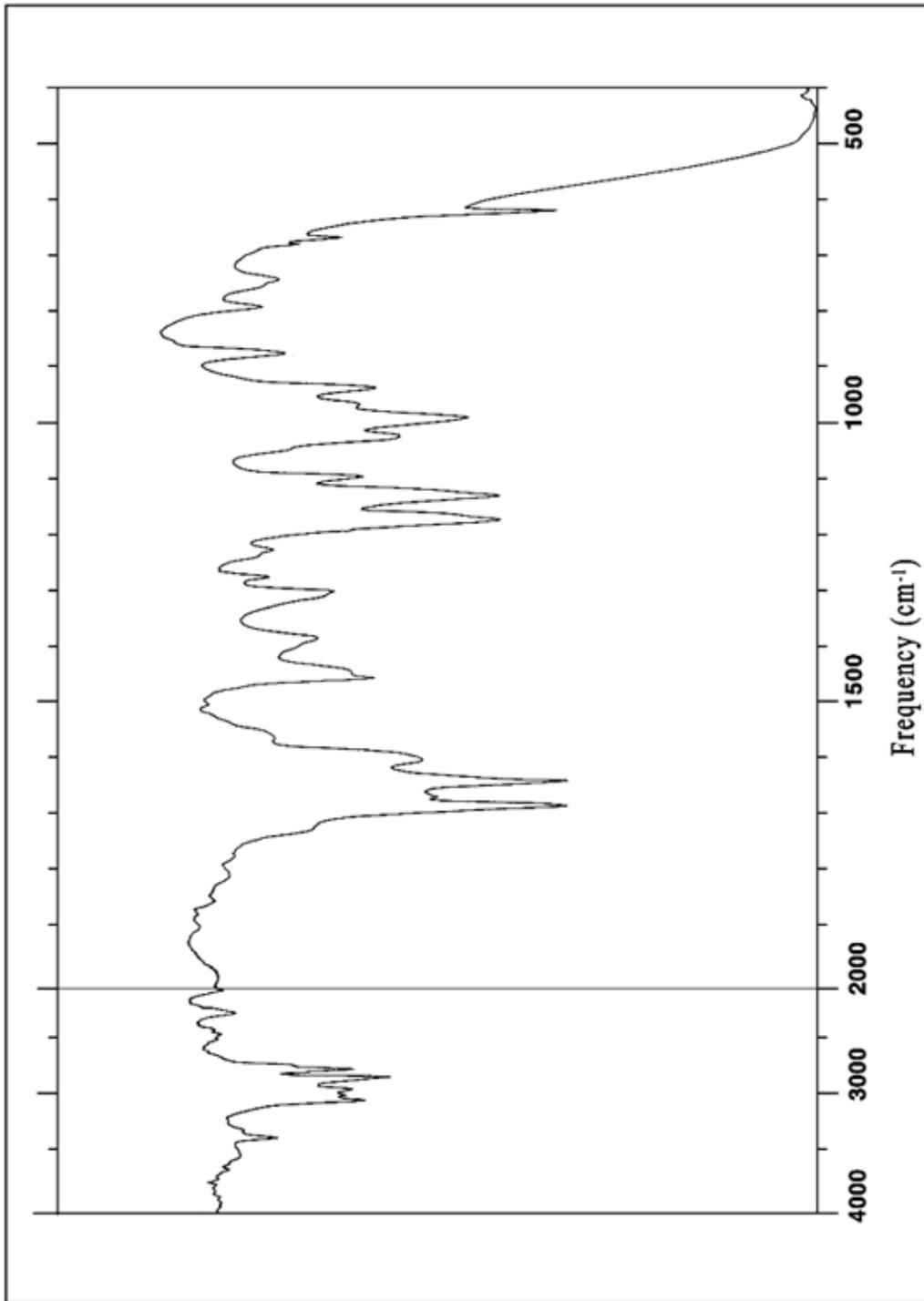


FIGURE I1
Infrared Absorption Spectrum of 2,4-Hexadienal

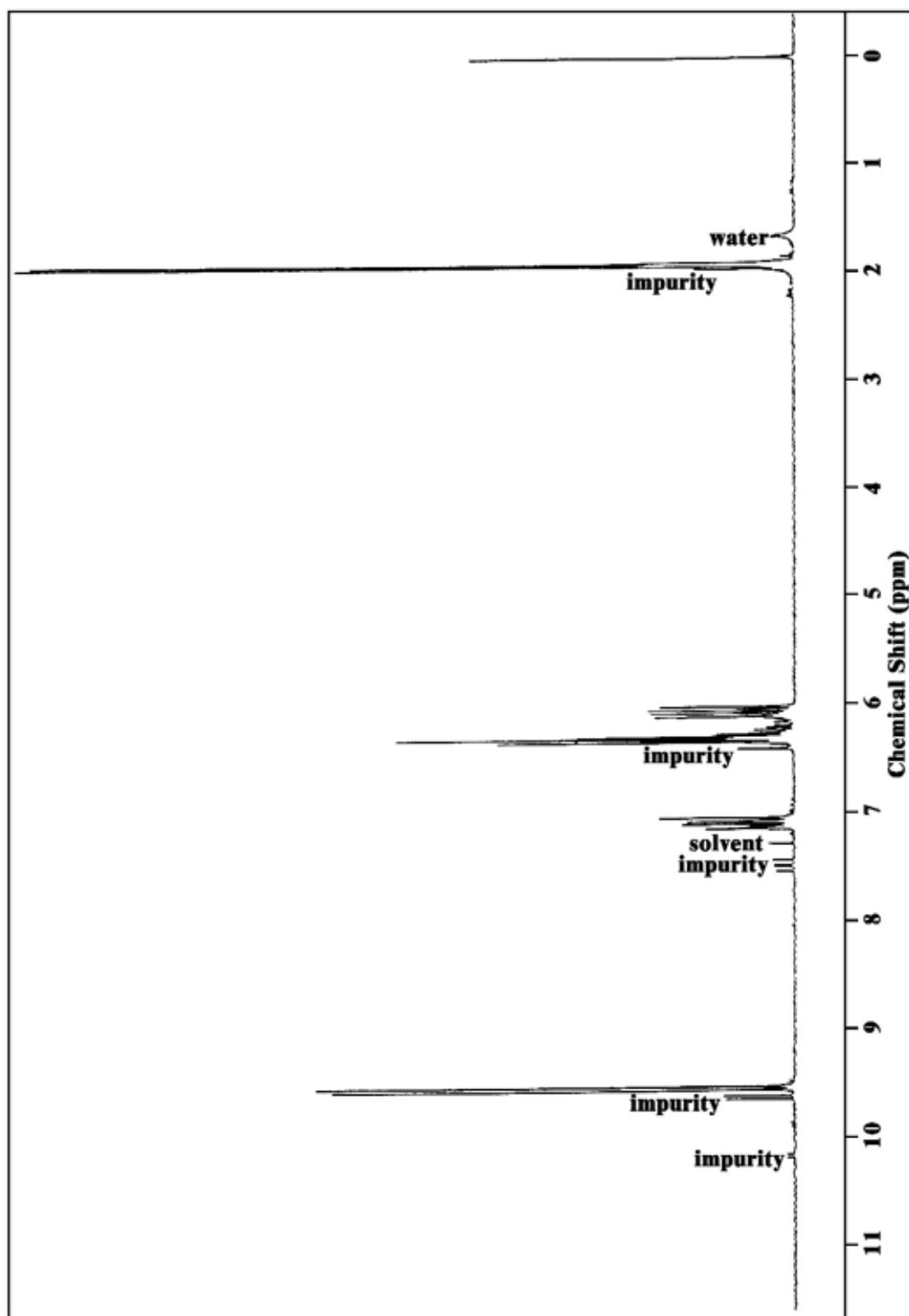


FIGURE I2
Nuclear Magnetic Resonance Spectrum of 2,4-Hexadienal

TABLE II
Gas Chromatography Systems Used in the Gavage Studies of 2,4-Hexadienal^a

Detection System	Column	Carrier Gas	Oven Temperature Program
System A Infrared spectroscopy	Supelco Nukol, 30 m × 0.25 mm 0.25- μ m film (Supelco, Inc., Bellefonte, PA)	Helium at 22.3 mL/minute	Isothermal at 80° C
System B Mass spectrometry	Supelco SPB-1, 60 m × 0.32 mm 1.0- μ m film (Supelco, Inc.)	Helium at 1.5 mL/minute	60° C for 2 minutes, then 10° C/minute to 230° C, held for 11 minutes
System C Flame ionization	DB-1 Megabore, 30 m × 0.53 mm (J&W Scientific)		50° C to 150° C at 5° C/minute; naphthalene was added as an internal standard
System D Flame ionization	J&W SE-30, 30 m × 0.25 mm 0.25- μ m film (J&W Scientific)	Helium at approximately 0.9 mL/minute	50° C to 150° C at 5° C/minute; naphthalene was added as an internal standard

^a Gas chromatographs were manufactured by Varian, Inc. (Palo Alto, CA) (systems A and B) and Hewlett-Packard (Palo Alto, CA) (systems C and D).

