NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDIES OF METHYLENE BLUE TRIHYDRATE

(CAS NO. 7220-79-3)

IN F344/N RATS AND B6C3F$_1$ MICE

(GAVAGE STUDIES)

National Toxicology Program
P.O. Box 12233
Research Triangle Park, NC 27709

May 2008

NTP TR 540

NIH Publication No. 08-4429

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

FOREWORD

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). Three agencies contribute resources to the program: NIEHS/NIH, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention (NIOSH/CDC), and the National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA) Established in 1978, the NTP is charged with coordinating toxicological testing activities, strengthening the science base in toxicology, developing and validating improved testing methods, and providing information about potentially toxic substances to health regulatory and research agencies, scientific and medical communities, and the public.

The Technical Report series began in 1976 with carcinogenesis studies conducted by the National Cancer Institute. In 1981, this bioassay program was transferred to the NTP. The studies described in the Technical Report series are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected substances in laboratory animals (usually two species, rats and mice). Substances selected for NTP toxicity and carcinogenicity studies are chosen primarily on the basis of human exposure, level of production, and chemical structure. The interpretive conclusions presented in NTP Technical Reports are based only on the results of these NTP studies. Extrapolation of these results to other species including characterization of hazards and risks to humans requires analyses beyond the intent of these reports. Selection per se is not an indicator of a substance's carcinogenic potential.

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CONTENTS

SUMMARY

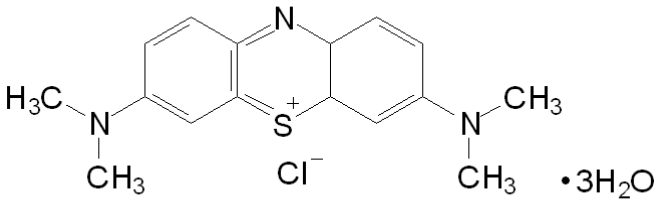
Background
Methylene blue trihydrate has a variety of medical uses, including the treatment of methemoglobinemia and psychiatric disorders and as a disinfectant and biological stain. We studied the effects of methylene blue trihydrate on male and female rats and mice to identify potential toxic or cancer-related hazards.

Methods
We deposited solutions containing methylene blue trihydrate in aqueous methylcellulose directly into the stomachs of male and female rats and mice. Groups of 50 male and female rats received 5, 25, or 50 milligrams of methylene blue trihydrate per kilogram body weight five days per week for two years; groups of 50 male and female mice received 2.5, 12.5, or 25 milligrams of methylene blue per kilogram of body weight for the same duration. Groups of animals receiving methylcellulose alone served as the control groups. At the end of the study, tissues from more than 40 sites were examined for every animal.

Results
The two highest dose groups of male and female rats weighed less than the control animals, while the two highest dose groups of female mice weighed more than their corresponding control group. In male and female rats and mice, the blood of the animals was affected, with animals receiving the highest doses experiencing methemoglobinemia and anemia. This caused secondary injury to the spleen in these animals. Cancer of the pancreatic islets was increased in male rats receiving methylene blue trihydrate, and some uncommon tumors of the small intestine were seen in exposed male mice. There was a slight increase in malignant lymphomas in exposed male and female mice.

Conclusions
We conclude that exposure to methylene blue caused pancreatic islet tumors in male rats and small intestine tumors in male mice. Malignant lymphomas in male and female mice were possibly associated with methylene blue trihydrate exposure. Methylene blue trihydrate caused blood abnormalities and anemia in male and female rats and mice.

ABSTRACT



METHYLENE BLUE TRIHYDRATE

CAS No. 7220-79-3

Chemical Formula: $C_{16}H_{24}ClN_3O_3S$ Molecular Weight: 373.9

Synonyms: Aizen methylene blue; basic blue 9 (8CI); C.I. 52015; methylthionine chloride; methylthioninium chloride; phenothiazine-5-ium, 3,7-bis, (dimethylamino)-, chloride; swiss blue; tetramethylthionine chloride
IUPAC Name: (7-dimethylaminophenothiazin-3-ylidene)-dimethyl-ammonium chloride trihydrate
IUPAC International Chemical Identifier: InChI=1/C16H18N3S.ClH.3H2O/c1-18(2)11-5-7-13-15(9-11)20-16-10-12(19(3)4)6-8-14(16)17-13;;;;/h5-10H,1-4H3;1H;3*1H2/q+1;;;;/p-1/fC16H18N3S.Cl.3H2O/h;1h;;;/qm;-1;;;
Canonical SMILES: CN(C)C1=CC2=C(C=C1)N=C3C=CC(=[N+](C)C)C=C3S2.O.O.O.[Cl-]
Trade Names: Desmoid piller, Desmoidpillen, Methylene Blue, Panatone, Urolene Blue, Vitableu

Methylene blue trihydrate has a variety of biomedical and biologically therapeutic applications. Methylene blue trihydrate was nominated by the National Cancer Institute (NCI) for carcinogenicity testing based on the numerous uses of this compound and the lack of long-term toxicity data, including epidemiological studies of methylene blue trihydrate, as well as the inadequate animal data on this compound. Male and female F344/N rats and B6C3F$_1$ mice were administered methylene blue trihydrate in 0.5% aqueous methylcellulose by gavage for 1 month, 3 months, or 2 years. Genetic toxicology studies were conducted using *Salmonella typhimurium, Escherichia coli,* cultured Chinese hamster ovary cells, mouse bone marrow cells, and mouse peripheral blood erythrocytes.

1-Month Study in Rats

Groups of 10 male and 10 female core study rats and groups of 10 male and 10 female clinical pathology study rats were administered methylene blue trihydrate in 0.5% aqueous methylcellulose solution by gavage at doses of 0, 125, 250, 500, 1,000, or 2,000 mg/kg, 5 days per week for 5 weeks. In the 500 mg/kg groups, one male died the first week of the study and one male and four females died the second week of the study. All rats in the 1,000 mg/kg group died by study day 10, and all rats in the 2,000 mg/kg group died by study day 6. Final mean body weights of male and female rats in the 250 and 500 mg/kg groups were significantly less than those of the vehicle controls. Dosed rats developed methemoglobinemia and a regenerative Heinz body anemia.

Significant increases in spleen weights occurred in all surviving dosed groups. There were also significant decreases in the thymus weights of 250 and 500 mg/kg males and 125 and 250 mg/kg females. Spleen lesions associated with methylene blue trihydrate administration included hematopoietic cell proliferation, pigmentation,

lymphoid depletion of the lymphoid follicles, and capsular fibrosis. Hyperplasia of the bone marrow occurred in all dosed groups of rats. Liver lesions associated with methylene blue exposure included centrilobular necrosis in rats dying early, hematopoietic cell proliferation, and Kupffer cell pigmentation with erythrophagocytosis.

1-Month Study in Mice

Groups of 10 male and 10 female core study mice were administered methylene blue trihydrate in 0.5% aqueous methylcellulose solution by gavage at doses of 0, 125, 250, 500, 1,000, or 2,000 mg/kg, 5 days per week for 5 weeks. None of the mice in the 500, 1,000, and 2,000 mg/kg groups survived to the end of the study. In the 250 mg/kg groups, two females died on days 16 and 18 and two males died on days 6 and 13. Mean body weights of surviving dosed mice were similar to those of the vehicle controls. Thinness, abnormal respiration, hypothermia, lethargy, ataxia, and ruffled fur were observed in a few surviving animals in the 250 mg/kg groups. Hypothermia and abnormal posture were observed in mice in the 500, 1,000, and 2,000 mg/kg groups. Dosed mice developed methemoglobinemia and a regenerative Heinz body anemia. Significant increases in spleen weights occurred in all surviving dosed groups of mice compared to vehicle controls. Significant decreases occurred in the thymus weights of 250 mg/kg males and females. The heart weights of 125 and 250 mg/kg females were significantly increased. Lesions in the spleen associated with methylene blue trihydrate administration included hematopoietic cell proliferation, pigmentation, and congestion. Liver lesions associated with methylene blue trihydrate administration included periportal degeneration, hematopoietic cell proliferation, and Kupffer cell pigmentation with eryth-rophagocytosis. The incidences of bone marrow pigmentation were significantly increased in all dosed groups of mice. Forestomach lesions that were related to methylene blue trihydrate administration included focal ulcer, inflammation, and squamous hyperplasia.

3-Month Study in Rats

Groups of 10 male and 10 female core study rats and groups of 20 male and 20 female clinical pathology study rats were administered methylene blue trihydrate in 0.5% aqueous methylcellulose solution by gavage at doses of 0, 25, 50, 100 or 200 mg/kg, 5 days per week for 14 weeks. Mean body weights of males in the 200 mg/kg group were significantly less than those of the vehicle controls. Dosed rats developed methemoglobinemia and a regenerative Heinz body anemia. Significant increases in spleen weights occurred in males and females administered 50 mg/kg or greater. Thymus and lung weights of 50, 100, and 200 mg/kg males (except relative lung weight at 100 mg/kg) were significantly less than those of the vehicle controls.

Spleen lesions in dosed rats included hematopoietic cell proliferation, congestion, lymphoid depletion of the lymphoid follicles, and capsular fibrosis. The incidences of bone marrow hyperplasia were significantly increased in groups administered 50 mg/kg or greater. There were no consistent effects of methylene blue trihydrate administration on reproductive system measures in male or female rats.

3-Month Study in Mice

Groups of 10 male and 10 female core study mice and groups of 20 male and 20 female clinical pathology study mice were administered methylene blue trihydrate in 0.5% aqueous methylcellulose solution by gavage at doses of 0, 25, 50, 100, or 200 mg/kg, 5 days per week for 14 weeks. Mean body weights of all dosed groups were similar to or only slightly less than those of the vehicle control groups. Dosed mice developed methemoglobinemia and a regenerative Heinz body anemia. Spleen weights of 100 and 200 mg/kg males and 50 mg/kg or greater females were significantly greater than those of the vehicle control groups. Heart weights were significantly increased in 200 mg/kg males. In females, there were significant decreases in thymus weights at 50 mg/kg or greater. Males had decreased sperm motility and increased epididymal sperm counts at 200 mg/kg.

In all dosed groups, the incidences of hematopoietic cell proliferation and pigmentation in the spleen were significantly greater than those in the vehicle controls. In the liver, the incidences of hematopoietic cell proliferation were significantly increased in males and females in the 100 and 200 mg/kg groups, and the incidences of Kupffer cell pigmentation were significantly increased in groups administered 50 mg/kg or greater. The incidences of bone marrow pigmentation were significantly increased in all dosed groups of mice except 25 mg/kg females.

2-Year Study in Rats

Groups of 50 male and 50 female rats were administered methylene blue trihydrate in 0.5% aqueous methylcellulose solution by gavage at doses of 0, 5, 25, or 50 mg/kg, 5 days per week for 2 years. Additional groups of 10 male and 10 female rats were administered the same doses for up to 18 months and were evaluated at 2 weeks and 3, 12, and 18 months for hematology. Survival of all dosed groups of rats was similar to that of the vehicle controls. Mean body weights of 25 and 50 mg/kg male rats were less than those of the vehicle controls after weeks 29 and 21, respectively. In the 25 and 50 mg/kg females, mean body weights were less after weeks 73 and 53. Dosed male and female rats developed methemoglobinemia, and females developed a regenerative Heinz body anemia.

The incidences of pancreatic islet cell adenoma and adenoma or carcinoma (combined) were increased in all dosed groups of males, were significantly increased in 25 mg/kg males, and exceeded the historical range in controls (all routes). The incidence of pancreatic islet cell hyperplasia was significantly increased in the 50 mg/kg males.

In the spleen, the incidence of hematopoietic cell proliferation in 50 mg/kg males was significantly increased; the incidences of capsular fibrosis were significantly increased in all dosed groups of males and in 5 and 50 mg/kg females.

2-Year Study in Mice

Groups of 50 male and 50 female mice were administered methylene blue trihydrate in a 0.5% aqueous methylcellulose solution by gavage at doses of 0, 2.5, 12.5, or 25 mg/kg, 5 days per week for 2 years. Additional groups of 30 male and 30 female mice were administered the same doses for up to 18 months and were evaluated at 2 weeks and 3, 12, or 18 months for hematology. Survival of dosed male and female groups exceeded that of the vehicle controls in a generally dose-related manner. Mean body weights of dosed female mice began to increase after weeks 29, 61, and 85, reaching final values that were 113%, 111%, and 106% of vehicle controls for the 2.5, 12.5, and 25 mg/kg groups, respectively. Dosed mice developed methemoglobinemia and a regenerative Heinz body anemia.

The incidences of carcinoma and of adenoma or carcinoma (combined) of the small intestine occurred with a positive trend in males. The incidences of malignant lymphoma occurred with a positive trend in females, and the incidence in 25 mg/kg males exceeded the historical control range.

The incidences of hematopoietic cell proliferation of the spleen were significantly increased in 12.5 and 25 mg/kg males and in 25 mg/kg females. The incidences of inflammation of the nose were significantly increased in 12.5 and 25 mg/kg females.

Genetic Toxicology

Methylene blue trihydrate was mutagenic in *Salmonella typhimurium* strains TA98 and TA100 with and without rat or hamster liver S9 activation enzymes; mutagenicity was also observed in *Escherichia coli* strain WP2 *urvA*/pKM101 with and without rat liver S9. In cytogenetic tests with cultured Chinese hamster ovary cells, methylene blue trihydrate induced sister chromatid exchanges and chromosomal aberrations with and without S9. However, in contrast to the positive results in the *in vitro* tests, no increase in the frequency of micronucleated erythrocytes was observed in bone marrow or blood samples collected from male mice and analyzed 48 hours after a single intraperitoneal injection of methylene blue trihydrate or in peripheral blood of male and female mice administered methylene blue trihydrate by gavage for 3 months. In the 3-month micronucleus tests, a dose-related increase in the percentage of reticulocytes among the total erythrocyte population was observed in both male and female mice.

Adjunct studies were conducted with three metabolites of methylene blue trihydrate: Azure A, Azure B, and Azure C. All three compounds were tested in the Ames assay, and all were positive, with and without rat liver S9 activation enzymes, in *Salmonella typhimurium* strains TA98 and TA100 and *Escherichia coli* strain WP2 *uvrA*/pKM101.