TABLE I2
High-Performance Liquid Chromatography Systems Used in the Gavage Studies of 2,4-Hexadienal

Detection System	Column	Solvent System
System 1 Ultraviolet (269 nm)	XPER-CHROM [®] C ₁₈ , 25 cm × 4.6 mm, 5-μm particle size (P.J. Cobert Associates, St. Louis, MO)	A) Water and B) methanol (70%A:30%B), flow rate 0.5 or 1.0 mL/minute
System 2 Ultraviolet (254 nm)	Zorbax C ₈ , 25 cm × 4.6 mm, 5-μm particle size (DuPont)	A) Acetonitrile and B) water (20%A:80%B), flow rate 1.5 mL/minute. Acetophenone was added as an internal standard.
System 3 Ultraviolet (254 nm)	Zorbax C ₈ , 25 cm × 4.6 mm, 5-μm particle size (DuPont)	A) Acetonitrile and B) water (25%A:75%B), flow rate 1.5 mL/minute

TABLE I3
Preparation and Storage of Dose Formulations in the Gavage Studies of 2,4-Hexadienal

16-Day Studies	14-Week Studies	2-Year Studies
Preparation 2,4-Hexadienal was added to corn oil and stirred until a homogeneous preparation was obtained. The dose formulations were prepared once during the study.	2,4-Hexadienal was added to corn oil under a nitrogen headspace and stirred with a magnetic stirrer until a homogeneous preparation was obtained. The dose formulations were prepared every 4 weeks.	Same as 14-week studies, except no nitrogen headspace was used during stirring.
Chemical Lot Number 90000345	P09653	P09653
Maximum Storage Time 35 days	35 days	35 days
Storage Conditions Stored refrigerated in amber glass containers under a nitrogen headspace	Same as 16-day studies	Same as 16-day studies
Study Laboratory Microbiological Associates, Inc. (Bethesda, MD)	Southern Research Institute (Birmingham, AL)	Southern Research Institute (Birmingham, AL)

TABLE I4
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 16-Day Gavage Studies of 2,4-Hexadienal

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration ^a (mg/mL)	Difference from Target (%)
October 30, 1995	November 3, 1995	1.2	1.71 ^b	+43
		3.6	4.69 ^b	+30
		10.8	13.9 ^b	+29
		32	36.5 ^b	+14
		96	124 ^b	+29
	November 3, 1995	1.2	1.32 ^c	+10
		3.6	3.65 ^c	+1
		10.8	11.2 ^c	+4
		32	26.9 ^c	-16
		96	96.8 ^c	+1
	December 20-21, 1995 ^d	1.2	1.32	+10
		3.6	3.53	-2
		10.8	11.1	+3
		32	36.3	+13
		96	89.0	-7
	December 20-21, 1995 ^e	1.2	1.30	+8
		3.6	3.50	-3
		10.8	10.9	+1
		32	29.5	-8
		96	88.4	-8

^a Results of duplicate analyses. Dosing volume=2.5 mL/kg; 1.2 mg/mL=3 mg/kg, 3.6 mg/mL=9 mg/kg, 10.8 mg/mL=27 mg/kg, 32 mg/mL=80 mg/kg, 96 mg/mL=240 mg/kg

^b High values were considered due to degradation of reference material; samples were reanalyzed.

^c Results of reanalysis

^d Animal room samples for rats

^e Animal room samples for mice

TABLE I5
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 14-Week Gavage Studies of 2,4-Hexadienal

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration ^a (mg/mL)	Difference from Target (%)		
Rats						
July 30-31, 1996	July 31-August 1, 1996	1.5	1.50	0		
		3	2.92	-3		
		6	6.00	0		
		12	12.1	+1		
		24	23.2	-3		
	August 7-9, 1996 ^b	1.5	1.65	+10		
		3	3.26	+9		
		6	6.66	+11		
		12	13.4	+12		
		24	25.2	+5		
	August 22-23, 1996 ^b	12	12.4	+3		
		August 21, 1996	August 22-23, 1996	1.5	1.47	-2
				3	3.01	0
				6	5.90	-2
				12	12.1	+1
24	24.1			0		
September 23-25, 1996 ^b	September 23-25, 1996 ^b	1.5	1.51	+1		
		3	3.00	0		
		6	5.90	-2		
		12	12.1	+1		
		24	23.9	0		
October 16, 1996	October 17, 1996	1.5	1.48	-1		
		3	3.01	0		
		6	6.04	+1		
		12	12.1	+1		
		24	24.1	0		
	November 12-14, 1996 ^b	November 12-14, 1996 ^b	1.5	1.47	-2	
			3	2.92	-3	
			6	5.94	-1	
			12	11.9	-1	
			24	23.2	-3	

TABLE I5
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 14-Week Gavage Studies of 2,4-Hexadienal

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)	
Mice					
July 30-31, 1996	July 31-August 1, 1996	0.75	0.814	+9	
		1.5	1.50	0	
		3	2.92	-3	
		6	6.00	0	
		12	12.1	+1	
	August 7-9, 1996 ^b	0.75	0.922	+23	
		1.5	1.68	+12	
		3	3.28	+9	
		6	6.65	+11	
		12	13.4	+12	
	August 22-23, 1996 ^b	0.75	0.803	+7	
		12	12.4	+3	
	August 21, 1996	August 22-23, 1996	0.75	0.743	-1
			1.5	1.47	-2
			3	3.01	0
6			5.90	-2	
12			12.1	+1	
September 23-25, 1996 ^b		0.75	0.704	-6	
		1.5	1.42	-5	
		3	2.83	-6	
		6	5.48	-9	
		12	11.4	-5	
October 16, 1996	October 17, 1996	0.75	0.758	+1	
		1.5	1.48	-1	
		3	3.01	0	
		6	6.04	+1	
		12	12.1	+1	
	November 12-14, 1996 ^b	0.75	0.709	-5	
		1.5	1.39	-7	
		3	2.84	-5	
		6	5.53	-8	
		12	11.5	-4	

^a Results of duplicate analyses. For rats, dosing volume=5 mL/kg; 1.5 mg/mL=7.5 mg/kg, 3 mg/mL=15 mg/kg, 6 mg/mL=30 mg/kg, 12 mg/mL=60 mg/kg, 24 mg/mL=120 mg/kg; for mice, dosing volume=10 mL/kg; 0.75 mg/mL=7.5 mg/kg, 1.5 mg/mL=15 mg/kg,

^b 3 mg/mL=30 mg/kg, 6 mg/mL=60 mg/kg, 12 mg/mL=120 mg/kg
 Animal room samples

TABLE I6
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 2-Year Gavage Studies of 2,4-Hexadienal

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration ^a (mg/mL)	Difference from Target (%)	
Rats					
July 8, 1997	July 8-9, 1997	4.5	4.81	+7	
		9	9.68	+8	
		18	19.4	+8	
	August 7-8, 1997 ^b	4.5	4.42	-2	
		9	8.87	-1	
		18	17.7	-2	
	September 30, 1997	October 1, 1997	4.5	4.55	+1
			9	9.03	0
			18	18.2	+1
November 24, 1997	November 25, 1997	4.5	4.65	+3	
		4.5	4.61	+2	
		9	9.41	+5	
		9	9.54	+6	
		18	19.0	+6	
		18	19.0	+6	
February 17, 1998	February 17-18, 1998	4.5	4.70	+4	
		9	9.33	+4	
		18	18.8	+4	
	March 24, 1998 ^b	4.5	4.57	+2	
		9	9.24	+3	
		18	18.5	+3	
April 14, 1998	April 15, 1998	4.5	4.55	+1	
		9	9.26	+3	
		18	18.5	+3	
July 7, 1998	July 7, 1998	4.5	4.35	-3	
		9	8.66	-4	
		18	17.5	-3	
September 2, 1998	September 2-3, 1998	4.5	4.43	-2	
		9	8.83	-2	
		18	17.8	-1	
	October 5, 1998 ^b	4.5	4.32	-4	
		9	8.76	-3	
		18	17.6	-2	
October 26, 1998	October 27, 1998	4.5	4.52	0	
		4.5	4.49	0	
		9	9.04	0	
		9	9.25	+3	
		18	18.1	+1	
		18	18.1	+1	