CONCLUSIONS

Under the conditions of these 2-year gavage studies, there was *some evidence of carcinogenic activity** of methylene blue trihydrate in male F344/N rats based on increased incidences of pancreatic islet cell adenoma and adenoma or carcinoma (combined). There was *no evidence of carcinogenic activity* in female F344/N rats administered 5, 25, or 50 mg/kg. There was *some evidence of carcinogenic activity* in male $B6C3F_1$ mice based on increased incidences of carcinoma and of adenoma or carcinoma (combined) in the small intestine. The increased incidence of malignant lymphoma in males receiving 25 mg/kg may have been related to the administration of methylene blue trihydrate. There was *equivocal evidence of carcinogenic activity* in female $B6C3F_1$ mice based on marginally increased incidences of malignant lymphoma.

Methylene blue trihydrate administration caused methemoglobinemia and a regenerative Heinz body anemia with secondary injury to other organs in rats and mice.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 12. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 14.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Methylene Blue Trihydrate

	Male F344/N Rats	Female F344/N Rats	Male $B6C3F_1$ Mice	Female $B6C3F_1$ Mice
Doses in 0.5% aqueous methylcellulose solution administered by gavage	0, 5, 25, or 50 mg/kg	0, 5, 25, or 50 mg/kg	0, 2.5, 12.5, or 25 mg/kg	0, 2.5, 12.5, or 25 mg/kg
Body weights	25 and 50 mg/kg groups less than the vehicle control group	25 and 50 mg/kg groups less than the vehicle control group	Dosed groups similar to the vehicle control group	12.5 and 25 mg/kg groups greater than the vehicle control group
Survival rates	31/50, 33/50, 39/50, 31/50	35/50, 32/50, 36/50, 35/50	35/50, 38/50, 38/50, 41/50	33/50, 40/50, 42/50, 43/50
Hematologic effects	Blood: Methemoglobinemia	Blood: Methemoglobinemia and Heinz body formation	Blood: Methemoglobinemia and Heinz body formation	Blood: Methemoglobinemia and Heinz body formation
Nonneoplastic effects	Pancreatic islets: hyperplasia (13/50, 13/50, 17/50, 26/50) Spleen: hematopoietic cell proliferation (11/50, 12/50, 17/50, 20/50); capsule fibrosis (1/50, 7/50, 12/50, 30/50)	Spleen: capsule fibrosis (8/49, 17/48, 12/49, 20/49)	Spleen: hematopoietic cell proliferation (14/49, 16/50, 25/49, 29/48)	Spleen: hematopoietic cell proliferation (23/47, 21/47, 31/49, 40/50)
Neoplastic effects	Pancreatic islets: adenoma (4/50, 9/50, 12/50, 8/50); adenoma or carcinoma (4/50, 9/50, 14/50, 8/50)	None	Small intestine: carcinoma (0/50, 1/50, 2/50, 4/50); adenoma or carcinoma (1/50, 2/50, 4/50, 6/50)	None
Equivocal findings	None	None	Malignant lymphoma: (2/50, 2/50, 2/50, 5/50)	Malignant lymphoma: (6/50, 4/50, 9/50, 12/50)
Level of evidence of carcinogenic activity	Some evidence	No evidence	Some evidence	Equivocal evidence
Genetic toxicology				
Salmonella typhimurium gene mutations:				
Methylene blue trihydrate	Positive in strains TA98 and TA100 with and without S9			
Methylene blue trihydrate (lot 68H3728)	Positive in strains TA98 and TA100 with and without S9 and in *Escherichia coli* WPM *uvrA*/pKM101 with and without S9			
Azure A, Azure B, and Azure C	Positive in strains TA98 and TA100 with and without S9 and in *Escherichia coli* WPM *uvrA*/pKM101 with and without S9			
Sister chromatid exchanges				
Cultured Chinese hamster ovary cells *in vitro:*	Positive with and without S9			
Chromosomal aberrations				
Cultured Chinese hamster ovary cells *in vitro:*	Positive with and without S9			
Micronucleated erythrocytes				
Mouse bone marrow *in vivo:*	Negative			
Mouse peripheral blood *in vivo:*	Negative in single dose and 3-month studies			

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 methylene blue trihydrate on June 12, 2006, 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.

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West Point, PA

Vernon Walker, D.V.M., Ph.D.
Lovelace Respiratory Institute
Albuquerque, NM

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* Did not attend

SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On June 12, 2006, the draft Technical Report on the toxicology and carcinogenesis studies of methylene blue trihydrate 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. D.W. Bristol, NIEHS, introduced the toxicology and carcinogenesis studies of methylene blue trihydrate by describing the uses and hematotoxicity of the chemical, the experimental design, and the results of the 2-year gavage studies. The proposed conclusions were *some evidence of carcinogenic activity* of methylene blue trihydrate in male F344/N rats, *no evidence of carcinogenic activity* in female F344/N rats, *some evidence of carcinogenic activity* in male B6C3F_1 mice, and *equivocal evidence of carcinogenic activity* in female B6C3F_1 mice.

Dr. Sikka, the first principal reviewer, agreed with the conclusions. He suggested making mention of the possible mutagenicity of the metabolites of methylene blue.

Dr. Crump, the second principal reviewer, felt that while the malignant lymphoma response in male mice was weak, it was supported by the increased incidences of malignant lymphoma in females. He also felt the increase in the incidences of pancreatic islet neoplasms in male rats was fairly weak, while the increased incidences of lung neoplasms, which were not mentioned in the conclusions, was comparable and might be considered equivocal. He noted that a statistical test for comparison with historical controls was being published and asked for future demonstration of examples of comparisons using that test.

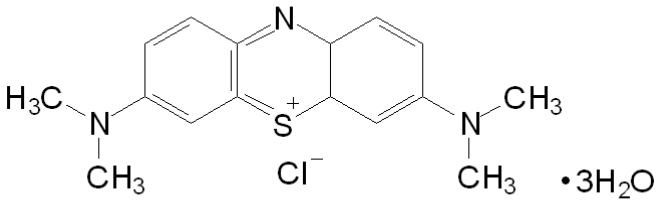
Dr. Bristol replied that a discussion of the mutagenicity of methylene blue trihydrate and its metabolites would be added to the report. Dr. G.E. Kissling, NIEHS, indicated that upon publication of the historical statistical analysis paper the test would be presented to the panel for consideration of inclusion in future reports. Dr. D.E. Malarkey, NIEHS, reported the historical average for male rat lung neoplasms was higher than the control rate seen in the present study. Dr. Birt asked if adding mention of the lung tumors would strengthen the some evidence call. Dr. J.R. Bucher, NIEHS, replied that if the response were considered equivocal it would be phrased as "may have been related" to chemical administration.

Dr. Daston mentioned that a number of negative trends in neoplasm incidences also occurred. Dr. Bristol replied that methylene blue trihydrate has some paradoxical properties, for example as a cause and a treatment for methemoglobinemia at different doses.

Dr. Sikka asked if the conclusion could carry any mention of the influence of possible mutagenicity. Dr. McQueen noted that the conclusion statement referred to interpretation of the rodent studies.

Dr. Soper moved, and Dr. Walker seconded, that the conclusions be accepted as written. The motion was passed unanimously with ten votes.

INTRODUCTION



METHYLENE BLUE TRIHYDRATE

CAS No. 7220-79-3

Chemical Formula: $C_{16}H_{24}ClN_3O_3S$ Molecular Weight: 373.9

Synonyms: Aizen methylene blue; basic blue 9 (8CI); C.I. 52015; methylthionine chloride; methylthioninium chloride; phenothiazine-5-ium, 3,7-bis, (dimethylamino)-, chloride; swiss blue; tetramethylthionine chloride
IUPAC Name: (7-dimethylaminophenothiazin-3-ylidene)-dimethyl-ammonium chloride trihydrate
IUPAC International Chemical Identifier: InChI=1/C16H18N3S.ClH.3H2O/c1-18(2)11-5-7-13-15(9-11)20-16-10-12(19(3)4)6-8-14(16)17-13;;;;/h5-10H,1-4H3;1H;3*1H2/q+1;;;;/p-1/fC16H18N3S.Cl.3H2O/h;1h;;;/qm;-1;;;
Canonical SMILES: CN(C)C1=CC2=C(C=C1)N=C3C=CC(=[N+](C)C)C=C3S2.O.O.O.[Cl-]
Trade Names: Desmoid piller, Desmoidpillen, Methylene Blue, Panatone, Urolene Blue, Vitableu

CHEMICAL AND PHYSICAL PROPERTIES

Methylene blue trihydrate occurs as odorless dark green crystals or crystalline powder with a bronze luster (*Merck*, 2001). It melts at 190° C, with decomposition, (MSDS, 1997) and is soluble in water and chloroform, but only sparingly so in alcohol (*Merck*, 2001). The structure is somewhat unusual in that the sulfur atom has an oxidation state of +4 but no sulfur-oxygen bonds.

PRODUCTION, USE, AND HUMAN EXPOSURE

Methylene blue was discovered in 1866, the first member of the phenothiazine family of dyes and redox indicators. It is one of a group of thiazinium halides, or phenothiazin-5-ium chloride compounds, that have a wide variety of uses, including biomedical applications and biological activity (Moura and Cordeiro, 2003). Most recently it has been used as an optical probe of biophysical systems, as an intercalator in nanoporous materials, as a redox mediator, and in photoelectrochromic imaging. It is synthesized commercially by oxidation of N,N-dimethyl-phenylenediamine with $Na_2Cr_2O_7$ in the presence of $Na_2S_2O_3$, followed by further oxidation in the presence of N,N-dimethylaniline. This and newer methods for the synthesis of phenothiazines are presented by Leventis *et al*. (1997).

Methylene blue is used in human and veterinary medicine for a number of therapeutic and diagnostic procedures including use as a stain in bacteriology, as a redox coloring agent, as a targeting agent for melanoma, as an antihemoglobinemic, and as an antiseptic and disinfectant (*Merck*, 2001). One of the most common clinical applications is for treating methemoglobinemia induced by overexposure to drugs, to industrial chemicals such as nitrophenols (ATSDR, 1992), or to environmental

poisons such as excessive nitrate in well water or cyanide compounds (Sills and Zinkkam, 1994; Christiansen *et al*., 1996). The recommended intravenous dosage for such treatment is 1 to 2 mg/kg body weight (Harvey, 1980). Methylene blue is used in the treatment of some psychiatric disorders because of the anxiolytic and antidepressant properties attributed to its ability to block activation of guanyl cyclase by nitric oxide (Naylor *et al*., 1986; Eroglu and Caglayan, 1997). In addition, it is being investigated as an adjuvant therapy in treatment of schizophrenia (Deutsch *et al*., 1997) and as a chemotherapeutic agent for use by direct intratumoral injection in combination with photodynamic therapy (Orth *et al*., 1998). Recently, methylene blue was recommended for use in biopsies performed to identify lymphoma, indicating that new biomedical uses continue to be found for this unusual chemical.

A National Occupational Exposure Survey indicates that between 1981 and 1983 an estimated 69,563 workers were potentially exposed to methylene blue in the workplace (NIOSH, 1990), but no information was reported on possible environmental exposure levels. No permissible exposure limits have been established by the Occupational Safety and Health Administration, the National Institute for Occupational Safety and Health, or the American Conference of Governmental Industrial Hygienists.

METABOLISM

Methylene blue, administered to rabbits by infusion, was rapidly and reversibly reduced to N,N,N′,N′-tetramethyl-10H-phenothiazine-3,7-diamine, better known as leucomethylene blue (Herter, 1904) (Figure 1). When biological samples are exposed to air, the leuco form is stable in urine, but in blood and other tissues it is rapidly oxidized to methylene blue (DiSanto and Wagner, 1972). In alkali solutions, methylene blue undergoes sequential N-demethylation to form trimethylthionine (Azure B) (Lillie, 1943; Singhal and Rabinowitch, 1967) (Figure 2). A brominated quinine imine (Figure 3) has been tentatively identified in urine from humans dosed with methylene blue (Plater, 2003). The author explains its occurrence by invoking reaction of endogenous bromide with an S-oxide of methylene blue and loss of the dimethylamino group. Metabolites that might be expected from more common metabolic pathways, such as mercapturic acids from reaction of the S-oxide with glutathione, have not been reported.

Methylene blue was administered intravenously to the penis vein of adult Sprague-Dawley rats at doses of 2, 5, 7.5, 10, 15, and 25 mg/kg (DiSanto and Wagner, 1972). Three minutes after injection, rats were sacrificed, blood was collected, and the heart, lungs, liver, and kidneys were examined for tissue uptake of methylene blue. The amount of methylene blue in the blood was negligible in comparison to the amount detected in the tissues studied. The uptake of methylene blue appeared to be rapid, and the selected tissues accounted for an average of 29.8% of the total administered dose.

In order to determine the human pharmacokinetics of methylene blue, seven adult male volunteers were administered 10 mg of methylene blue orally in the form of a gelatin capsule (DiSanto and Wagner, 1972). The subjects were fasted overnight preceding dosing and for 4 hours postdosing. An average of 74% (range 53% to 97%) of the dose was recovered in the urine, 78% of which was in the leuco form. Some methylene blue was also present. Analysis of plasma fractions of whole blood that were spiked with methylene blue accounted for 40% to 50% of the spiked concentration, indicating that methylene blue rapidly binds to red blood cells.

TOXICITY

Experimental Animals

The oral LD_{50} of methylene blue has been estimated as 1,180 mg/kg in rats and 3,500 mg/kg in mice (Lewis, 1992); the intraperitoneal LD_{50} as 150 mg/kg in mice and 180 mg/kg in rats; and the intravenous LD_{50} as 77 mg/kg in mice, 1,250 mg/kg in rats, and 42.3 mg/kg in sheep (Burrows, 1984; Lewis, 1992). Acute toxic effects that have been described in animals exposed to methylene blue include hemoconcentration, hypothermia, acidosis, hypercapnia, hypoxia, increases in blood pressure, changes in respiratory frequency and amplitude, corneal injury, conjunctival damage, and Heinz body formation (Christiansen, 1980; NTP, 1990).

Humans

In humans, large doses of methylene blue (approximately 500 mg) administered intravenously have been reported to cause nausea, abdominal and chest pain, cyanosis, methemoglobinemia, sweating, dizziness, headache, and confusion (Harvey, 1980). Numerous reports have demonstrated toxicity in infants exposed to methylene blue trihydrate during prenatal or perinatal diagnostic or therapeutic procedures, including hyperbilirubinemia,

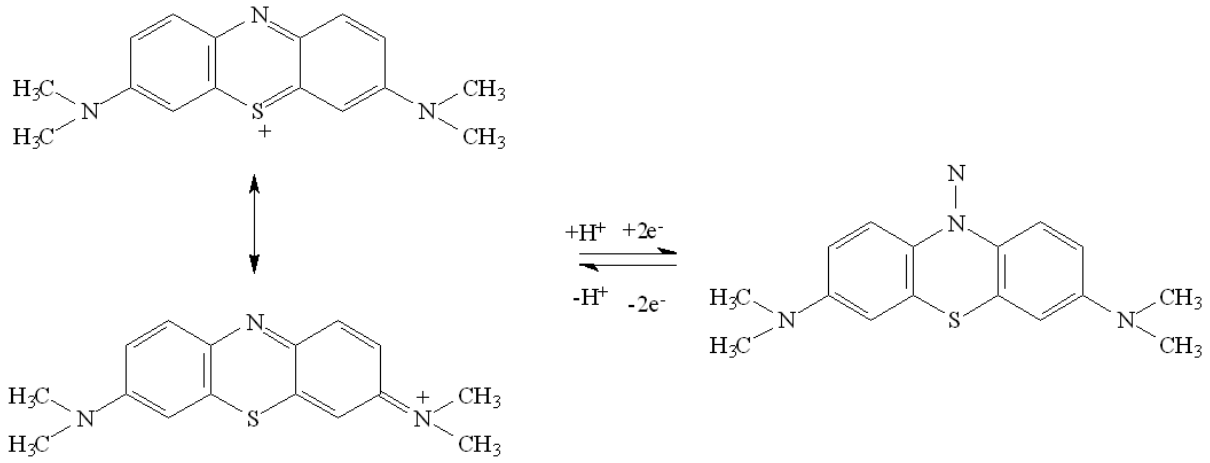


FIGURE 1
Reduction of Methylene Blue to N,N,N′,N′-tetramethyl-10H-phenothiazine-3,7-diamine (Leucomethylene Blue)

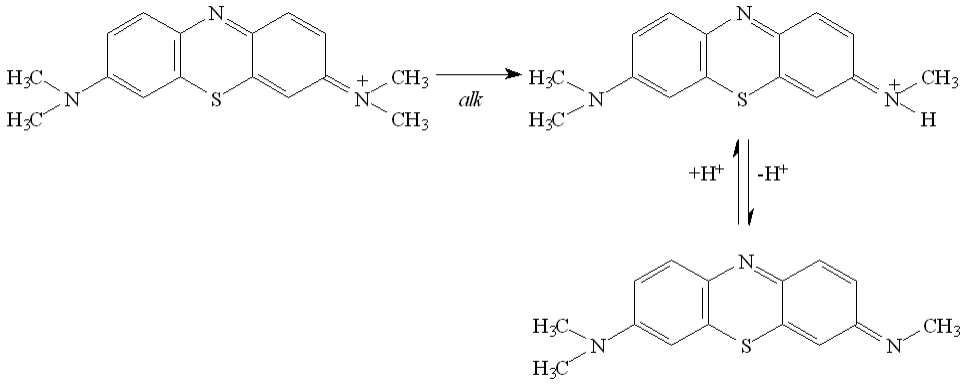


FIGURE 2
N-demethylation of Methylene Blue to Trimethylthionine (Azure B)

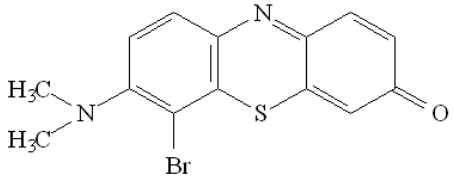


FIGURE 3
Structure of a Brominated Quinine Imine

anemia, Heinz bodies, erythrocytic blister cells, skin discoloration, and photosensitization (Sills and Zinkham, 1994; Porat *et al.*, 1996).

REPRODUCTIVE TOXICITY

Experimental Animals

Methylene blue has been shown to be a reproductive toxicant in animals and humans. Telford *et al*. (1962) reported an increase in resorptions in female rats exposed to methylene blue in the diet during gestation. Maternal toxicity was also reported in this study, but the investigators did not report evidence of teratogenicity in the offspring.

Humans

Coddington *et al.* (1989) and Sheynkin *et al*. (1999) demonstrated that the compound markedly reduced the motility of human sperm *in vitro*.

CARCINOGENICITY

Experimental Animals

In one 2-year study conducted in Wistar rats, five animals per sex were exposed to methylene blue in the diet at a concentration of 4% (Willheim and Ivy, 1953). Although no evidence of tumor induction was observed in this study, the number of animals used was considered to be too small to obtain a valid statistical estimate of the tumor-causing capability of methylene blue.

Humans

No data on the carcinogenicity of methylene blue trihydrate in humans were found in the literature.

GENETIC TOXICOLOGY

Methylene blue trihydrate was shown to be mutagenic when testing was conducted in the absence of light (i.e., without photoactivation) in a variety of *Salmonella typhimurium* tester strains, inducing both base-substitution and frameshift mutations (Chung *et al*., 1981; Yamaguchi, 1981; Lunn and Sansane, 1991); mutagenic activity or induction of DNA damage was also reported in a number of strains of *Escherichia coli* (McCarroll *et al*., 1981; Mohn *et al*., 1984; Webb and Hass, 1984). Methylene blue trihydrate, activated with light, did not induce gene conversion in the yeast *Saccharomyces cerevisiae* (Ito and Kobayashi, 1977), and no induction of gene mutations was seen in *S. cerevisiae* treated with a single concentration of 20 µg/mL methylene blue in the absence of photodynamic activation (Tuite *et al*., 1981). Methylene blue trihydrate did not induce sex-linked recessive lethal mutations in male *Drosophila melanogaster* treated either by injection (Sobels, 1954) or feeding (Clark, 1953). However, in a modified *D. melanogaster* somatic mutation and recombination test that employed white light for activation of photosensitizers, photoactivated methylene blue trihydrate induced high levels of homologous mitotic recombination (Smijs *et al*., 2004). Positive results were reported in a number of *in vitro* mutagenicity or DNA damage-inducing test systems using photodynamically activated methylene blue, presumably the result of singlet oxygen production (Brendel, 1973; Gutter *et al*., 1977; Epe *et al*., 1988, 1989, 1993; McBride *et al*., 1992).

Methylene blue trihydrate was shown to intercalate into calf thymus DNA (Lee *et al*., 1973) and to bind to calf thymus DNA in an orientation perpendicular to the helix axis, coplanar with the bases, at low methylene blue/DNA binding ratios and low ionic strengths (Norden and Tjerneld, 1982). Villanueva *et al*. (1993) reported that methylene blue induced light dose-dependent increases in DNA-protein crosslinks (calf thymus DNA, calf thymus histone Type II), and they attributed this activity to production of singlet oxygen.

Published results from tests measuring induction of sister chromatid exchanges in cultured hamster lung V79 cells (Popescu *et al.*, 1977; Speit and Vogel, 1979) or Syrian hamster BHK-1 cells (MacRae *et al*., 1980) treated with methylene blue trihydrate were negative. Negative results were also reported in tests for induction of chromosomal aberrations in Chinese hamster cells (Popescu *et al*., 1977). Negative results were reported in a second test for induction of chromosomal aberrations, but protocol deficiencies including a brief exposure time, small number of cells scored, and use of a single concentration of methylene blue trihydrate renders these results inconclusive (Au and Hsu, 1979).

Despite extensive *in vitro* studies of the mutagenic and DNA damaging ability of methylene blue trihydrate, only one published *in vivo* study was identified, and that report showed no significant increases in sister chromatid exchanges in bone marrow cells of adult Chinese hamsters that were administered a single dose of

12 mg/kg methylene blue trihydrate by intraperitoneal injection (Speit, 1982).

Information on the mutagenicity of five methylene blue metabolites is available and, although limited, data are consistent with methylene blue trihydrate genotoxicity data. Toluidine blue was mutagenic in a variety of *S. typhimurium* tester strains, with and without S9 (Dunipace *et al*., 1992), but did not induce mutations in the yeast *S. cerevisiae*, even with photodynamic activation (Ito and Kobayashi, 1977). Exposure to toluidine blue plus visible light produced DNA-protein crosslinks and singlet oxygen production in isolated calf thymus DNA (Villanueva *et al*., 1993). The metabolite thionine was negative in the *D. melanogaster* sex-linked recessive lethal assay (Clark, 1953). Four metabolites (toluidine blue and Azures A, B, and C) induced chromosomal damage in cultured Chinese hamster ovary cells in the absence of exogenous metabolic activation (Au and Hsu, 1979).

Study Rationale

Methylene blue trihydrate was selected for toxicity and carcinogenicity studies because of the potential for exposure of humans and animals to high doses of the drug in the treatment of various conditions in human and veterinary medicine. The National Cancer Institute, the nominating agency, noted a lack of studies of the chronic toxicity of methylene blue in experimental animals and also a lack of epidemiology studies evaluating the safety of the drug for human use. Therefore, the National Toxicology Program (NTP) performed a series of toxicity and carcinogenesis studies in rodents with special emphasis on hematologic effects.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION

Methylene Blue Trihydrate

Methylene blue trihydrate was obtained from Aldrich Chemical Company (Milwaukee, WI) in two lots (PY01917JX and 10306AF) and Sigma Chemical Company (St. Louis, MO) in one lot (68H3728) (Appendix I). Lots PY01917JX, 10306AF, and 68H3728 were used in the 1-month, 3-month, and 2-year studies, respectively. Identity and purity analyses were conducted by the study laboratories, Battelle Columbus Operations (Columbus, OH; lots PY01917JX and 10306AF) and Southern Research Institute (Birmingham, AL; lot 68H3728), and by the analytical chemistry laboratory, Research Triangle Institute (Research Triangle Park, NC; lot 68H3728). Galbraith Laboratories, Inc. (Knoxville, TN), conducted melting point determination, elemental analyses, and Karl Fischer titration (lot 68H3728). Reports on analyses performed in support of the studies on methylene blue trihydrate are on file at the National Institute of Environmental Health Sciences.

Lots PY01917JX and 10306AF of methylene blue trihydrate, a green crystalline powder, were identified by the study laboratory using infrared (IR) spectroscopy. Lot 68H3728 was identified by the analytical chemistry laboratory and the study laboratory using IR and proton nuclear magnetic resonance spectroscopy (NMR). IR spectra were consistent with the literature spectra (*Aldrich*, 1981a,b) of methylene blue trihydrate, and IR and NMR spectra obtained from lot 68H3728 were consistent with spectra of a reference sample (different lot of methylene blue trihydrate) obtained from Aldrich Chemical Co. and a reference sample from the same lot.

The purities of lots PY01917JX and 10306AF were accepted as determined by the manufacturer using titration, melting point determination, elemental analyses, and ultraviolet/visible (UV/Vis) chromatography. The purity of lot 10306AF was determined by the study laboratory using high-performance liquid chromatography (HPLC). The purity of lot 68H3728 was determined by the study laboratory using HPLC and by the analytical chemistry laboratory using elemental analysis, Karl Fischer titration, melting point determination, UV/Vis chromatography, and HPLC. The analytical chemistry laboratory performed additional analyses using HPLC/mass spectrometry (MS) to identify the major impurity detected by HPLC.

For lot PY01917JX, elemental analyses showed good agreement between theoretical and observed percentages by weight for carbon, hydrogen, and nitrogen; water content was 16.1%; and the melting point was 192° C. The UV/Vis spectrum was consistent with the structure of methylene blue trihydrate. The overall purity of lot PY01917JX was determined to be greater than 96%.

For lot 10306AF, elemental analyses showed good agreement between theoretical and observed percentages by weight for carbon, hydrogen, and nitrogen; water content was 16.1%; and the melting point was 192° C. The UV/Vis spectrum was consistent with the structure of methylene blue trihydrate. HPLC, at 290 nm, indicated one major peak and three impurities with relative peak areas of 0.16%, 0.12%, and 2.8%; at 665 nm, there was one major peak and one impurity with a relative area of 3.2%.

For lot 68H3728, elemental analyses showed good agreement between theoretical and observed percentages by weight for carbon, hydrogen, nitrogen, sulfur, and chlorine; water content was 16.55%; and the melting point was between 185° and 186° C, consistent for the chemical with water content of 16.55%. UV/Vis spectra were consistent with the structure of methylene blue trihydrate. HPLC indicated one major peak and three impurities with relative peak areas of 0.16%, 0.21%, and 6.55%. A second HPLC analysis, designed to detect more impurities, indicated similar results. Additional analysis using HPLC/MS indicated that this impurity was very similar to methylene blue trihydrate with the exception of one methyl group replaced by a proton. HPLC indicated 102% relative purity compared to a reference standard from the same lot and greater than 94% purity using calculated peak areas. The overall

purity of lot 68H3728 was determined to be greater than 91%. Stability studies conducted by the analytical chemistry laboratory demonstrated that the bulk chemical could be stored at room temperature (25° C).

For lots PY01917JX and 10306AF, the bulk chemical was reanalyzed at the end of each study by the study laboratory using HPLC. For lot 68H3728, periodic reanalyses were conducted at least every 26 weeks and at the end of the study by the study laboratory using HPLC. No degradation of the bulk chemical was observed.

Methylcellulose

For the 2-year studies, methylcellulose was obtained from Aldrich Chemical Company in two lots (11414HU and 128H0668). Identity was confirmed using IR; spectra were consistent with the structure of methylcellulose. The methoxyl content (29.6% and 32.7%, respectively) was determined by Galbraith Laboratories, Inc., according to specifications given in NTP (1992).