TABLE I6
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 2-Year Gavage Studies of 2,4-Hexadienal

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
Rats (continued)				
January 18, 1999	January 19, 1999	4.5	4.82	+7
		9	9.68	+8
		18	18.8	+4
April 13, 1999	April 14-16 and 19, 1999	4.5	4.20	-7
		9	9.16	+2
		18	19.3	+7
	May 14-15 and 17-18, 1999 ^b	4.5	4.53	+1
		9	10.1	+12
		18	20.8	+16
June 8, 1999	June 9, 1999	4.5	4.41	-2
		9	9.15	+2
		18	18.4	+2
Mice				
July 8, 1997	July 8-9, 1997	3	3.13	+4
		6	6.28	+5
		12	12.7	+6
	August 7, 1997 ^b	3	2.78	-7
		6	5.55	-7
		12	11.2	-7
September 30, 1997	October 1, 1997	3	2.97	-1
		6	5.93	-1
		12	11.9	-1
November 24, 1997	November 25, 1997	3	3.08	+3
		6	6.25	+4
		12	12.2	+2
February 17, 1998	February 17-18, 1998	3	3.13	+4
		6	6.26	+4
		12	12.4	+3
	March 20, 1998 ^b	3	2.96	-1
		6	5.99	0
		12	12.0	0
April 14, 1998	April 15, 1998	3	3.05	+2
		6	6.06	+1
		12	12.2	+2
July 7, 1998	July 7, 1998	3	2.96	-1
		6	5.87	-2
		12	11.8	-2

TABLE I6
Results of Analyses of Dose Formulations Administered to Rats and Mice
in the 2-Year Gavage Studies of 2,4-Hexadienal

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
Mice (continued)				
September 2, 1998	September 2-3, 1998	3	2.94	-2
		6	5.76	-4
		12	12.3	+3
	October 2, 1998 ^b	3	2.88	-4
		6	5.62	-6
		12	12.0	0
October 26, 1998	October 27, 1998	3	2.99	0
		6	6.01	0
		12	12.2	+2
January 18, 1999	January 19, 1999	3	3.40 ^c	+13
		6	6.52	+9
		12	12.8	+7
January 21, 1999	January 21, 1999	3	2.98 ^d	-1
April 13, 1999	April 14-16 and 19, 1999	3	2.70	-10
		6	5.54	-8
		12	11.6	-3
	May 13, 1999 ^b	3	2.89	-4
		6	6.02	0
		12	12.8	+7
June 8, 1999	June 9, 1999	3	3.02	+1
		6	5.92	-1
		12	12.0	0

^a Results of duplicate analyses. For rats, dosing volume=5 mL/kg; 4.5 mg/mL=22.5 mg/kg, 9 mg/mL=45 mg/kg, 18 mL/kg=90 mg/kg; for mice, dosing volume=10 mL/kg; 3 mL/kg=30 mg/kg, 6 mL/kg=60 mg/kg, 12 mL/kg=120 mg/kg

^b Animal room samples

^c Remixed; not used in study

^d Results of remix

APPENDIX J
INGREDIENTS, NUTRIENT COMPOSITION,
AND CONTAMINANT LEVELS
IN NTP-2000 RAT AND MOUSE RATION

TABLE J1	Ingredients of NTP-2000 Rat and Mouse Ration	268
TABLE J2	Vitamins and Minerals in NTP-2000 Rat and Mouse Ration	268
TABLE J3	Nutrient Composition of NTP-2000 Rat and Mouse Ration	269
TABLE J4	Contaminant Levels in NTP-2000 Rat and Mouse Ration	270

TABLE J1
Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground hard winter wheat	22.26
Ground #2 yellow shelled corn	22.18
Wheat middlings	15.0
Oat hulls	8.5
Alfalfa meal (dehydrated, 17% protein)	7.5
Purified cellulose	5.5
Soybean meal (49% protein)	5.0
Fish meal (60% protein)	4.0
Corn oil (without preservatives)	3.0
Soy oil (without preservatives)	3.0
Dried brewer's yeast	1.0
Calcium carbonate (USP)	0.9
Vitamin premix ^a	0.5
Mineral premix ^b	0.5
Calcium phosphate, dibasic (USP)	0.4
Sodium chloride	0.3
Choline chloride (70% choline)	0.26
Methionine	0.2

^a Wheat middlings as carrier

^b Calcium carbonate as carrier

TABLE J2
Vitamins and Minerals in NTP-2000 Rat and Mouse Ration^a

	Amount	Source
Vitamins		
A	4,000 IU	Stabilized vitamin A palmitate or acetate
D	1,000 IU	D-activated animal sterol
K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl acetate	100 IU	
Niacin	23 mg	
Folic acid	1.1 mg	
<i>d</i> -Pantothenic acid	10 mg	<i>d</i> -Calcium pantothenate
Riboflavin	3.3 mg	
Thiamine	4 mg	Thiamine mononitrate
B ₁₂	52 μg	
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	<i>d</i> -Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

^a Per kg of finished product

TABLE J3
Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	13.2 ± 0.40	12.5 – 14.2	27
Crude fat (% by weight)	8.1 ± 0.25	7.6 – 8.6	27
Crude fiber (% by weight)	9.3 ± 0.71	7.9 – 10.3	27
Ash (% by weight)	5.0 ± 0.16	4.7 – 5.3	27
Amino Acids (% of total diet)			
Arginine	0.731 ± 0.050	0.670 – 0.800	8
Cystine	0.224 ± 0.012	0.210 – 0.240	8
Glycine	0.684 ± 0.041	0.620 – 0.740	8
Histidine	0.333 ± 0.018	0.310 – 0.350	8
Isoleucine	0.524 ± 0.046	0.430 – 0.590	8
Leucine	1.061 ± 0.061	0.960 – 1.130	8
Lysine	0.708 ± 0.056	0.620 – 0.790	8
Methionine	0.401 ± 0.035	0.350 – 0.460	8
Phenylalanine	0.598 ± 0.036	0.540 – 0.640	8
Threonine	0.501 ± 0.051	0.430 – 0.590	8
Tryptophan	0.126 ± 0.014	0.110 – 0.150	8
Tyrosine	0.390 ± 0.056	0.280 – 0.460	8
Valine	0.640 ± 0.049	0.550 – 0.690	8
Essential Fatty Acids (% of total diet)			
Linoleic	3.97 ± 0.284	3.59 – 4.54	8
Linolenic	0.30 ± 0.042	0.21 – 0.35	8
Vitamins			
Vitamin A (IU/kg)	5,394 ± 1,157	3,280 – 7,790	27
Vitamin D (IU/kg)	1,000 ^a		
α-Tocopherol (ppm)	82.2 ± 14.08	62.2 – 107.0	8
Thiamine (ppm) ^b	7.6 ± 0.87	6.1 – 9.3	27
Riboflavin (ppm)	5.6 ± 1.12	4.20 – 7.70	8
Niacin (ppm)	74.3 ± 5.94	66.4 – 85.8	8
Pantothenic acid (ppm)	22.5 ± 3.96	17.4 – 29.1	8
Pyridoxine (ppm) ^b	9.04 ± 2.37	6.4 – 12.4	8
Folic acid (ppm)	1.64 ± 0.38	1.26 – 2.32	8
Biotin (ppm)	0.333 ± 0.15	0.225 – 0.704	8
Vitamin B ₁₂ (ppb)	68.7 ± 63.0	18.3 – 174.0	8
Choline (ppm) ^c	3,155 ± 325	2,700 – 3,790	8
Minerals			
Calcium (%)	0.965 ± 0.045	0.871 – 1.050	27
Phosphorus (%)	0.546 ± 0.025	0.496 – 0.592	27
Potassium (%)	0.659 ± 0.022	0.627 – 0.691	8
Chloride (%)	0.357 ± 0.027	0.300 – 0.392	8
Sodium (%)	0.189 ± 0.019	0.160 – 0.212	8
Magnesium (%)	0.199 ± 0.009	0.185 – 0.213	8
Sulfur (%)	0.178 ± 0.021	0.153 – 0.209	8
Iron (ppm)	160 ± 14.7	135 – 177	8
Manganese (ppm)	50.3 ± 4.82	42.1 – 56.0	8
Zinc (ppm)	50.7 ± 6.59	43.3 – 61.1	8
Copper (ppm)	6.29 ± 0.828	5.08 – 7.59	8
Iodine (ppm)	0.461 ± 0.187	0.233 – 0.843	8
Chromium (ppm)	0.542 ± 0.128	0.330 – 0.707	8
Cobalt (ppm)	0.23 ± 0.049	0.20 – 0.30	8