Preparation and Analysis of Dose Formulations

The vehicle was prepared by mixing methylcellulose with heated, deionized water. The dose formulations were prepared once for the 1-month studies and every four weeks for the 3-month and 2-year studies. The dose formulations were stored in sealed amber glass bottles at room temperature (25° C) for up to 28 (1-month studies) or 35 days (3-month and 2-year studies).

Prior to the 1-month studies, the study laboratory performed solubility, homogeneity, resuspendibility, and gavageability studies. Homogeneity was confirmed with the recommendation that dose formulations be stirred continuously while sampling and during administration; resuspendibility was confirmed; gavageability was confirmed; and stability was confirmed for up to 29 days for dose formulations stored in sealed glass bottles, protected from light at 5° and 25° C, and for 3 hours at simulated animal room conditions.

Prior to the 3-month studies, the study laboratory conducted homogeneity, gavageability, and stability studies of dose formulations using HPLC. Homogeneity and gavageability were confirmed, and stability was confirmed for up to 35 days for dose formulations stored in amber glass bottles, protected from light at 5° and 25° C, and for 3 hours at simulated animal room conditions.

Prior to the 2-year studies, the analytical chemistry laboratory tested the solubility, homogeneity, and stability of dose formulations. To check the solubility of methylene blue trihydrate in 0.5% methylcellulose, a 15.5 mg/mL dose formulation was visually examined after storage at 5° C for 24 hours. Homogeneity and stability studies were performed using HPLC. Solubility and homogeneity were confirmed, and stability was confirmed for up to 35 days for dose formulations stored in amber glass containers, sealed with Teflon®-lined lids and protected from light at –20°, 5°, and 22° C, and for up to 3 hours at simulated animal room conditions.

Periodic analyses of the dose formulations of methylene blue trihydrate in 0.5% methylcellulose were conducted at the study laboratories using HPLC. Dose formulations were analyzed using HPLC once for the 1-month studies; animal room samples were also analyzed. Four of five dose formulations were within 10% of the target concentrations; all five animal room samples were within 10% of target concentrations. Dose formulations were analyzed twice for the 3-month studies; animal room samples were also analyzed. All dose formulations analyzed were within 10% of the target concentrations; 10 of 16 rat animal room samples and all eight mouse animal room samples were within 10% of the target concentrations. Dose formulations were analyzed every 3 months for the 2-year studies; animal room samples were also analyzed. All 33 dose formulations analyzed were within 10% of the target concentrations; all rat and mice animal room samples were within 10% of target concentrations.

1-Month Studies

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Charles River Laboratories (Raleigh, NC). On receipt, the rats and mice were 6 weeks old. Animals were quarantined for 11 to 14 days and were 7 to 8 weeks old on the first day of the studies. Blood samples were collected from five male and five female rats and mice at study start. Serologic analyses of the blood samples were performed 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 methylene blue trihydrate by gavage in a 0.5% aqueous methylcellulose solution at doses of 0, 125, 250, 500, 1,000, or 2,000 mg/kg once daily, 5 days per week for 5 weeks. Feed and water were

available *ad libitum*. Rats were housed five per cage and mice were housed individually. Clinical findings were recorded weekly for rats and mice. The animals were weighed initially, weekly, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

Neurobehavioral evaluations were conducted at the end of weeks 2 and 4 on all surviving core study rats. Functional measurements to assess neuromuscular, autonomic, and sensory activity domains were taken.

Blood was collected from the retroorbital sinus of 10 male and 10 female clinical pathology study rats per group on day 4 and from 10 male and 10 female core study rats and mice per group at the end of the 1-month studies. After blood collection on day 4, the clinical pathology study rats were euthanized with carbon dioxide and the spleen was collected, weighed, and preserved in 10% neutral buffered formalin; the core study rats and mice were euthanized by carbon dioxide and necropsied. All blood samples were collected no earlier than 0.5 hours after dosing. The blood for clinical chemistry samples was collected into microcollection serum separator tubes (Sarstedt, Inc., Nümbrecht, Germany), and serum was obtained by centrifugation (approximately 3,000 rpm for 15 minutes). Blood was also collected into microcollection tubes containing potassium-EDTA (Sarstedt, Inc.) for hematology samples; the samples were gently inverted on an aliquot mixer to prevent clotting prior to analyses. Blood dilution for methemoglobin determinations were performed within approximately 15 minutes after the samples were collected. The parameters measured are listed in Table 1.

Necropsies were performed on all core study rats and mice. The heart, right kidney, liver, lung, spleen, right testis, and thymus were weighed. Histopathologic examinations were performed on all vehicle control animals, all animals in the highest dose groups with at least 60% survivors (500 mg/kg, 1,000 mg/kg, and 2,000 mg/kg rats; 250, 500, 1,000, and 2,000 mg/kg mice), and all animals that died early. Because of evidence of red blood cell damage and increased tissue pigmentation, spleen, kidney, and liver sections from 10 rats and 12 mice were stained using a Prussian blue technique for the presence of iron to confirm the presence of hemosiderin (Stefanski *et al.*, 1990). Table 1 lists the tissues and organs examined.

3-Month Studies

The 3-month studies were conducted to evaluate the cumulative toxic effects of repeated exposure to methylene blue trihydrate and to determine the appropriate doses to be used in the 2-year studies.

Male and female F344/N rats and $B6C3F_1$ mice were obtained from Taconic Laboratory Animals and Services, Inc. (Germantown, NY). On receipt, the rats and mice were 5 weeks old. Animals were quarantined for 12 to 15 days and were 7 weeks old on the first day of the studies. Blood samples were collected from five male and five female rats and mice at the start of the 3-month studies and from five male and five female sentinel rats and mice four weeks after the start of the studies and at study termination. Serologic analyses of the blood samples were performed 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 20 male and 20 female clinical pathology study rats and mice were administered methylene blue trihydrate by gavage in a 0.5% aqueous methylcellulose solution at doses of 0, 25, 50, 100, or 200 mg/kg once daily 5 days per week for 14 weeks. 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 weekly for rats and mice. The animals were weighed initially, weekly, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

During weeks 1 and 6 of the 3-month study, 10 male and 10 female rats and mice per group were bled for hematology and clinical chemistry determinations. Animals were anesthetized with a carbon dioxide/oxygen mixture. Blood was drawn by cardiac puncture in mice and from the retroorbital sinus in rats. After blood collection, animals were euthanized with carbon dioxide and discarded without necropsy, except for five control and five high dose female rats that were maintained until study termination and then necropsied with tissue collection. At terminal sacrifice on study day 92, 10 male and 10 female core study rats and mice per group and selected remaining clinical pathology study 200 mg/kg female rats were bled for hematology and clinical chemistry determinations and the animals were euthanized and necropsied. The blood for clinical chemistry samples was collected into microcollection serum

separator tubes (Sarstedt, Inc., Nümbrecht, Germany) and serum obtained by centrifugation (approximately 3,000 rpm for 15 minutes). Blood was also collected into microcollection tubes containing potassium-EDTA (Sarstedt, Inc.) for hematology samples; the samples were gently inverted on an aliquot mixer to prevent clotting prior to analyses. The parameters measured are listed in Table 1.

At the end of the 3-month studies, samples were collected for sperm motility and vaginal cytology evaluations on rats and mice in the vehicle control, 50, 100, and 200 mg/kg groups and selected remaining clinical pathology 200 mg/kg female rats. The parameters evaluated are listed in Table 1. For 12 consecutive days prior to scheduled terminal sacrifice, the vaginal vaults of the females were moistened with 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 right 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.

Necropsies were performed on all core study rats and mice. The heart, right kidney, liver, lung, spleen, right testis, and thymus 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. While tissues from the remaining five clinical pathology study and five 200 mg/kg female rats were collected at necropsy, the tissues were not processed or examined microscopically due to inadequate survival in the female 200 mg/kg group. Complete histopathologic examinations were performed on all core study groups of rats and mice. Spleen, kidney, and liver sections from 12 mice having pigmentation were stained using a Prussian blue technique for the presence of iron to confirm the presence of hemosiderin (Stefanski *et al*., 1990).

2-YEAR STUDIES

Study Design

Groups of 50 male and 50 female rats and mice were administered methylene blue trihydrate in a 0.5% aqueous methylcellulose solution by gavage at doses of 0, 5, 25, or 50 mg/kg (rats) or 0, 2.5, 12.5, or 25 mg/kg (mice) once daily, 5 days per week for 2 years. Additional groups of 10 male and 10 female rats and 30 male and 30 female mice were administered the same doses for up to 18 months and were evaluated at 2 weeks and 3, 12, and 18 months for hematology.

Source and Specification of Animals

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Taconic Farms, Inc. (Germantown, NY), for use in the 2-year studies. Rats and mice were quarantined for 12 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 6 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

Rats were housed three (males) or five (females) per cage, and mice were housed individually (males) or five per cage (females). Feed and water were available *ad libitum*. Cages were changed once (male mice) or twice weekly, and racks were rotated every 2 weeks. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is provided in Appendix J.

Clinical Examinations and Pathology

Animals were observed twice daily. Clinical findings for core study animals were recorded every 4 weeks beginning with week 5. Body weights for core study animals were recorded on day 1, weekly for the first 13 weeks, at 4-week intervals thereafter, and at terminal sacrifice.

Blood was taken from the retroorbital sinus of 10 male and 10 female hematology study rats at 2 weeks and 3, 12, and 18 months; the rats were sacrificed and discarded at 18 months. Blood was drawn from the retroorbital sinus of 10 male and 10 female hematology study mice at 2 weeks or 3 months or 12 and 18 months; the mice were sacrificed and discarded at 2 weeks, 3 months, or 18 months. Blood was collected from each animal into a tube containing EDTA and transported to the clinical pathology laboratory after collection. At each collection interval, methemoglobin analyses were initiated within 30 minutes of blood collection; the remaining hematology analyses, including reticulocyte counts, were performed within approximately 6 hours of blood collection. Hematology analyses and reticulocyte counts were conducted on the day of sample collection. At 2 weeks and 3 months, the automated hematology analyses, excluding the methemoglobin and reticulocyte assays, were conducted using the Technicon H·1™ hematology analyzer (Technicon Corporation, Tarrytown, NY) with reagents manufactured by R&D Systems, Inc. (Minneapolis, MN), Bayer, Inc. (Tustin, CA), and Fisher Scientific (Norcross, GA); the reticulocyte analyses were conducted at 2 weeks and 3 months using a Coulter Model EPICS XL Flow Cytometer (Coulter Corporation, Miami, FL) with reagents manufactured by Coulter Corporation and Molecular Probes (Eugene, OR). At 12 and 18 months, hematology analyses, including the reticulocyte analyses, were conducted using an ADVIA 120 Hematology System Analyzer (Bayer Diagnostics, Tarrytown, NY) with reagents manufactured and/or supplied by Bayer, Inc., and Fisher Scientific. At all four intervals, the methemoglobin analyses were conducted using a Beckman DU spectrophotometer with reagents manufactured by Baker Chemical Company (Phillipsburg, NJ) and Fisher Scientific. Blood smears were prepared within approximately 2 hours of sample collection for Heinz body enumeration and for evaluation of platelet and erythrocyte morphology by light microscopy. The parameters measured are listed in Table 1.

At 3, 12, and 18 months, five male and five female core study rats and mice were randomly selected from each dose group for urine collection. After four consecutive days of dosing, the animals were placed into metabolism cages, and urine was collected over ice for approximately 24 hours. Creatinine concentration and volume were determined by standard methods for each animal, and the remainder of each urine sample was frozen and shipped to an NTP-designated analytical laboratory. The parameters measured are listed in Table 1.

Complete necropsies and microscopic examinations were performed on all core study 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 4 to 6 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 1.

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 bone marrow, harderian gland, liver, pancreas, and spleen of rats and mice; eye of rats; nose of mice; lung and testis of male rats; mammary gland of female rats; lung and small intestine of male mice; and mandibular and mesenteric lymph nodes and thymus 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 1
Experimental Design and Materials and Methods in the Gavage Studies of Methylene Blue Trihydrate

1-Month Studies	3-Month Studies	2-Year Studies
Study Laboratory		
Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)	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		
Charles River Laboratories (Raleigh, NC)	Taconic Laboratory Animals and Services, Inc. (Germantown, NY)	Taconic Farms, Inc. (Germantown, NY)
Time Held Before Studies		
Rats: 11 (males) or 12 days (females) Mice: 13 (males) or 14 days (females)	Rats: 12 (males) or 13 days (females) Mice: 14 (males) or 15 days (females)	12 days
Average Age When Studies Began		
7-8 weeks	7 weeks	6 weeks
Date of First Dose		
Rats: May 4 (males) or 5 (females), 1992 Mice: May 6 (males) or 7 (females), 1992	Rats: October 5 (males) or 6 (females), 1993 Mice: October 7 (males) or 8 (females), 1993	Rats: June 26, 2000 Mice: July 10, 2000
Duration of Dosing		
5 days/week for up to 5 weeks	5 days/week for up to 14 weeks	5 days/week for up to 106 weeks
Date of Last Dose		
Rats: June 2 (males) or 3 (females), 1992 Mice: June 4 (males) or 5 (females), 1992	Rats: January 4 (males) or 5 (females), 1994 Mice: January 6 (males) or 7 (females), 1994	Rats: July 1, 2002 Mice: July 15, 2002
Necropsy Dates		
Rats: June 2-3, 1992 Mice: June 4-5, 1992	Rats: January 4-5, 1994 Mice: January 6-7, 1994	Rats: June 24-July 2, 2002 Mice: July 8-16, 2002
Average Age at Necropsy		
11-12 weeks	19 weeks	110-111 weeks
Size of Study Groups		
Core studies: 10 male and 10 female rats and mice Clinical pathology studies: 10 male and 10 female rats	Core studies: 10 male and 10 female rats and mice Clinical pathology studies: 20 male and 20 female rats and mice	Core studies: 50 male and 50 female rats and mice Hematology studies: 10 male and 10 female rats; 30 male and 30 female mice
Method of Distribution		
Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 1-month studies	Same as 1-month studies
Animals per Cage		
Rats: 5 Mice: 1	Rats: 5 Mice: 1 (males) or 5 (females)	Rats: 3 (males) or 5 (females) Mice: 1 (males) or 5 (females)
Method of Animal Identification		
Tail tattoo	Tail tattoo	Tail tattoo

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Methylene Blue Trihydrate

1-Month Studies	3-Month Studies	2-Year Studies
Diet		
NIH-07 open formula pellets (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed once weekly	Same as 1-month studies	Irradiated NTP-2000 open formula wafers (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed once weekly
Water		
Tap water (Columbus municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available *ad libitum*	Same as 1-month studies	Tap water (Birmingham municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available *ad libitum*
Cages		
Polycarbonate solid-bottom (Lab Products, Inc., Garfield, NJ), changed at least twice weekly	Same as 1-month studies	Polycarbonate solid-bottom (Lab Products, Inc., Maywood, NJ), changed once (male mice) or twice weekly
Bedding		
Sani-Chips® hardwood chips (P.J. Murphy Forest Products, Inc., Montville, NJ), changed once (mice) or twice (rats) weekly	Sani-Chips® hardwood chips (P.J. Murphy Forest Products, Inc., Montville, NJ), changed once (male mice) or twice (female mice and rats) weekly	Heat-treated irradiated hardwood chips (P.J. Murphy Forest Products, Inc., Montville, NJ), changed once (male mice) or twice (female mice and rats) weekly
Rack Filters		
Reemay® spun-bonded polyester (Andico, Birmingham, AL); changed every 2 weeks	Reemay® spun-bonded polyester (Andico, Birmingham, AL); changed every 2 weeks	Reemay® spun-bonded polyester (Andico, Birmingham, AL); changed every 2 weeks
Racks		
Stainless steel (Lab Products; Maywood, NJ), changed every 2 weeks	Stainless steel (Lab Products; Maywood, NJ), changed every 2 weeks	Stainless steel (Lab Products; Maywood, NJ), changed every 2 weeks
Animal Room Environment		
Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room/Chamber air changes: 10/hours	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room/Chamber air changes: 10/hours	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room/Chamber air changes: 10/hours
Doses		
0, 125, 250, 500, 1,000, or 2,000 mg/kg in 0.5% methylcellulose by gavage (dosing volume 10 mL/kg)	0, 25, 50, 100, or 200 mg/kg in 0.5% methylcellulose by gavage (dosing volumes 5 mL/kg for rats and 10 mL/kg for mice)	Rats: 0, 5, 25, or 50 mg/kg in 0.5% methylcellulose by gavage (dosing volume 5 mL/kg) Mice: 0, 2.5, 12.5, or 25 mg/kg in 0.5% methylcellulose by gavage (dosing volume 10 mL/kg)
Type and Frequency of Observation		
Observed twice daily; core study animals were weighed and clinical findings were recorded initially, weekly for 4 weeks, and at the end of the studies.	Observed twice daily; core study animals were weighed and clinical findings were recorded initially, weekly, and at the end of the studies.	Observed twice daily; core study animals were weighed initially, weekly for 13 weeks, monthly thereafter, and at the end of the studies. Clinical findings were recorded monthly for core study animals.
Method of Sacrifice		
CO_2 asphyxiation	Same as 1-month studies	Same as 1-month studies

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Methylene Blue Trihydrate

1-Month Studies	3-Month Studies	2-Year Studies
Necropsy Necropsies were performed on core study animals. Organs weighed: heart, right kidney, liver, lung, spleen, right testis, and thymus.	Necropsies were performed on core study animals and five each from vehicle control and 200 mg/kg female rats. Organs weighed: heart, right kidney, liver, lung, spleen, right testis, and thymus.	Necropsies were performed on core study animals.
Clinical Pathology Blood was collected from the retroorbital sinus of 10 male and 10 female clinical pathology study rats per group on day 4 and of 10 male and 10 female core study rats and mice per group at the end of the 1-month studies. ***Hematology:*** automated hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; erythrocyte and platelet morphology; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; leukocyte count and differentials; methemoglobin; and Heinz bodies ***Clinical Chemistry:*** urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and total bile acids	Blood was collected from 10 male and 10 female clinical pathology study rats and mice per group during weeks 1 and 6, from selected clinical pathology study animals at the end of the studies, and from 10 male and 10 female core study rats and mice per group at the end of the studies. Blood was collected from the retroorbital sinus of rats and by cardiac puncture in mice. ***Hematology:*** automated hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; erythrocyte and platelet morphology; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; leukocyte count and differentials; methemoglobin; and Heinz bodies ***Clinical Chemistry:*** urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, and total bile acids	Blood was collected from the retroorbital sinus of 10 male and 10 female hematology study rats and mice per group at 2 weeks and 3, 12, and 18 months. At 3, 12, and 18 months, five male and five female core study rats and mice from each dose group were placed in metabolism cages for 24-hour urine collection. ***Hematology:*** automated and manual hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; erythrocyte and platelet morphology; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; leukocyte count and differentials; methemoglobin; and Heinz bodies ***Urinalysis:*** creatinine and volume

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Methylene Blue Trihydrate

1-Month Studies	3-Month Studies	2-Year Studies
Histopathology		
Complete histopathology was performed on all vehicle control animals; 500, 1,000, 2,000 mg/kg rats; and 250, 500, 1,000, and 2,000 mg/kg mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eye, gallbladder (mice only), harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung and mainstem bronchi, lymph nodes (mandibular, and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicles, thymus, thyroid gland, trachea, urinary bladder, and uterus. The following target organs were identified and examined at lower dose levels: rats - bone marrow, heart, small intestine (duodenum and ileum), kidney, liver, nose, spleen, stomach (forestomach and glandular), thymus, ovary, and urinary bladder; mice - bone marrow, heart, kidney, liver, lymph nodes (mandibular and mesenteric), spleen, stomach (forestomach), thymus, and urinary bladder	Complete histopathology was performed on all 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, eye, gallbladder (mice only), harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung and mainstem bronchi, lymph nodes (mandibular, and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicles, thymus, thyroid gland, trachea, urinary bladder, and uterus.	Complete histopathology was performed on all 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, eye, gallbladder (mice only), harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung and mainstem bronchi, lymph nodes (mandibular, and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicles, thymus, thyroid gland, trachea, urinary bladder, and uterus.
Sperm Motility and Vaginal Cytology		
None	At the end of the studies, sperm samples were collected from male animals in the 0, 50, 100, and 200 mg/kg groups for sperm motility evaluations. The following parameters were evaluated: spermatid heads per testis and per gram testis, sperm counts, and epididymal spermatozoal motility and concentration. 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 administered 0, 50, 100, or 200 mg/kg for vaginal cytology evaluations. The percentage of time spent in the various estrous cycle stages and estrous cycle length were evaluated.	None

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Methylene Blue Trihydrate

1-Month Studies	3-Month Studies	2-Year Studies
Neurobehavioral Studies Neurobehavioral evaluations were performed on all surviving rats at the end of weeks 2 and 4. The functional observational battery was performed to assess neuromuscular, autonomic, and sensory activities of the rats.	None	None

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, A4, B1, B3, C1, C4, D1, and D4 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A2, B2, C2, and D2) 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 A2, B2, C2, and D2 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, only to site-specific, lesion-free 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 B6C3F_1 mice (Portier *et al.,* 1986). Bailer and Portier (1988) showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range from 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use

of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams (1993).

Tests of significance included pairwise comparisons of each dosed group with controls and a test for an overall dose 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, urinalysis, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) (as modified by Williams, 1986) 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 (1957) 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 and fat than the NIH-07 diet previously used in toxicity and carcinogenicity studies (Rao, 1996, 1997). The current NTP historical database contains all 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, including the present study. However, because the database for gavage studies, including all vehicles, is small, only the historical database of studies by all routes and all vehicles was used to evaluate incidences for the present study.

Quality Assurance Methods

The 1-month, 3-month, 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 and specimens from the 2-year studies were submitted to the NTP Archives, they were inspected by NTP Archives and Pathology Support Contract staff for conformance with NTP specifications (NTP, 1992) and professional standards. The corresponding study reports were audited retrospectively by the NTP Quality Assurance Office through its Quality Assurance Support Contract. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report. NTP Quality Assurance audited all changes made to diagnoses in pathology tables following completion of the PWG review process and will audit this Technical Report before it is published. Quality assurance audit procedures and findings are presented in reports that are on file at NIEHS.

Genetic Toxicology

The genetic toxicology of methylene blue trihydrate was assessed by testing the ability of the chemical to

induce mutations in various strains of *Salmonella typhimurium,* sister chromatid exchanges and chromosomal aberrations in cultured Chinese hamster ovary cells, micronucleated erythrocytes in mouse bone marrow, and increases in the frequency of micronucleated erythrocytes in mouse peripheral blood. Micronuclei (literally "small nuclei" or Howell-Jolly bodies are biomarkers of induced structural or numerical chromosomal alterations and are formed when acentric fragments or whole chromosomes fail to incorporate into either of two daughter nuclei during cell division (Schmid, 1975; Heddle *et al*., 1983). 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

1-MONTH STUDY

Rats exposed to 125 or 250 mg/kg methylene blue trihydrate survived to the end of the study (Table 2). In the 500 mg/kg groups, four females died on days 11 to 13 and two males died on days 7 and 12. All rats in the 1,000 mg/kg group died by study day 10, and all rats in the 2,000 mg/kg group died by study day 6. The deaths were associated with severe acute hypoxia. Final mean body weights of male rats in the 250 and 500 mg/kg groups were significantly less (approximately 8% and 11%, respectively) than those of the vehicle control group. In 250 and 500 mg/kg females, the final mean body weights were also significantly less than controls (5% and 7%, respectively). Mean body weight gains of 500 mg/kg males and of 250 and 500 mg/kg females were significantly less than those of the vehicle controls. Blue staining of the urogenital area, tail, and fur from excretion of test material in the urine and feces was observed in all dosed groups. Thinness, abnormal respiration, hypothermia, hypoxia, lethargy, ataxia, abnormal posture, ruffled fur, and blue discoloration of the nasal and footpad regions were observed in the 1,000 and 2,000 mg/kg groups. No significant neurological deficits were observed in rats exposed to methylene blue trihydrate at 2 weeks or at the end of the study (data not shown).

TABLE 2
Survival and Body Weights of Rats in the 1-Month Gavage Study of Methylene Blue Trihydrate

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	147 ± 1	233 ± 2	86 ± 2	
125	10/10	146 ± 2	226 ± 4	80 ± 5	97
250	10/10	145 ± 2	213 ± 4**	68 ± 4	92
500	8/10[c]	146 ± 2	208 ± 6**	63 ± 5**	89
1,000	0/10[c]	146 ± 2	—	—	—
2,000	0/10[d]	147 ± 2	—	—	—
Female					
0	10/10	106 ± 2	146 ± 2	40 ± 1	
125	10/10	107 ± 1	143 ± 2	36 ± 1	98
250	10/10	106 ± 1	139 ± 2*	34 ± 2**	95
500	6/10[e]	108 ± 1	136 ± 4**	27 ± 3**	93
1,000	0/10[c]	110 ± 1	—	—	—
2,000	0/10[d]	106 ± 1	—	—	—

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' test
** $P \leq 0.01$
[a] Number of animals surviving at 1 month/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 deaths: 1, 2
[d] Week of deaths: 1
[e] Week of deaths: 2

The hematology and clinical chemistry data for rats in the 1-month study of methylene blue trihydrate are listed in Tables 3 and F1. The primary responses to administration of methylene blue trihydrate were development of a methemoglobinemia, increased Heinz body formation, and development of a macrocytic, hyperchromic, responsive anemia. A dose-related methemoglobinemia, evidenced by increased methemoglobin concentrations, occurred in all dosed groups at day 4 and persisted at day 30. At day 30, the 500 mg/kg groups had an approximate 100% increase in methemoglobin concentration. Heinz body formation responded similarly. Significant dose-related increases in Heinz bodies occurred in the 500, 1,000, and 2,000 mg/kg groups at day 4 and in all surviving dosed groups, except 125 mg/kg females, at day 30. In the 500 mg/kg groups at day 30, more than 20% of the erythrocytes had Heinz bodies compared to no Heinz bodies observed in erythrocytes of vehicle control animals.

The anemia occurred by day 30 and was apparent in all surviving male and female dosed groups. The 500 mg/kg animals demonstrated the most severe erythron change, evidenced by an approximate 10%, 6% and 28.5% decrease in the hematocrit, hemoglobin, and erythrocyte counts, respectively. The macrocytosis was evidenced by the increase in mean cell volume, and the hyperchromia was indicated by the increased mean cell hemoglobin concentration. A hematopoietic response was indicated by increased numbers of circulating reticulocytes and nucleated erythrocytes. There was an apparent increase in leukocyte counts involving the neutrophil, lymphocyte, and monocyte cell types. There was histopathologic evidence of an inflammatory process that may help explain the increase in the leukon, particularly the neutrophil counts observed in dosed males and females.

Increases in alanine aminotransferase and sorbitol dehydrogenase activities occurred in a dose-related fashion in females; dose-related increases in bile salt concentrations occurred in males and females. These are markers of liver injury and would be consistent with a hepatocellular effect and, possibly, the hepatic necrosis observed histologically. At day 4, there was a transient decrease in albumin and, consequently, total protein concentrations in higher-dose males and females. The mechanism for this transient serum protein decrease was not evident.

Enlarged spleens were observed at necropsy in dosed rats, except in the 2,000 mg/kg groups that all died by day 6. The cause of death for two rats administered 2,000 mg/kg was attributed to duodenal perforation. Significant increases in absolute and relative spleen weights occurred in all surviving dosed groups (Table G1). There were also significant decreases in absolute and relative thymus weights of 250 and 500 mg/kg males and 125 and 250 mg/kg females; the absolute thymus weight of 500 mg/kg females was also significantly decreased. In males, there were significant increases in relative weights of the heart, testis, and kidney in the 250 and 500 mg/kg groups and relative liver weights in all dosed groups. In females, significant increases in relative kidney and liver weights occurred in all dosed groups and in relative heart weight and absolute liver weight in the 500 mg/kg group.