^a From formulation

^b As hydrochloride

^c As chloride

TABLE J4
Contaminant Levels in NTP-2000 Rat and Mouse Ration^a

	Mean ± Standard Deviation ^b	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.20 ± 0.129	0.10 – 0.50	27
Cadmium (ppm)	0.04 ± 0.012	0.04 – 0.10	27
Lead (ppm)	0.09 ± 0.038	0.06 – 0.25	27
Mercury (ppm)	<0.02		27
Selenium (ppm)	0.17 ± 0.032	0.13 – 0.28	27
Aflatoxins (ppb)	<5.00		27
Nitrate nitrogen (ppm) ^c	15.4 ± 7.93	9.04 – 39.6	27
Nitrite nitrogen (ppm) ^c	<0.61		27
BHA (ppm) ^d	1.1 ± 0.35	1.0 – 2.5	27
BHT (ppm) ^d	1.0 ± 0.13	1.0 – 1.7	27
Aerobic plate count (CFU/g)	10 ± 1.9	10 – 20	27
Coliform (MPN/g)	0.1 ± 0.6	0 – 3	27
<i>Escherichia coli</i> (MPN/g)	<10		27
<i>Salmonella</i> (MPN/g)	Negative		27
Total nitrosoamines (ppb) ^e	4.8 ± 1.84	2.1 – 8.8	27
<i>N</i> -Nitrosodimethylamine (ppb) ^e	1.9 ± 0.90	1.0 – 5.1	27
<i>N</i> -Nitrosopyrrolidine (ppb) ^e	2.9 ± 1.4	1.0 – 5.6	27
Pesticides (ppm)			
α-BHC	<0.01		27
β-BHC	<0.02		27
γ-BHC	<0.01		27
δ-BHC	<0.01		27
Heptachlor	<0.01		27
Aldrin	<0.01		27
Heptachlor epoxide	<0.01		27
DDE	<0.01		27
DDD	<0.01		27
DDT	<0.01		27
HCB	<0.01		27
Mirex	<0.01		27
Methoxychlor	<0.05		27
Dieldrin	<0.01		27
Endrin	<0.01		27
Telodrin	<0.01		27
Chlordane	<0.05		27
Toxaphene	<0.10		27
Estimated PCBs	<0.20		27

TABLE J4
Contaminant Levels in NTP-2000 Rat and Mouse Ration

	Mean ± Standard Deviation	Range	Number of Samples
Pesticides (ppm) (continued)			
Ronnel	<0.01		27
Ethion	<0.02		27
Trithion	<0.05		27
Diazinon	<0.10		27
Methyl chlorpyrifos	0.100 ± 0.083	0.020 – 0.368	27
Methyl parathion	<0.02		27
Ethyl parathion	<0.02		27
Malathion	0.303 ± 0.540	0.020 – 2.810	27
Endosulfan I	<0.01		27
Endosulfan II	<0.01		27
Endosulfan sulfate	<0.03		27

^a All samples were irradiated. CFU=colony-forming units; MPN=most probable number; BHC=hexachlorocyclohexane or benzene hexachloride

^b For values less than the limit of detection, the detection limit is given as the mean.

^c Sources of contamination: alfalfa, grains, and fish meal

^d Sources of contamination: soy oil and fish meal

^e All values were corrected for percent recovery.

APPENDIX K

SENTINEL ANIMAL PROGRAM

METHODS	274
RESULTS	275

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 rats and mice during the 14-week and 2-year studies. Blood from each animal was collected and allowed to clot, and the serum was separated. The samples were processed appropriately and sent to BioReliance Corporation (Rockville, MD) for determination of antibody titers. 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

RATS

14-Week Study

ELISA

Mycoplasma arthritis

Study termination

Mycoplasma pulmonis

Study termination

PVM (pneumonia virus of mice)

Study termination

RCV/SDA

(rat coronavirus/sialodacryoadenitis virus)

Study termination

Sendai

Study termination

Hemagglutination Inhibition

H-1 (Toolan's H-1 virus)

Study termination

KRV (Kilham rat virus)

Study termination

2-Year Study

ELISA

M. arthritis

6, 12, and 18 months, study termination

M. pulmonis

6, 12, and 18 months, study termination

PVM

6, 12, and 18 months, study termination

RCV/SDA

6, 12, and 18 months, study termination

Sendai

6, 12, and 18 months, study termination

Immunofluorescence Assay

Parvovirus

6, 12, and 18 months, study termination

MICE**14-Week Study**

ELISA

Ectromelia virus	Study termination
EDIM (epizootic diarrhea of infant mice)	Study termination
GDVII (mouse encephalomyelitis virus)	Study termination
LCM (lymphocytic choriomeningitis virus)	Study termination
Mouse adenoma virus-FL	Study termination
MHV (mouse hepatitis virus)	Study termination
<i>M. arthritidis</i>	Study termination
<i>M. pulmonis</i>	Study termination
PVM	Study termination
Reovirus 3	Study termination
Sendai	Study termination

Immunofluorescence Assay

MCMV (mouse cytomegalovirus)	Study termination
------------------------------	-------------------

Hemagglutination Inhibition

K (papova virus)	Study termination
MVM (minute virus of mice)	Study termination
Polyoma virus	Study termination

2-Year Study

ELISA

Ectromelia virus	6, 12, and 18 months, study termination
EDIM	6, 12, and 18 months, study termination
GDVII	6, 12, and 18 months, study termination
LCM	6, 12, and 18 months, study termination
Mouse adenoma virus-FL	6, 12, and 18 months, study termination
MHV	6, 12, and 18 months, study termination
<i>M. arthritidis</i>	6, 12, and 18 months, study termination
<i>M. pulmonis</i>	6, 12, and 18 months, study termination
PVM	6, 12, and 18 months, study termination
Reovirus 3	6, 12, and 18 months, study termination
Sendai	6, 12, and 18 months, study termination

Immunofluorescence Assay

GDVII	12 months, study termination
LCM	12 months, study termination
Mouse adenoma virus-FL	12 months, study termination
MCMV	6, 12, and 18 months, study termination
Parvovirus	6, 12, and 18 months, study termination
PVM	12 months, study termination

RESULTS

All test results were negative.

APPENDIX L
MEASURES OF 2,4-HEXADIENAL-INDUCED
OXIDATIVE STRESS IN THE FORESTOMACH
OF F344/N RATS

INTRODUCTION	278
MATERIALS AND METHODS	278
RESULTS	278
REFERENCES	279
TABLE L1 Glutathione Concentrations in the Forestomach of F344/N Rats in the 28-Day Gavage Study of 2,4-Hexadienal	280
TABLE L2 Malondialdehyde Concentrations in the Forestomach of F344/N Rats in the 28-Day Gavage Study of 2,4-Hexadienal	281

MEASURES OF 2,4-HEXADIENAL-INDUCED OXIDATIVE STRESS IN THE FORESTOMACH OF F344/N RATS

INTRODUCTION

These studies were performed to evaluate whether oral administration of 2,4-hexadienal to F344/N rats induces the formation of the lipid peroxidation product malondialdehyde in the forestomach and/or affects the defensive antioxidant glutathione system. The studies were conducted at SRI International, Menlo Park, CA.

MATERIALS AND METHODS

Forestomach samples were collected from groups of 10 male and 10 female F344/N rats administered 0, 90, or 120 mg/kg 2,4-hexadienal in corn oil by gavage for 28 days to measure the concentrations of reduced glutathione (GSH), oxidized glutathione (GSSG), and malondialdehyde (MDA).