TABLE 3
Selected Hematology Parameters for Rats in the 1-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg	1,000 mg/kg	2,000 mg/kg
Male						
Day 4	9	7	10	8	10	6
Day 30	10	10	10	8	0	0
Hematocrit (%)						
Day 4	43.0 ± 0.5	42.0 ± 0.4	44.2 ± 0.5	43.9 ± 0.7	43.5 ± 0.7	42.2 ± 2.2
Day 30	46.1 ± 0.3	43.6 ± 0.5**	42.6 ± 0.5**	40.9 ± 0.9**		
Hemoglobin (g/dL)						
Day 4	14.3 ± 0.1	14.2 ± 0.1	14.5 ± 0.1	14.3 ± 0.3	14.2 ± 0.2	14.6 ± 0.8
Day 30	15.7 ± 0.2	14.9 ± 0.1**	14.7 ± 0.2**	14.6 ± 0.2**		
Erythrocytes (10^6/μL)						
Day 4	6.87 ± 0.07	6.83 ± 0.05	7.15 ± 0.09	7.15 ± 0.13	7.02 ± 0.12	6.80 ± 0.37
Day 30	8.26 ± 0.08	7.35 ± 0.08**	6.46 ± 0.10**	5.82 ± 0.21**		
Reticulocytes (10^6/μL)						
Day 4	0.41 ± 0.03	0.40 ± 0.03	0.37 ± 0.02	0.40 ± 0.03	0.35 ± 0.03	0.33 ± 0.06
Day 30	0.24 ± 0.03	0.48 ± 0.04**	0.69 ± 0.09**	0.99 ± 0.05**		
Nucleated erythrocytes (10^3/μL)						
Day 4	0.09 ± 0.04	0.08 ± 0.03	0.07 ± 0.06	0.09 ± 0.05	0.08 ± 0.03	0.27 ± 0.08
Day 30	0.02 ± 0.01	0.18 ± 0.06**	0.96 ± 0.16**	1.07 ± 0.21**		
Mean cell volume (fL)						
Day 4	62.6 ± 0.3	61.5 ± 0.3	61.9 ± 0.3	61.3 ± 0.3	62.0 ± 0.3	62.1 ± 0.3
Day 30	55.8 ± 0.2	59.3 ± 0.2**	66.1 ± 0.8**	70.5 ± 1.4**		
Mean cell hemoglobin concentration (g/dL)						
Day 4	33.3 ± 0.3	33.8 ± 0.4	32.7 ± 0.2	32.6 ± 0.2	32.7 ± 0.2	34.5 ± 0.4
Day 30	34.1 ± 0.1	34.1 ± 0.2	34.4 ± 0.2	35.9 ± 0.3**		
Methemoglobin (g/dL)						
Day 4	0.25 ± 0.02[b]	0.42 ± 0.03**[b]	0.62 ± 0.04**	1.00 ± 0.06**[b]	1.18 ± 0.08**	1.13 ± 0.03**
Day 30	0.36 ± 0.03	0.59 ± 0.02**	0.63 ± 0.05**	0.68 ± 0.05**		
Heinz bodies (%)						
Day 4	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0	57.7 ± 15.2**[b]	95.0 ± 0.4**	95.2 ± 0.8**
Day 30	0.0 ± 0.0	1.7 ± 0.8**	20.2 ± 1.9**	26.6 ± 1.2**[c]		

TABLE 3
Selected Hematology Parameters for Rats in the 1-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg	1,000 mg/kg	2,000 mg/kg
Female						
Day 4	10	10	10	10	10	5
Day 30	10	10	10	6	0	0
Hematocrit (%)						
Day 4	44.0 ± 0.5	44.0 ± 0.4	45.0 ± 0.3	45.8 ± 0.3	43.4 ± 1.3	37.4 ± 2.3
Day 30	46.6 ± 0.4	43.7 ± 0.4**	41.8 ± 0.6**	42.3 ± 0.2**		
Hemoglobin (g/dL)						
Day 4	14.5 ± 0.2	14.6 ± 0.1	14.7 ± 0.1	14.9 ± 0.1	14.7 ± 0.3	13.5 ± 0.8
Day 30	15.5 ± 0.1	14.5 ± 0.1**	14.2 ± 0.1**	14.8 ± 0.1**		
Erythrocytes (10^6/µL)						
Day 4	6.86 ± 0.10	6.88 ± 0.08	7.10 ± 0.05	7.24 ± 0.07*	6.87 ± 0.20	5.90 ± 0.34
Day 30	7.74 ± 0.06	6.70 ± 0.05**	6.11 ± 0.10**	5.61 ± 0.13**		
Reticulocytes (10^6/µL)						
Day 4	0.36 ± 0.01	0.37 ± 0.02	0.39 ± 0.02	0.39 ± 0.03	0.28 ± 0.02	0.27 ± 0.04
Day 30	0.12 ± 0.01	0.23 ± 0.02**	0.35 ± 0.02**	0.55 ± 0.04**		
Nucleated erythrocytes (10^3/µL)						
Day 4	0.02 ± 0.01	0.05 ± 0.02	0.05 ± 0.02	0.07 ± 0.06	0.17 ± 0.08	0.15 ± 0.08
Day 30	0.00 ± 0.00	0.06 ± 0.03*	0.24 ± 0.10**	3.17 ± 1.13**		
Mean cell volume (fL)						
Day 4	64.2 ± 0.3	63.9 ± 0.2	63.4 ± 0.2	63.2 ± 0.3	63.2 ± 0.3	63.3 ± 0.3
Day 30	60.3 ± 0.2	65.3 ± 0.3**	68.4 ± 0.4**	75.6 ± 1.6**		
Mean cell hemoglobin concentration (g/dL)						
Day 4	33.0 ± 0.2	33.2 ± 0.2	32.6 ± 0.1	32.5 ± 0.2	34.1 ± 0.5	36.1 ± 0.4*
Day 30	33.3 ± 0.1	33.3 ± 0.2	34.1 ± 0.3	34.9 ± 0.2**		
Methemoglobin (g/dL)						
Day 4	0.23 ± 0.03	0.77 ± 0.03**	1.05 ± 0.06**	1.48 ± 0.06**	1.31 ± 0.03**	1.60 ± 0.23**
Day 30	0.35 ± 0.04	0.62 ± 0.03**	0.70 ± 0.04**	0.72 ± 0.11**		
Heinz bodies (%)						
Day 4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	7.2 ± 2.1**	77.6 ± 11.9**	95.6 ± 0.5**
Day 30	0.0 ± 0.0	0.1 ± 0.1	10.8 ± 2.4**	20.1 ± 1.4**		

* 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. No data presented for the 1,000 and 2,000 mg/kg groups on day 30 due to 100% mortality.
[b] n=10
[c] n=7

All dosed rats had intense blue discoloration of tissues. Spleen lesions associated with methylene blue trihydrate administration included hematopoietic cell proliferation, pigmentation, lymphoid depletion of the lymphoid follicles, and capsular fibrosis (Table 4). The incidences of hematopoietic cell proliferation were significantly increased in males and females in the 125, 250, 500, and 1,000 mg/kg groups. Also, the severity of this lesion generally increased from minimal to moderate with increasing dose concentration. This lesion was characterized by proliferation of hematopoietic cells, predominantly of the erythroid series, in the red pulp of the spleen. The presence of hematopoietic cell proliferation correlated with the enlarged spleens observed at necropsy. The incidence of minimal to mild splenic pigmentation was significantly increased in male and female rats in the 125, 250, and 500 mg/kg groups. Pigment in the spleen was characterized by the presence of numerous macrophages containing golden-brown refractile granules in the red pulp. The pigment was confirmed as hemosiderin and is evidence for the destruction of damaged erythrocytes. Lymphoid follicle depletion was diagnosed in the 1,000 and 2,000 mg/kg groups of rats dying early in the study and consisted

TABLE 4
Incidences of Selected Nonneoplastic Lesions in Rats in the 1-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg	1,000 mg/kg	2,000 mg/kg
Male						
Spleen[a]	10	10	10	9	10	10
Hematopoietic Cell Proliferation[b]	0	10** (1.0)[c]	10** (1.9)	9** (2.2)	8** (1.5)	1 (1.0)
Pigmentation	0	10** (1.9)	10** (1.9)	7** (1.0)	2 (2.0)	0
Lymphoid Follicle, Depletion Cellular	0	0	0	0	9** (2.2)	9** (2.4)
Capsule, Fibrosis	0	0	9** (1.0)	7** (1.0)	0	0
Bone Marrow	10	10	10	10	10	10
Hyperplasia	0	8** (1.3)	10** (2.0)	10** (2.0)	10** (1.9)	10** (2.0)
Liver	10	10	10	9	10	10
Centrilobular Necrosis	0	0	0	1 (3.0)	3 (3.0)	7** (2.9)
Hematopoietic Cell Proliferation	0	7** (1.0)	10** (1.0)	9** (1.0)	3 (1.0)	0
Kupffer Cell Pigmentation	0	0	0	5* (1.0)	4* (1.3)	0
Thymus	10	10	10	9	9	9
Necrosis	0	0	0	1 (3.0)	7** (2.4)	9** (2.1)
Kidney	10	10	10	9	10	10
Renal Tubule, Pigmentation	0	0	0	6** (1.0)	0	0
Stomach, Glandular	10	10	10	9	10	10
Inflammation, Chronic, Active	0	0	0	1 (2.0)	1 (2.0)	0
Necrosis	0	0	0	0	1 (4.0)	0
Intestine Small, Duodenum	10	10	10	9	10	10
Necrosis	0	0	0	0	0	6** (2.7)
Serosa, Inflammation	0	0	0	0	0	4* (2.3)
Heart	10	10	10	9	10	10
Inflammation, Chronic, Active	8 (1.1)	6 (1.0)	6 (1.0)	3 (1.0)	6 (2.0)	4 (1.3)
Epididymis	10	0	0	9	10	10
Hypospermia	0	0	0	0	5* (1.0)	10** (1.0)

TABLE 4
Incidences of Selected Nonneoplastic Lesions in Rats in the 1-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg	1,000 mg/kg	2,000 mg/kg
Female						
Spleen	10	10	10	10	10	10
Hematopoietic Cell Proliferation	0	10** (1.0)	10** (2.0)	10** (2.4)	5* (2.6)	0
Pigmentation	0	10** (1.8)	10** (1.8)	9** (1.4)	3 (1.3)	0
Lymphoid Follicle, Depletion Cellular	0	0	0	0	6** (1.5)	9** (2.2)
Capsule, Fibrosis	0	0	7** (1.0)	4* (1.0)	0	0
Bone Marrow	10	10	10	10	10	10
Hyperplasia	0	10** (1.8)	10** (1.9)	10** (2.1)	10** (2.1)	10** (2.0)
Liver	10	10	10	10	10	10
Centrilobular Necrosis	0	0	0	4* (3.0)	3 (3.0)	9** (2.1)
Hematopoietic Cell Proliferation	0	2 (1.0)	10** (1.0)	10** (1.0)	4* (1.3)	0
Kupffer Cell Pigmentation	0	0	1 (1.0)	9** (1.0)	4* (1.0)	0
Thymus	10	10	10	10	10	10
Necrosis	0	0	0	4* (3.5)	8** (2.1)	9** (3.2)
Kidney	10	10	10	10	10	10
Renal Tubule, Pigmentation	0	0	0	8** (1.0)	1 (1.0)	0
Stomach, Glandular	10	10	10	10	10	10
Necrosis	0	0	0	0	0	4* (3.5)
Serosa, Inflammation	0	0	0	0	1 (1.0)	3 (2.0)
Intestine Small, Duodenum	10	10	10	9	10	10
Necrosis	0	0	0	0	0	7** (3.1)
Serosa, Inflammation	0	0	0	0	0	4* (2.0)
Heart	10	10	10	10	10	10
Inflammation, Chronic Active	1 (1.0)	3 (1.0)	2 (1.0)	7** (2.0)	7** (2.3)	3 (1.0)

* 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

of necrosis and a loss of lymphocytes from the white pulp resulting in a reduced or indistinct mantle of lymphocytes around the splenic arteries. Spleen capsular fibrosis was diagnosed in most 250 and 500 mg/kg rats that survived to the end of the study. Capsular fibrosis consisted of focal thickening of the splenic capsule comprised of variable amounts of fibrosis and accumulations of mononuclear cells.

Hyperplasia of the bone marrow occurred in all rats except two 125 mg/kg males; severity of bone marrow hyperplasia was slightly greater in dosed females (minimal to moderate) than in males (minimal to mild) (Table 4). Hyperplasia consisted of increased quantities of erythroid and myeloid elements of the bone marrow.

Liver lesions associated with methylene blue exposure included centrilobular necrosis, hematopoietic cell proliferation, and Kupffer cell pigmentation with eryth-rophagocytosis (Table 4). Mild to moderate centrilobular necrosis was observed in some early-death males and females in the 500, 1,000 and 2,000 mg/kg groups. These lesions, characterized by coagulation necrosis of hepatocytes in the centrilobular region of the liver, were consistent with hypoxia resulting from anemia. The incidence of centrilobular necrosis was highest in the 2,000 mg/kg groups. Hematopoietic cell proliferation was observed in all 250 and 500 mg/kg males and females, in most 125 mg/kg males, and in a few 125 mg/kg females and 1,000 mg/kg males and females. This lesion increased significantly in males administered 125, 250, or 500 mg/kg and in females administered 250, 500, or 1,000 mg/kg; severity was slightly greater in females (minimal to mild versus minimal in males). This lesion consisted of small foci of hematopoietic cells, predominantly erythroid precursors, in the sinusoids scattered throughout the liver. The incidences of Kupffer cell lesions, consisting of pigment accumulation and erythrophagocytosis, were significantly increased in the 500 and 1,000 mg/kg groups. The lesions had golden to brown granular pigment consistent with hemosiderin deposition and were slightly more severe in males (minimal to mild versus minimal in females). The pigment was confirmed as hemosiderin. In addition to pigment, Kupffer cells often contained one to multiple red blood cells and erythrophagocytosis. The liver lesions were consistent with anemia and red blood cell destruction.
Necrosis of the thymus was observed in groups of animals that died during the study (Table 4). The incidences of thymic necrosis were increased significantly in rats administered 1,000 or 2,000 mg/kg and in females administered 500 mg/kg; severity was mild to moderate in males and mild to marked in females. The lesions consisted of a loss of lymphocytes in both the cortex and medulla of the thymus. There was a decrease in the number of lymphocytes as well as frank necrosis of the lymphocytes. The necrosis was indicated by the presence of numerous pyknotic and karyorrhectic lymphocyte nuclei and the presence of cellular fragments with macrophages. Thymic necrosis was not considered a direct effect of methylene blue trihydrate.

In the kidney, incidences of pigmentation of renal tubules in 500 mg/kg males and females were significantly increased (Table 4). Pigmentation was minimal in severity and consisted of fine brownish granules of hemosiderin in the renal tubular epithelium of the proximal tubules in the outer cortex. Renal hemosiderin pigmentation was consistent with the destruction of damaged erythrocytes.

Lesions considered related to methylene blue trihydrate administration were observed in the glandular stomach and in the duodenum of some animals that died early (Table 4). The lesions included moderate to marked inflammation and necrosis of the glandular stomach in males and mild to marked inflammation and necrosis of the duodenum in females.

Increased incidences and severities of chronic active inflammation in the heart occurred in the 500 and 1,000 mg/kg females; increased severity occurred in the 1,000 mg/kg males (Table 4). Minimal hypospermia was observed in the epididymis of males in the 1,000 and 2,000 mg/kg groups; the lesion consisted of decreased amounts of sperm in the lumen of the epididymis. No testicular lesions were observed in these animals.

Dose Selection Rationale for the 3-Month Study: Based on the effects on the hematopoietic system and the early deaths at doses of 500 mg/kg or greater in the 1-month study, doses of 25, 50, 100, and 200 mg/kg were selected for the 3-month study in rats.

3-MONTH STUDY

One male rat in the 25 mg/kg group, one female in the 100 mg/kg group, and four females in the 200 mg/kg group died during the study due to gavage errors (Table 5). Mean body weights of males in the 200 mg/kg group were significantly less than those of the vehicle controls. Mean body weights of females in all dosed groups were similar to those of vehicle controls. Blue staining of the urine, urogenital area, tail, and fur was observed in all dosed groups. The staining resulted from test material in the urine and feces and was not a toxic effect of methylene blue trihydrate administration.

The hematology and clinical chemistry data for rats in the 3-month study of methylene blue trihydrate are listed in Tables 6 and F2. Similar to the 1-month rat study, the primary responses to chemical administration were development of a methemoglobinemia, increased Heinz body formation, and development of a macrocytic, responsive anemia. In this study, the highest dose was 200 mg/kg. A dose-related methemoglobinemia, evidenced by increased methemoglobin concentrations, occurred in all but the 25 mg/kg males at week 1, persisted throughout the study, and involved all dosed groups at month 3. Increased Heinz body formation occurred in the 100 and 200 mg/kg males and females at week 6, persisting to study termination.

TABLE 5
Survival and Body Weights of Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	125 ± 5	330 ± 4	205 ± 4	
25	9/10[c]	125 ± 4	337 ± 7	213 ± 6	102
50	10/10	123 ± 4	325 ± 6	202 ± 5	98
100	10/10	124 ± 5	321 ± 7	197 ± 5	97
200	10/10	125 ± 4	309 ± 6*	183 ± 3**	94
Female					
0	10/10	106 ± 3	198 ± 3	92 ± 2	
25	10/10	105 ± 3	200 ± 4	95 ± 2	101
50	10/10	105 ± 3	197 ± 3	93 ± 2	100
100	9/10[d]	104 ± 3	189 ± 3	86 ± 2	95
200	6/10[e]	105 ± 2	193 ± 4	90 ± 4	97

* Significantly different (P≤0.05) from the vehicle control group by Williams' test
** P≤0.01
[a] Number of animals surviving at 14 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] Week of death: 11
[d] Week of death: 2
[e] Week of deaths: 2, 4, 4, 11

TABLE 6
Selected Hematology Parameters for Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Week 1	9	10	10	9	9
Week 6	8	8	10	10	9
Month 3	10	9	10	10	10
Hematocrit (%)					
Week 1	41.9 ± 0.5	43.0 ± 0.6	42.3 ± 0.6	42.6 ± 0.5	42.2 ± 1.0
Week 6	47.0 ± 0.4	43.5 ± 0.4**	44.1 ± 0.4**	44.4 ± 0.5**	44.5 ± 0.7*
Month 3	47.0 ± 0.5	45.8 ± 0.6	46.5 ± 0.5	46.9 ± 0.4	45.1 ± 0.4*
Hemoglobin (g/dL)					
Week 1	14.0 ± 0.1	14.3 ± 0.2	14.0 ± 0.1	14.2 ± 0.2	13.6 ± 0.3
Week 6	16.5 ± 0.1	15.1 ± 0.1**	15.2 ± 0.2**	15.3 ± 0.2**	15.1 ± 0.1**
Month 3	16.1 ± 0.2	15.8 ± 0.2	15.8 ± 0.2	15.9 ± 0.1	15.3 ± 0.1**
Erythrocytes (10^6/μL)					
Week 1	6.86 ± 0.09	7.04 ± 0.10	6.98 ± 0.07	7.05 ± 0.08	6.76 ± 0.15
Week 6	8.66 ± 0.05	8.02 ± 0.09**	7.94 ± 0.08**	7.72 ± 0.11**	6.80 ± 0.17**
Month 3	8.91 ± 0.08	8.48 ± 0.13**	8.34 ± 0.10**	8.16 ± 0.09**	7.52 ± 0.10**
Reticulocytes (10^5/μL)					
Week 1	3.36 ± 0.18	3.48 ± 0.24	3.86 ± 0.18	3.60 ± 0.30	4.86 ± 0.43**
Week 6	1.64 ± 0.12	2.86 ± 0.22**	3.52 ± 0.14**	4.56 ± 0.29**	8.83 ± 0.86**
Month 3	1.94 ± 0.15	2.92 ± 0.20**	3.38 ± 0.26**	4.22 ± 0.23**	4.93 ± 0.28**
Nucleated erythrocytes/100 leukocytes					
Week 1	0.44 ± 0.18	0.00 ± 0.00	0.60 ± 0.27	0.33 ± 0.17	2.22 ± 0.55*
Week 6	0.00 ± 0.00	0.50 ± 0.27	0.60 ± 0.22*	2.00 ± 0.56**	4.67 ± 1.01**
Month 3	0.20 ± 0.13	0.78 ± 0.32	1.20 ± 0.49	3.00 ± 0.88**	5.30 ± 1.63**
Mean cell volume (fL)					
Week 1	61.1 ± 0.4	61.0 ± 0.3	60.8 ± 0.4	60.6 ± 0.4	62.6 ± 0.3
Week 6	54.3 ± 0.2	54.1 ± 0.4	55.6 ± 0.3**	57.5 ± 0.4**	65.4 ± 1.1**
Month 3	52.9 ± 0.2	54.0 ± 0.2**	55.9 ± 0.3**	57.6 ± 0.2**	59.9 ± 0.5**
Mean cell hemoglobin concentration (g/dL)					
Week 1	33.5 ± 0.2	33.3 ± 0.2	33.2 ± 0.3	33.4 ± 0.2	32.3 ± 0.2**
Week 6	35.2 ± 0.1	34.8 ± 0.2*	34.5 ± 0.2**	34.6 ± 0.2**	34.1 ± 0.4**
Month 3	34.3 ± 0.2	34.4 ± 0.2	34.1 ± 0.1	33.9 ± 0.2	33.9 ± 0.1
Methemoglobin (g/dL)					
Week 1	0.40 ± 0.03[b]	0.46 ± 0.02	0.64 ± 0.03**	0.71 ± 0.05**[b]	0.88 ± 0.04**[b]
Week 6	0.45 ± 0.03	0.51 ± 0.03	0.63 ± 0.04**	0.74 ± 0.04**	0.80 ± 0.04**
Month 3	0.45 ± 0.02	0.57 ± 0.02**	0.66 ± 0.03**	0.80 ± 0.02**	0.84 ± 0.05**
Heinz bodies (%)					
Week 1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 1.4
Week 6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2**	15.2 ± 2.3**
Month 3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.2**	12.7 ± 1.3**

TABLE 6
Selected Hematology Parameters for Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female					
Week 1	10	10	10	10	10
Week 6	9	10	10	10	9
Month 3	10	10	10	9	10
Hematocrit (%)					
Week 1	43.4 ± 0.5	43.6 ± 0.6	43.9 ± 0.3	43.9 ± 0.7	42.2 ± 0.7
Week 6	44.8 ± 0.6	42.8 ± 0.4	43.3 ± 0.4	42.1 ± 0.6*	43.8 ± 0.6
Month 3	46.4 ± 0.6	45.9 ± 0.5	44.6 ± 0.2**	44.2 ± 0.4**	44.4 ± 0.4**
Hemoglobin (g/dL)					
Week 1	14.7 ± 0.1	14.6 ± 0.2	14.7 ± 0.1	14.5 ± 0.2	13.8 ± 0.2
Week 6	15.6 ± 0.2	14.9 ± 0.2*	14.8 ± 0.2**	14.4 ± 0.2**	14.9 ± 0.1**
Month 3	15.7 ± 0.1	15.3 ± 0.2*	15.0 ± 0.1**	14.9 ± 0.1**	14.9 ± 0.2**
Erythrocytes (10^6/μL)					
Week 1	7.06 ± 0.09	7.13 ± 0.11	7.14 ± 0.04	7.22 ± 0.11	6.81 ± 0.14
Week 6	7.69 ± 0.13	7.15 ± 0.07**	7.04 ± 0.07**	6.71 ± 0.10**	6.61 ± 0.09**
Month 3	7.88 ± 0.10	7.51 ± 0.08*	7.26 ± 0.06**	7.07 ± 0.06**	6.93 ± 0.10**
Reticulocytes (10^5/μL)					
Week 1	2.23 ± 0.14	1.97 ± 0.14	2.39 ± 0.09	2.50 ± 0.20	3.81 ± 0.48**
Week 6	1.34 ± 0.10	2.48 ± 0.23**	2.40 ± 0.12**	3.86 ± 0.28**	4.48 ± 0.45**
Month 3	1.42 ± 0.09	2.26 ± 0.19**	2.65 ± 0.25**	3.30 ± 0.14**	4.21 ± 0.26**
Nucleated erythrocytes/100 leukocytes					
Week 1	0.80 ± 0.33	0.20 ± 0.20	0.40 ± 0.22	1.60 ± 0.52	2.90 ± 1.14
Week 6	0.22 ± 0.15	1.00 ± 0.39	1.80 ± 0.55**	3.80 ± 1.27**	2.44 ± 0.97**
Month 3	0.30 ± 0.21	1.30 ± 0.50	1.00 ± 0.33	3.44 ± 1.23**	7.40 ± 2.27**
Mean cell volume (fL)					
Week 1	61.6 ± 0.5	61.3 ± 0.5	61.4 ± 0.3	60.8 ± 0.3	62.2 ± 0.4
Week 6	58.7 ± 0.4	59.9 ± 0.2*	61.7 ± 0.3**	62.9 ± 0.3**	66.3 ± 0.5**
Month 3	59.0 ± 0.2	61.2 ± 0.7**	61.5 ± 0.3**	62.7 ± 0.2**	64.3 ± 0.6**
Mean cell hemoglobin concentration (g/dL)					
Week 1	33.8 ± 0.2	33.4 ± 0.2	33.5 ± 0.3	32.9 ± 0.3*	32.6 ± 0.2**
Week 6	34.7 ± 0.2	34.7 ± 0.1	34.2 ± 0.2*	34.2 ± 0.2*	33.9 ± 0.3*
Month 3	33.8 ± 0.3	33.4 ± 0.4	33.6 ± 0.2	33.7 ± 0.2	33.6 ± 0.3
Methemoglobin (g/dL)					
Week 1	0.36 ± 0.03	0.45 ± 0.03*	0.51 ± 0.02**	0.68 ± 0.04**	0.78 ± 0.03**
Week 6	0.33 ± 0.02	0.46 ± 0.02**	0.57 ± 0.03**	0.63 ± 0.03**	0.83 ± 0.07**
Month 3	0.36 ± 0.02	0.64 ± 0.03**	0.73 ± 0.05**	0.86 ± 0.04**	1.10 ± 0.07**
Heinz bodies (%)					
Week 1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
Week 6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 0.5**	5.0 ± 1.1**
Month 3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.2**	14.0 ± 2.3**

* 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=10

A trend suggesting a developing anemia occurred by week 6, persisted to month 3, and was apparent in all dosed groups. While the hematocrit, hemoglobin, and erythrocyte count values demonstrated a decreased erythron, the erythrocyte count was affected most consistently and in a dose-related fashion. The macrocytosis was evidenced by the increase in mean cell volume. But, unlike the 1-month study, no increase in mean cell hemoglobin concentration occurred, which probably reflected the lower doses used in the 3-month study. A hematopoietic response was indicated by increased numbers of circulating reticulocytes and nucleated erythrocytes. Similar to the 1-month study, increased bile salt concentrations occurred in a dose-related fashion as early as week 6 (males) and at month 3 and may represent some hepatocellular effect. In this study, however, there were no corresponding increases in alanine aminotransferase or sorbitol dehydrogenase activities.

No biologically significant alterations were observed in the reproductive system endpoints of dosed males or females (Tables H1 and H2).

Increases in absolute and relative spleen weights were significant in groups administered 50 mg/kg or greater (Table G2). Increased spleen weights correlated with gross and microscopic findings. Splenic enlargement that was observed at necropsy in 100 mg/kg males and in 200 mg/kg males and females was considered related to methylene blue trihydrate administration. Statistically significant increases occurred in absolute liver weights of 200 mg/kg females, relative liver weights of 100 and 200 mg/kg females, relative liver and testis weights of 50, 100, and 200 mg/kg males, and relative kidney weights of 100 and 200 mg/kg males. Absolute and relative thymus and lung weights of 50, 100, and 200 mg/kg males (except relative lung weight at 100 mg/kg) were significantly less than those of the vehicle controls. Changes in liver, testes, thymus, lung, and kidney weights did not correlate with any microscopic findings.