Animals were sacrificed 1, 4, or 24 hours after the last dose by exsanguination following intraperitoneal injection of sodium pentobarbital. The forestomach was excised, rinsed repeatedly in phosphate-buffered saline to remove stomach contents, weighed, immediately immersed in liquid nitrogen, and stored at approximately -80°C . Tissues from three male and three female rats were collected for the MDA analyses, and tissues from four male and four female rats were collected for the glutathione analyses.

Samples collected from the vehicle control and 120 mg/kg groups were analyzed first to determine whether a treatment-related effect could be observed. Forestomach samples were homogenized in the presence of a buffer containing butylated hydroxyanisole prior to analysis. Glutathione analyses were performed by the method of Toyooka, *et al.* (1989) using separation on high-performance liquid chromatography (HPLC) after successive derivatizations of GSH and GSSG with different fluorescent reagents. Malondialdehyde concentrations were measured by the method of Fukunaga *et al.* (1998) using separation on HPLC after derivatizations of MDA with thiobarbituric acid.

Nonparametric bootstrap trend tests developed by Peddada *et al.* (2001) were used to detect dose-related trends in the forestomach concentrations of GSH, GSSG, GSH + GSSG, and MDA and in the ratio of GSH to GSSG. The data were first converted in terms of ranks. Using the rank data, the means of the ranks of the dosed and vehicle control groups were estimated using the isotonic regression methodology. Similar to the method of Peddada *et al.* (2001), the trend test statistic was then obtained by taking the difference between the largest estimated mean rank and the smallest estimated mean rank. Critical values of this trend test were obtained by the bootstrap methodology. For the GSH, GSSG, GSH + GSSG, and MDA data, positive trends were tested (time point \times sex) to evaluate the potential effect of the compound on GSH, GSSG, and GSH + GSSG, as well as on increased levels of the lipid peroxidation endproduct MDA. Conversely, negative trends were tested for the ratio of GSH to GSSG in order to evaluate the potential effect of the compound on oxidative stress.

RESULTS

The concentration of GSH increased significantly in males at 1 and 4 hours postdosing and in females at 4 and 24 hours postdosing. The concentration of GSSG increased significantly in males at all three timepoints and in females at 4 and 24 hours postdosing. The concentration of GSH + GSSG increased significantly in males at 4 hours postdosing and in females at 4 and 24 hours postdosing. There was a significant reduction of the GSH/GSSG ratio in males at 4 hours postdosing and no significant trend at other times.

No statistically significant changes in the concentration of MDA were observed in the forestomach of male or female rats.

REFERENCES

- Fukunaga, K., Yoshida, M., and Nakazono, N. (1998). A simple, rapid, highly sensitive and reproducible quantification method for plasma malondialdehyde by high performance liquid chromatography. *Biomed. Chromatogr.* **12**, 300-303.
- Peddada, S., Prescott, K., and Conway, M. (2001). Tests for order restrictions in binary data. *Biometrics* **57**, 1219-1227.
- Toyooka, T., Furukawa, F., Suzuki, T., Saito, Y., Takahashi, M., Hayashi, Y., Uzu, S., and Imai, K. (1989). Determination of thiols and disulfides in normal rat tissues and hamster pancreas treated with N-nitrosobis(2-oxopropyl)amine using 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole and ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate. *Biomed. Chromatogr.* **4**, 166-172.

TABLE L1
Glutathione Concentrations in the Forestomach of F344/N Rats
in the 28-Day Gavage Study of 2,4-Hexadienal^a

	Time (hours after last dose)	Vehicle Control	90 mg/kg ^b	120 mg/kg ^b	P value ^c
Male					
Reduced glutathione					
	1	1,510 ± 23.8	1,764 ± 342 (117)	3,308 ± 1,335 (219)	0.026
	4	1,741 ± 522	1,619 ± 231 (93)	2,799 ± 841 (161)	0.029
	24	1,064 ± 148	3,051 ± 3,359 (287)	3,451 ± 1,905 (324)	0.064
Oxidized glutathione					
	1	545 ± 124	2,269 ± 499 (416)	1,606 ± 92.8 (295)	0.013
	4	687 ± 182	2,288 ± 247 (333)	2,419 ± 803 (352)	0.012
	24	641 ± 128	867 ± 37 (135)	1,381 ± 207 (215)	0.034
Reduced glutathione/oxidized glutathione ratio					
	1	2.85 ± 0.69	0.83 ± 0.34 (29)	2.09 ± 0.95 (73)	0.079
	4	2.73 ± 1.48	0.71 ± 0.12 (26)	1.16 ± 0.04 (43)	0.026
	24	1.72 ± 0.48	3.6 ± 4.03 (210)	2.50 ± 1.22 (145)	0.990
Reduced glutathione + oxidized glutathione					
	1	2,055 ± 100	4,033 ± 187 (196)	4,914 ± 1,242 (239)	0.059
	4	2,428 ± 341	3,908 ± 362 (161)	5,218 ± 1,644 (215)	0.011
	24	1,706 ± 125	3,918 ± 3,322 (230)	4,833 ± 1,978 (283)	0.052
Female					
Reduced glutathione					
	1	1,038 ± 440	1,219 ± 748 (117)	1,231 ± 943 (119)	0.570
	4	935 ± 298	2,079 ± 635 (222)	2,231 ± 680 (239)	0.029
	24	1,300 ± 175	6,246 ± 2,619 (482)	4,903 ± 807 (377)	0.050
Oxidized glutathione					
	1	871 ± 235	2,142 ± 51.7 (246)	1,826 ± 358 (210)	0.086
	4	726 ± 170	2,183 ± 2.4 (300)	1,081 ± 276 (149)	0.034
	24	412 ± 170	2,539 ± 1,535 (616)	1,478 ± 1,161 (358)	0.049
Reduced glutathione/oxidized glutathione ratio					
	1	1.3 ± 0.72	0.57 ± 0.36 (44)	0.64 ± 0.42 (49)	0.122
	4	1.3 ± 0.26	0.95 ± 0.29 (73)	2.22 ± 1.01 (171)	0.990
	24	3.5 ± 0.48	3.34 ± 4.03 (94)	4.49 ± 1.21 (127)	0.990
Reduced glutathione + oxidized glutathione					
	1	1,910 ± 299	3,362 ± 696 (176)	3,057 ± 1,206 (160)	0.079
	4	1,661 ± 453	4,263 ± 638 (257)	3,312 ± 470 (199)	0.020
	24	1,712 ± 502	8,803 ± 1,503 (514)	6,380 ± 1,968 (373)	0.046

^a n=4 at each time point; concentrations are given in nmoles/gram forestomach tissue (mean ± standard deviation).

^b Value in parenthesis is percent of vehicle control.

^c Trend test, significant at P≤0.05 (Peddada *et al.*, 2001)

TABLE L2
Malondialdehyde Concentrations in the Forestomach of F344/N Rats
in the 28-Day Gavage Study of 2,4-Hexadienal^a

	Time (hours after last dose)	Vehicle Control	90 mg/kg ^b	120 mg/kg ^b	P value ^c
Male					
Malondialdehyde	1	3.17 ± 1.03	2.35 ± 0.16 (74)	3.15 ± 0.48 (99)	0.455
	4	4.92 ± 2.46	5.23 ± 1.07 (106)	3.23 ± 0.43 (66)	0.596
	24	4.45 ± 1.76	2.82 ± 0.49 (63)	4.61 ± 1.54 (103)	0.999
Female					
Malondialdehyde	1	4.31 ± 2.38	5.67 ± 4.76 (132)	2.24 ± 0.05 (52)	0.999
	4	2.54 ± 0.88	4.13 ± 0.92 (163)	3.45 ± 1.09 (136)	0.156
	24	5.73 ± 0.43	3.48 ± 0.05 (61)	4.79 ± 0.81 (84)	0.999

^a n=3 at each time point; concentrations are given in nmoles/gram forestomach tissue (mean ± standard deviation).

^b Value in parenthesis is percent of vehicle control.

^c Trend test, significant at P ≤ 0.05 (Peddada *et al.*, 2001)

APPENDIX M

DNA ADDUCT CHARACTERIZATION STUDIES

INTRODUCTION	284
MATERIALS AND METHODS	284
RESULTS	285
REFERENCES	286
TABLE M1 DNA Adducts in the Liver of Male F344/N Rats in the 2-Year Gavage Study of 2,4-Hexadienal	287
TABLE M2 DNA Adducts in the Forestomach of Male F344/N Rats in the 2-Year Gavage Study of 2,4-Hexadienal	288
TABLE M3 DNA Adducts in the Forestomach of Male B6C3F₁ Mice in the 2-Year Gavage Study of 2,4-Hexadienal	289

DNA ADDUCT CHARACTERIZATION STUDIES

INTRODUCTION

Acrolein and crotonaldehyde are ubiquitous pollutants in the environment and also products of lipid peroxidation. In the past several years, Chung *et al.* (1996) and Nath *et al.* (1996) have studied acrolein-, crotonaldehyde- and *trans*-4-hydroxy-2-nonenal-derived 1,*N*²-propanodeoxyguanosine and related cyclic adducts. Chung *et al.* (1996) showed by a ³²P-postlabeling-high performance liquid chromatography (HPLC) assay that the cyclic propano-dG adducts are present in tissue DNA of humans and untreated rodents at relatively high levels. Collective results indicate that oxidized polyunsaturated fatty acids are an important endogenous source of propano adducts. Lipid peroxidation can be stimulated by enals, such as 2,4-hexadienal, by depleting endogenous glutathione. The hypothesis that treatment with this dienal can result in an increase in the endogenous formation of acrolein and crotonaldehyde-derived cyclic DNA adducts in the target tissues was tested here.

DNA adduct analysis was performed on samples of liver and forestomach tissue from male F344/N rats and forestomach tissue from B6C3F₁ mice administered 0, 90 (rats only), or 120 (mice only) mg 2,4-hexadienal/kg body weight by gavage. Vehicle control male rats were treated for 118 days; all other rats and mice were treated for 90 days. The studies were conducted at the American Health Foundation, Valhalla, NY.

MATERIALS AND METHODS

To isolate DNA, approximately 400 mg of tissue was homogenized for 10 to 15 seconds in 3 mL Tris EDTA-NaCl buffer (0.01 M-0.001 M-1.0 M; pH 7.0); 100 µL of 10% sodium dodecylsulfate was then mixed into the sample, which was vortexed and incubated at 37° C for 30 minutes. The mixture was then extracted by manual shaking for 5 minutes with 3 mL chloroform:isoamyl alcohol (24:1) (Sevag procedure) and then centrifuged at 5,000 rpm for 12 minutes at 4° C. The supernatant and 2 units of ribonuclease A were placed in glass vials and incubated at 37° C. After 60 minutes, 4 units of protease were added and incubation was continued at 37° C for 40 minutes. The sample was then extracted twice with 3 mL chloroform:isoamyl alcohol (24:1), and the DNA was precipitated with 150 µL 5 M NaCl and 3 mL cold alcohol at -20° C for approximately 60 minutes or overnight for complete precipitation. Following centrifugation at 3,500 × g for 15 minutes, the supernatant was discarded, and the DNA was washed twice with 3 mL 70% alcohol for 5 minutes and then air dried by turning the tubes upside down for 5 minutes. The DNA was quantified and checked spectrophotometrically for purity.

After isolation, the DNA was digested to mononucleotides with desalted micrococcal nuclease (MN) and spleen phosphodiesterase (SPD). Digestion was carried out in a 50:50 mixture of sodium succinate buffer (150 mM, pH 6.0) and calcium chloride (50 mM) at 37° C for 3.5 hours; ideally, the DNA solution was approximately 2 µg DNA/µL.

Deoxyguanosine-3'-monophosphate (dGp) was quantified in the filtered digest using HPLC performed with a Phenomenex C₁₈ reverse phase 4.6 mm × 250 mm column with 5 µm particle size using a Waters 994 Photodiode Array Detector (Waters-Millipore, Milford, MA) and a solvent system of A) 50 mM NaH₂PO₄, pH 5.8 and B) methanol:water (50:50), 0% to 30% B in 15 minutes, at a flow rate of 1.0 mL/minute. This C₁₈ column was used in all subsequent HPLC analyses; the composition of phase A and the phase-transition gradient was varied in other HPLC procedures.

In an HPLC prepurification step to remove most of the unmodified (normal) nucleotides from the adducts, a similar HPLC system using photodiode array detection, 1 mM Tris-HCl as phase A, and a gradient program of 0% to 30% B in 30 minutes was used. The HPLC analysis identified retention times for Acr-dG and Cro-dG 3'-monophosphates and showed that deoxyadenosine-3'-monophosphate (dAp) could be used as an internal marker for collection of Acr-dG.

For subsequent assays, 10 fmole standard and positive control rat liver samples were analyzed along with the experimental DNA digests. Ideally, the fractions used for ^{32}P -postlabeling contained approximately 100 μg DNA. Prior to ^{32}P -postlabeling, unmodified nucleotides in the fractions were dephosphylated using a nuclease P1 enrichment step in which 12 μL nuclease P1 (4 $\mu\text{g}/\mu\text{L}$), 2.5 μL sodium acetate (1 M, pH 5.0), 5.5 μL zinc chloride (1 mM), and 20 μL water were added to the fractions. Each mixture was vortexed, spun in a microcentrifuge for 30 seconds at maximum speed, and incubated for 60 minutes at 37° C. To facilitate the reaction, the samples were briefly vortexed and spun again 15 minutes after the start of incubation. At the end of the reaction, 6.5 μL Tris base (500 mM) was added to the samples, which were then vortexed and dried.

To perform ^{32}P -postlabeling, a mixture containing 6.65 μL water, 2 μL [γ - ^{32}P]-ATP (10 $\mu\text{Ci}/\mu\text{L}$); Amersham Pharmacia Biotech, Piscataway, NJ), 2 μL kinase buffer (dithiothreitol/spermidine/ MgCl_2 /bicine), and 0.35 μL T_4 polynucleotide kinase was added to each of the dried samples. The samples were vortexed, spun for 30 seconds, and incubated at 37° C. After 40 minutes, 3 μL apyrase (20 mU/ μL) was added to each sample, and incubation was continued for 20 minutes at 37° C.

After the apyrase incubation, the samples were spotted onto polyetheleneimine cellulose thin layer chromatography (TLC) sheets; the TLC sheets were developed in 2.25 M ammonium formate (pH 3.5). The sheets were then removed, air dried, spotted with radioactive ink (^{99}Tc , ammonium pertechnetate), and autoradiographed in steel cassettes on Kodak XAR film for 20 minutes (Ewen Parker X-rays, Elmsford, NY). The film was developed, fixed, and dried, and the adduct areas were marked in ink. The hot chromatogram was placed on the film atop a light box, and the adduct areas on the sheet were marked with a soft pencil. The adduct areas were cut from the chromatograms, put into glass vials, and extracted with 2 mL isopropanol:ammonium hydroxide (6.5 M:3 N) by shaking in a water bath for 15 minutes at 37° C. The extract was filtered, dried, reconstituted in distilled water, and refiltered. The extract was spiked with Acr-dG and Cro-dG bisphosphate ultraviolet (UV) markers and purified by reverse-phase HPLC on two columns in series using procedures as described above except that phase A (50 mM NaH_2PO_4) was at pH 5.2, and the phase gradient was 0% to 40% B in 50 minutes. The second, larger acrolein adduct peak (Acr-dG 2+3) and both Cro-dG peaks were collected; these Acr-dG and Cro-dG fractions were dried and repurified using an ion-pair HPLC analysis similar to those described above except that phase A (25 mM triethylamine phosphate) was at pH 6.5, and the phase gradient was 0% to 45% B in 45 minutes.

The purified adducts were analyzed by reverse-phase radioflow HPLC, with small amounts of extra UV standards added if needed to guarantee observable UV peaks. In this reverse phase HPLC system, a Flow One β -RAM radiodetector, 10 mM sodium citrate phase A (pH 5.0), and 0% to 60% B in 60 minutes gradient were used. The radioactivity of the standard and the sample (dpm for each isomer) were determined, and the values were corrected for radioactive decay for the number of days from the reference date of the [^{32}P]-ATP to the date of analysis. Using the recoveries of the standards and the volumes of the labeled fractions, the total adduct in the sample and nmole adduct per mole deoxyguanosine were calculated.

RESULTS

Following 90 days of administration, there was no significant difference in the concentration of DNA adducts detected in liver samples of vehicle control and 90 mg/kg male rats (Table M1). In rat forestomach samples, Acr-dG 3 concentrations appeared to be greater in the treated group than in the vehicle control group, although the difference was not significant ($P=0.079$). While neither Cro-dG 1 nor Cro-dG 2 were detected in forestomach tissue from vehicle control rats, Cro-dG 2 was present in tissue from rats dosed with 90 mg/kg (Table M2). These results suggest that treatment with 2,4-hexadienal may increase cyclic adduct formation in rat forestomach DNA via a lipid peroxidation pathway. In mouse forestomach tissue, no significant change in concentration of the Acr-dG 3 adduct was detected following 90 days of exposure to 120 mg/kg 2,4-hexadienal. Cro-dG adduct concentrations appeared to be greater in samples from the vehicle control group than in those from the 120 mg/kg group ($P=0.0010$ for Cro-dG 1; $P=0.0011$ for Cro-dG 2) (Table M3).

REFERENCES

Chung, F.L., Chen, H.J., and Nath, R.G. (1996). Lipid peroxidation as a potential endogenous source for the formation of exocyclic DNA adducts. *Carcinogenesis* **17**, 2105-2111.

Nath, R.G., Ocando, J.E., and Chung, F.L. (1996). Detection of 1, N2-propanodeoxyguanosine adducts as potential endogenous DNA lesions in rodent and human tissues. *Cancer Res.* **56**, 452-456.

TABLE M1
DNA Adducts in the Liver of Male F344/N Rats in the 2-Year Gavage Study of 2,4-Hexadienal^a

Group	Sample Code	Acrolein-dG 3	Crotonaldehyde-dG 1	Crotonaldehyde-dG 2
Vehicle control	VM431	233	3	6
	VM432	223	5	7
	VM433	185	4	15
	VM434	147 ^b	1	10
	VM435	—	—	—
Mean ± standard deviation		197 ± 39	3 ± 2	10 ± 4
Positive control		146	9	31
90 mg/kg	HM411	294	6	16
	HM412	106	0	10
	HM413	130	0	14
	HM414	181	0	15
	HM415	55	0	8
Mean ± standard deviation		153 ± 87	1 ± 3	13 ± 3
Positive control		602	20	68
		P=0.40 ^c	P=0.23	P=0.25

^a Data are given as nmoles adduct/mole of deoxyguanosine (dG); samples were collected from rats sacrificed after 90 days (90 mg/kg group) or 118 days (vehicle control group) of treatment.

^b Lost in final analysis, no radioactive peaks

^c Comparison (t-test) between the vehicle control and dosed group

TABLE M2
DNA Adducts in the Forestomach of Male F344/N Rats in the 2-Year Gavage Study of 2,4-Hexadienal^a

Group	Sample Code	Acrolein-dG 3	Crotonaldehyde-dG 1	Crotonaldehyde-dG 2
Vehicle control	VM431	0	0	0
	VM432	2.26	0	0
	VM433	0	0	0
	VM434	0.60	0	0
	VM435	0	0	0
Mean ± standard deviation		0.57 ± 0.98		
90 mg/kg	HM411	1.14	0	1.97
	HM412	1.46	0	1.20
	HM413	4.75	0	0
	HM414	10.22	0	1.87
	HM415	2.73	0	2.91
Mean ± standard deviation		4.06 ± 3.72		1.59 ± 1.08
Positive control		22.4	11.8	2.7
		P=0.079 ^b	NA ^c	P=0.011

^a Data are given as nmoles adduct/mole of deoxyguanosine (dG); samples were collected from rats sacrificed after 90 days (90 mg/kg group) or 118 days (vehicle control group) of treatment.

^b Comparison (t-test) between the vehicle control and dosed group

^c Not applicable

TABLE M3
DNA Adducts in the Forestomach of Male B6C3F₁ Mice in the 2-Year Gavage Study of 2,4-Hexadienal^a

Group	Sample Code	Acrolein-dG 3	Crotonaldehyde-dG 1	Crotonaldehyde-dG 2
Vehicle Control	VM431	20.8	17.7	6.4
	VM432	15.9	6.8	2.0
	VM433	29.9	4.5	2.3
	VM434	7.1	13.7	5.4
	VM435	18.2	9.0	2.9
Mean ± standard deviation		18.4 ± 8.3	10.4 ± 5.3	3.8 ± 2.0
120 mg/kg	HM436	1.3	1.2	0.6
	HM437	28.1 _b	4.2	0.0
	HM438	—	—	1.4
	HM439	9.5	2.0	0.0
	HM440	9.3	2.9	1.8
	HM441 ^c	940.1	16.0	10.4
	HM442	13.2	2.1	0.0
	HM443	10.5	0.0	0.0
	HM444	12.0	1.6	0.9
	HM445	17.3	0.0	1.6
Mean ± standard deviation		12.6 ± 7.7	1.7 ± 1.4	0.7 ± 0.7
Positive control		20.5	16.9	2.9
		P=0.2299 ^d	P=0.0010	P=0.0011

^a Data are given as nmoles adduct/mole of deoxyguanosine (dG); samples were collected from mice sacrificed after 90 days of treatment.

^b Fraction lost in HPLC purification procedure

^c Sample excluded from the statistical analysis

^d Comparison (t-test) between the vehicle control and dosed group

National Toxicology Program Technical Reports

Printed as of October 2003

Environmental Health Perspectives (EHP) maintains the library of NTP Technical Reports in electronic and print format. To gain access to these reports, contact EHP online at <http://ehp.niehs.nih.gov> or call 866-541-3841 or 919-653-2590.

Chemical	TR No.	Chemical	TR No.
Acetaminophen	394	Chloroprene	467
Acetonitrile	447	1-Chloro-2-Propanol	477
Acrylonitrile	506	Chlorpheniramine Maleate	317
Agar	230	C.I. Acid Orange 3	335
Allyl Glycidyl Ether	376	C.I. Acid Orange 10	211
Allyl Isothiocyanate	234	C.I. Acid Red 14	220
Allyl Isovalerate	253	C.I. Acid Red 114	405
1-Amino-2,4-Dibromoanthraquinone	383	C.I. Basic Red 9 Monohydrochloride	285
2-Amino-4-Nitrophenol	339	C.I. Direct Blue 15	397
2-Amino-5-Nitrophenol	334	C.I. Direct Blue 218	430
11-Aminoundecanoic Acid	216	C.I. Disperse Blue 1	299
<i>dl</i> -Amphetamine Sulfate	387	C.I. Disperse Yellow 3	222
Ampicillin Trihydrate	318	C.I. Pigment Red 3	407
Asbestos, Amosite (Hamsters)	249	C.I. Pigment Red 23	411
Asbestos, Amosite (Rats)	279	C.I. Solvent Yellow 14	226
Asbestos, Chrysotile (Hamsters)	246	Cobalt Sulfate Heptahydrate	471
Asbestos, Chrysotile (Rats)	295	Coconut Oil Acid Diethanolamine Condensate	479
Asbestos, Crocidolite	280	Codeine	455
Asbestos, Tremolite	277	Comparative Initiation/Promotion Studies (Mouse Skin)	441
L-Ascorbic Acid	247	Corn Oil, Safflower Oil, and Tricaprylin	426
AZT and AZT/ α -Interferon A/D	469	Coumarin	422
Barium Chloride Dihydrate	432	Cytembena	207
Benzaldehyde	378	D&C Red No. 9	225
Benzene	289	D&C Yellow No. 11	463
Benzethonium Chloride	438	Decabromodiphenyl Oxide	309
Benzofuran	370	Diallyl Phthalate (Mice)	242
Benzyl Acetate (Gavage)	250	Diallyl Phthalate (Rats)	284
Benzyl Acetate (Feed)	431	4,4'-Diamino-2,2'-Stilbenedisulfonic Acid, Disodium Salt	412
Benzyl Alcohol	343	2,4-Diaminophenol Dihydrochloride	401
<i>o</i> -Benzyl- <i>p</i> -Chlorophenol (Gavage)	424	1,2-Dibromo-3-Chloropropane	206
<i>o</i> -Benzyl- <i>p</i> -Chlorophenol (Mouse Skin)	444	1,2-Dibromoethane	210
2-Biphenylamine Hydrochloride	233	2,3-Dibromo-1-Propanol	400
2,2-Bis(Bromomethyl)-1,3-Propanediol	452	1,2-Dichlorobenzene (<i>o</i> -Dichlorobenzene)	255
Bis(2-Chloro-1-Methylethyl) Ether	239	1,4-Dichlorobenzene (<i>p</i> -Dichlorobenzene)	319
Bisphenol A	215	<i>p,p'</i> -Dichlorodiphenyl sulfone	501
Boric Acid	324	2,4-Dichlorophenol	353
Bromodichloromethane	321	2,6-Dichloro- <i>p</i> -Phenylenediamine	219
Bromoethane	363	1,2-Dichloropropane	263
1,3-Butadiene	288	1,3-Dichloropropene (Telone II)	269
1,3-Butadiene	434	Dichlorvos	342
<i>t</i> -Butyl Alcohol	436	Dietary Restriction	460
Butyl Benzyl Phthalate	213	Diethanolamine	478
Butyl Benzyl Phthalate	458	Di(2-Ethylhexyl) Adipate	212
<i>n</i> -Butyl Chloride	312	Di(2-Ethylhexyl) Phthalate	217
<i>t</i> -Butylhydroquinone	459	Diethyl Phthalate	429
γ -Butyrolactone	406	Diglycidyl Resorcinol Ether	257
Caprolactam	214	3,4-Dihydrocoumarin	423
<i>d</i> -Carvone	381	1,2-Dihydro-2,2,4-Trimethylquinoline (Monomer)	456
Citral	505	Dimethoxane	354
Chloral Hydrate	502	3,3'-Dimethoxybenzidine Dihydrochloride	372
Chloral Hydrate	503	N,N-Dimethylaniline	360
Chlorinated and Chloraminated Water	392	3,3'-Dimethylbenzidine Dihydrochloride	390
Chlorendic Acid	304	Dimethyl Hydrogen Phosphite	287
Chlorinated Paraffins: C ₂₃ , 43% Chlorine	305	Dimethyl Methylphosphonate	323
Chlorinated Paraffins: C ₁₂ , 60% Chlorine	308	Dimethyl Morpholinophosphoramidate	298
Chlorinated Trisodium Phosphate	294	Dimethylvinyl Chloride	316
2-Chloroacetophenone	379	Diphenhydramine Hydrochloride	355
<i>p</i> -Chloroaniline Hydrochloride	351	5,5-Diphenylhydantoin	404
CS ₂	377	Emodin	493
Chlorobenzene	261	Ephedrine Sulfate	307
Chlorodibromomethane	282	Epinephrine Hydrochloride	380
Chloroethane	346	1,2-Epoxybutane	329
2-Chloroethanol	275	Erythromycin Stearate	338
3-Chloro-2-Methylpropene	300	Ethyl Acrylate	259

Chemical	TR No.	Chemical	TR No.
Ethylbenzene	466	<i>o</i> -Nitroanisole	416
Ethylene Glycol	413	<i>p</i> -Nitrobenzoic Acid	442
Ethylene Glycol Monobutyl Ether	484	Nitrofurantoin	341
Ethylene Oxide	326	Nitrofurazone	337
Ethylene Thiourea	388	Nitromethane	461
Eugenol	223	<i>p</i> -Nitrophenol	417
FD&C Yellow No. 6	208	<i>o</i> -Nitrotoluene	504
Fumonisin B ₁	496	<i>p</i> -Nitrotoluene	498
Furan	402	Ochratoxin A	358
Furfural	382	Oleic Acid Diethanolamine Condensate	481
Furfuryl Alcohol	482	Oxazepam (Mice)	443
Furosemide	356	Oxazepam (Rats)	468
Gallium Arsenide	492	Oxymetholone	485
Geranyl Acetate	252	Oxytetracycline Hydrochloride	315
Glutaraldehyde	490	Ozone and Ozone/NNK	440
Glycidol	374	Penicillin VK	336
Guar Gum	229	Pentachloroanisole	414
Gum Arabic	227	Pentachloroethane	232
HC Blue 1	271	Pentachloronitrobenzene	325
HC Blue 2	293	Pentachlorophenol, Purified	483
HC Red 3	281	Pentachlorophenol, Technical Grade	349
HC Yellow 4	419	Pentaerythritol Tetranitrate	365
Hexachlorocyclopentadiene	437	Phenolphthalein	465
Hexachloroethane	361	Phenylbutazone	367
2,4-Hexadienal	509	Phenylephrine Hydrochloride	322
4-Hexylresorcinol	330	N-Phenyl-2-Naphthylamine	333
Hydrochlorothiazide	357	<i>o</i> -Phenylphenol	301
Hydroquinone	366	Polybrominated Biphenyl Mixture (Firemaster FF-1) (Gavage)	244
8-Hydroxyquinoline	276	Polybrominated Biphenyl Mixture (Firemaster FF-1) (Feed)	398
Indium Phosphide	499	Polysorbate 80 (Glycol)	415
Iodinated Glycerol	340	Polyvinyl Alcohol	474
Isobutene	487	Primidone	476
Isobutyl Nitrite	448	Probenecid	395
Isobutyraldehyde	472	Promethazine Hydrochloride	425
Isophorone	291	Propylene	272
Isoprene	486	1,2-Propylene Oxide	267
Lauric Acid Diethanolamine Condensate	480	Propyl Gallate	240
<i>d</i> -Limonene	347	Pyridine	470
Locust Bean Gum	221	Quercetin	409
60-Hz Magnetic Fields	488	Riddelliine	508
Magnetic Field Promotion	489	Resorcinol	403
Malonaldehyde, Sodium Salt	331	Rhodamine 6G	364
Manganese Sulfate Monohydrate	428	Rotenone	320
D-Mannitol	236	Roxarsone	345
Marine Diesel Fuel and JP-5 Navy Fuel	310	Salicylazosulfapyridine	457
Melamine	245	Scopolamine Hydrobromide Trihydrate	445
2-Mercaptobenzothiazole	332	Sodium Azide	389
Mercuric Chloride	408	Sodium Fluoride	393
Methacrylonitrile	497	Sodium Nitrite	495
8-Methoxypsoralen	359	Sodium Xylenesulfonate	464
α -Methylbenzyl Alcohol	369	Stannous Chloride	231
Methyl Bromide	385	Succinic Anhydride	373
Methyl Carbamate	328	Talc	421
Methyldopa Sesquihydrate	348	Tara Gum	224
Methylene Chloride	306	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -Dioxin (Dermal)	201
4,4'-Methylenedianiline Dihydrochloride	248	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -Dioxin (Gavage)	209
Methyleugenol	491	1,1,1,2-Tetrachloroethane	237
Methyl Methacrylate	314	Tetrachloroethylene	311
N-Methylolacrylamide	352	Tetracycline Hydrochloride	344
Methylphenidate Hydrochloride	439	Tetrafluoroethylene	450
Mirex	313	1-Trans-Delta ⁹ -Tetrahydrocannabinol	446
Molybdenum Trioxide	462	Tetrahydrofuran	475
Monochloroacetic Acid	396	Tetrakis(Hydroxymethyl)Phosphonium Sulfate	296
Monuron	266	Tetrakis(Hydroxymethyl)Phosphonium Chloride	296
Nalidixic Acid	368	Tetranitromethane	386
Naphthalene (Mice)	410	Theophylline	473
Naphthalene (Rats)	500	4,4-Thiobis(6- <i>t</i> -Butyl- <i>m</i> -Cresol)	435
Nickel (II) Oxide	451	Titanocene Dichloride	399
Nickel Sulfate Hexahydrate	454	Toluene	371
Nickel Subsulfide	453	2,4- & 2,6-Toluene Diisocyanate	251
<i>p</i> -Nitroaniline	418	Triamterene	420

Chemical	TR No.	Chemical	TR No.
Tribromomethane	350	Vanadium Pentoxide	507
Trichloroethylene	243	4-Vinylcyclohexene	303
Trichloroethylene	273	4-Vinyl-1-Cyclohexene Diepoxide	362
1,2,3-Trichloropropane	384	Vinylidene Chloride	228
Tricresyl Phosphate	433	Vinyl Toluene	375
Triethanolamine	449	Xylenes (Mixed)	327
Tris(2-Chloroethyl) Phosphate	391	2,6-Xylidine	278
Tris(2-Ethylhexyl) Phosphate	274	Zearalenone	235
Turmeric Oleoresin (Curcumin)	427	Ziram	238