Chemical-related microscopic lesions were observed in the spleen and bone marrow of males and females (Table 7). Spleen lesions included hematopoietic cell proliferation, congestion, lymphoid depletion of the lymphoid follicles, and capsular fibrosis. Hematopoietic cell proliferation was diagnosed in males and females in groups administered 50 mg/kg or greater. This lesion was characterized by proliferation of hematopoietic cells, predominantly of the erythroid series, in the red pulp of the spleen. Splenic congestion occurred in most dosed males and females and was characterized by enlargement of the spleen as a result of dilated sinusoids filled with blood in the red pulp. The presence of hematopoietic cell proliferation and congestion correlated with the enlarged spleens observed at necropsy. Lymphoid depletion of the lymphoid follicles in the white pulp was diagnosed in males and females in the 100 and 200 mg/kg groups and consisted of loss of lymphocytes from the white pulp resulting in a reduced or indistinct mantle of lymphocytes around the splenic arteries. This lesion was not considered to be a direct toxic effect of methylene blue trihydrate. The incidence of capsular fibrosis was significantly increased in the 200 mg/kg groups. Capsular fibrosis consisted of focal thickening of the splenic capsule resulting from variable amounts of fibrosis and accumulations of mononuclear cells. The incidences of bone marrow hyperplasia were significantly increased in the 50, 100, and 200 mg/kg groups and consisted of an increase in the numbers of both erythroid and myeloid elements of the bone marrow.

Dose Selection Rationale for the 2-Year Study: The dose concentrations selected for the 2-year study were 5, 25, and 50 mg/kg. In the 3-month study, the hematopoietic system was the major target of methylene blue trihydrate induced toxicity. Dose-related increased severity of regenerative anemia was observed in all groups administered methylene blue trihydrate. At the 50 mg/kg concentration, minimal regenerative anemia was observed and it was considered that this concentration would not affect the longevity of rats or cause overt toxicity in a 2-year study. The lowest dose concentration selected was within the range of human therapeutic use of methylene blue trihydrate (HSDB, 2006).

TABLE 7
Incidences of Selected Nonneoplastic Lesions in Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Spleen[a]	10	10	10	10	10
Hematopoietic Cell Proliferation[b]	0	0	10** (2.0)	9** (2.0)	10** (2.0)
Congestion	0	9** (1.6)[c]	10** (2.0)	10** (2.0)	10** (1.9)
Lymphoid Follicle, Depletion Cellular	0	0	0	2 (1.0)	5* (1.4)
Capsule, Fibrosis	1 (1.0)	2 (1.0)	0	1 (1.0)	9** (1.0)
Bone Marrow	10	10	10	10	10
Hyperplasia	0	0	8** (2.0)	10** (2.0)	10** (3.0)
Female					
Spleen	10	10	10	10	10
Hematopoietic Cell Proliferation	0	0	8** (1.9)	9** (2.0)	10** (2.0)
Congestion	0	9** (1.7)	10** (2.0)	9** (2.0)	9** (1.8)
Lymphoid Follicle, Depletion Cellular	0	0	0	1 (2.0)	9** (1.6)
Capsule, Fibrosis	0	0	0	3 (1.0)	7** (1.0)
Bone Marrow	10	10	10	10	10
Hyperplasia	0	0	4* (1.3)	9** (1.8)	10** (2.9)

* 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 8 and in the Kaplan-Meier survival curves (Figure 4). Survival of all dosed groups of rats was similar to that of the vehicle controls.

TABLE 8
Survival of Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Male				
Animals initially in study	50	50	50	50
Accidental deaths[a]	0	0	1	2
Moribund	13	7	5	10
Natural deaths	6	10	5	7
Animals surviving to study termination	31[e]	33	39	31[f]
Percent probability of survival at end of study[b]	62	66	80	65
Mean survival (days)[c]	693	694	711	674
Survival analysis[d]	P=0.750N	P=0.813N	P=0.067N	P=0.957N
Female				
Animals initially in study	50	50	50	50
Accidental deaths[a]	0	0	0	1
Other[a]	0	1	0	0
Moribund	9	13	5	3
Natural deaths	6	4	9	11
Animals surviving to study termination	35	32	36[e]	35
Percent probability of survival at end of study	70	65	72	72
Mean survival (days)	696	697	693	658
Survival analysis	P=0.895N	P=0.806	P=1.000N	P=1.000

[a] Censored from survival analyses
[b] Kaplan-Meier determinations
[c] Mean of all deaths (uncensored, censored, and terminal sacrifice).
[d] The result of the life table trend test (Tarone, 1975) is in the vehicle control column, and the results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns. A negative trend or lower mortality in a dosed group is indicated by **N**.
[e] Includes two animals that died last week of study
[f] Includes one animal that died last week of study

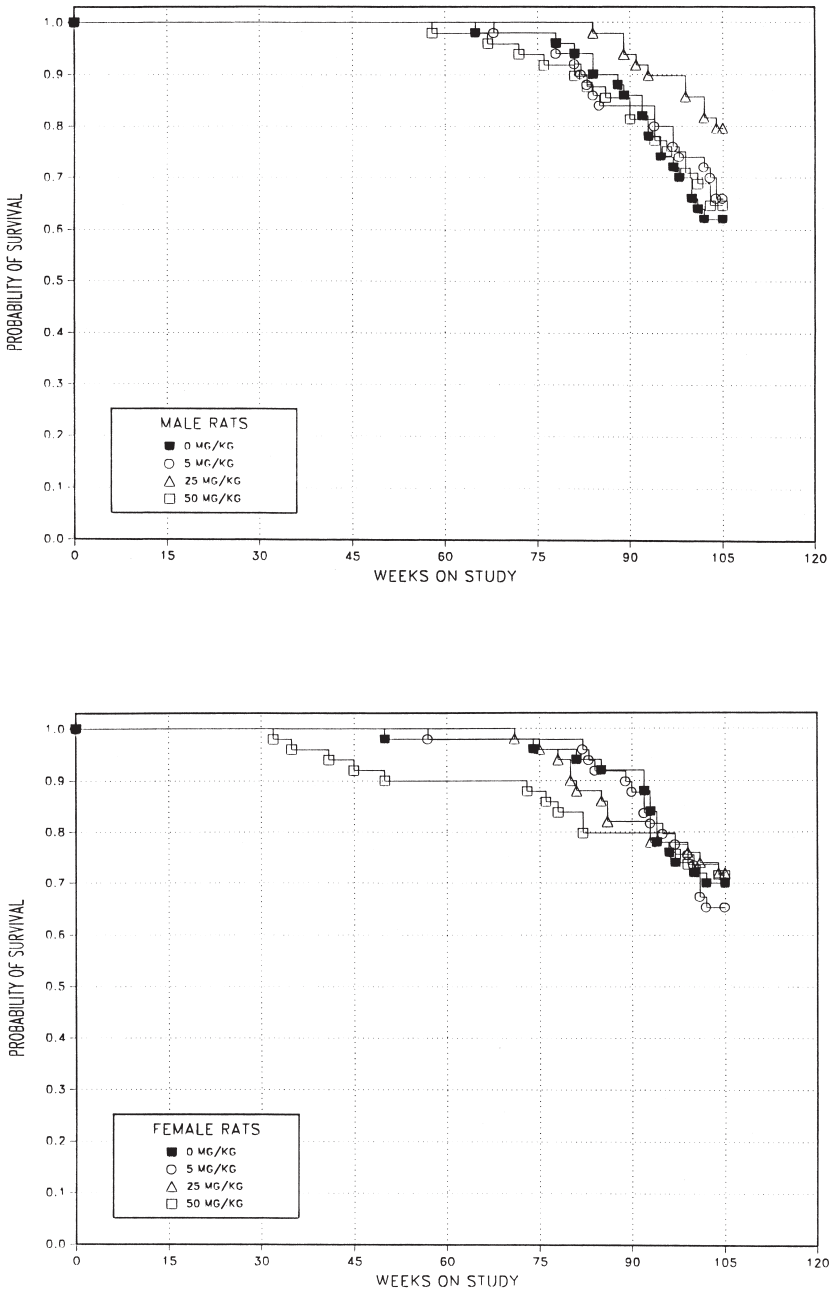


FIGURE 4
Kaplan-Meier Survival Curves for Male and Female Rats Administered Methylene Blue Trihydrate by Gavage for 2 Years

Body Weights and Clinical Findings

Mean body weights of 25 and 50 mg/kg male rats were less than those of the vehicle controls after weeks 29 and 21, respectively; mean body weights of these groups at the end of the study were 91% and 87% that of the vehicle controls, respectively (Table 9 and Figure 5). In the 25 and 50 mg/kg females, mean body weights were less after weeks 73 and 53 and the final mean body weights were 91% and 88% that of the vehicle controls (Table 10 and Figure 5). The incidences of unrelated eye abnormalities were increased in all dosed groups of males and correlated with dose in male rats, but only when the miscellaneous clinical observations were combined (vehicle controls, 2/50; 5 mg/kg, 5/50; 25 mg/kg, 7/50; 50 mg/kg, 8/50). There were no clinical findings related to the administration of methylene blue trihydrate in females, and the relationship of those in males to methylene blue trihydrate was uncertain.

Clinical Pathology

The hematology and urinalysis data for rats in the 2-year study of methylene blue trihydrate are listed in Table F3. As in the 1- and 3-month studies, the primary responses to methylene blue trihydrate administration were the development of a methemoglobinemia, increased Heinz body formation, and development of a macrocytic, responsive anemia. Because the highest dose in this study was 50 mg/kg, the changes were not as dramatic. A dose-related increase in methemoglobin concentrations occurred in the 50 mg/kg males and females at month 3. By month 6, the 25 mg/kg males and females were also affected; both groups remained affected at month 18. The occurrence of Heinz bodies diminished in this study, and increased numbers only occurred in the 25 and 50 mg/kg females at month 18. The severity of decreases in the erythron also diminished in this study. Small decreases in the hematocrit, hemoglobin, and erythrocyte count values occurred fairly consistently in the 25 and 50 mg/kg males and females as early as month 3 and persisted through month 18. A hematopoietic response was indicated by increased numbers of circulating nucleated erythrocytes and/or reticulocytes, and the small increase in mean cell volume probably reflects the increased presence of the larger immature erythrocytes.

Urinary Excretion

Urine was collected over a 24-hour period from five male and five female rats from the core study groups after 3, 12, and 18 months of dosing. Urine samples were sent to an analytical chemistry support contractor where they were analyzed for methylene blue and its metabolites using HPLC-PDA (600 nm). The analyses found that methylene blue, leucomethylene blue, trimethylthionine, and leucotrimethylthionine were present in the urine. Trimethylthionine was present at the highest concentrations, while unchanged methylene blue was present at somewhat lower concentrations. The leuco forms of both substances were present at concentrations approximately equal to their oxidized forms. Concentrations of methylene blue and its metabolites were higher at the 18-month time point compared to the 3-month time point, but no time-dependent increases in urine concentrations were observed for female rats (data not shown) (NIEHS, 2006a,b).

TABLE 9
Mean Body Weights and Survival of Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

Weeks on Study	Vehicle Control		5 mg/kg			25 mg/kg			50 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	111	50	109	99	50	109	99	50	110	99	50
2	150	50	150	100	50	150	100	50	151	101	50
3	181	50	181	100	50	179	99	50	185	102	50
4	206	50	205	100	50	204	99	50	212	103	50
5	230	50	227	99	50	225	98	50	233	101	50
6	249	50	246	99	50	243	98	50	252	102	50
7	264	50	260	98	50	258	98	50	267	101	50
8	280	50	274	98	50	272	97	50	280	100	50
9	293	50	287	98	50	284	97	50	291	100	50
10	306	50	300	98	50	297	97	50	303	99	50
11	317	50	312	99	50	309	97	50	314	99	50
12	328	50	324	99	50	320	97	50	324	99	50
13	338	50	334	99	50	328	97	50	332	98	50
17	368	50	363	99	50	356	97	50	353	96	50
21	391	50	387	99	50	379	97	50	370	95	50
25	407	50	404	99	50	391	96	50	381	94	50
29	424	50	418	99	50	404	95	50	396	94	49
33	441	50	431	98	50	413	94	50	406	92	49
37	454	50	444	98	50	425	94	50	414	91	49
41	468	50	459	98	50	437	94	50	421	90	49
45	477	50	469	98	50	446	94	50	430	90	49
49	489	50	483	99	50	460	94	50	441	90	49
53	495	50	487	99	50	461	93	50	445	90	49
57	503	50	495	99	50	471	94	50	448	89	49
61	499	50	505	101	50	470	94	50	442	89	48
65	509	50	503	99	50	462	91	50	445	87	48
69	514	49	505	98	49	474	92	50	453	88	47
73	519	49	502	97	49	472	91	49	454	88	45
77	519	49	510	98	49	475	92	49	453	87	44
81	521	47	512	98	46	475	91	49	455	87	43
85	512	45	505	99	42	478	93	48	452	88	42
89	514	44	510	99	42	473	92	46	453	88	41
93	506	40	503	99	42	473	94	45	452	89	39
97	507	36	507	100	38	470	93	44	448	88	36
101	500	32	497	100	37	455	91	42	436	87	33
Mean for weeks											
1-13	250		247	99		244	98		250	100	
14-52	435		429	99		412	95		401	92	
53-101	509		503	99		470	92		449	88	

TABLE 10
Mean Body Weights and Survival of Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

Weeks on Study	Vehicle Control		5 mg/kg			25 mg/kg			50 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	96	50	96	101	50	97	102	50	96	101	50
2	118	50	118	100	50	120	102	50	120	102	50
3	131	50	132	101	50	134	103	50	135	104	50
4	141	50	143	102	50	143	102	50	145	103	50
5	151	50	154	102	50	154	102	50	154	102	50
6	160	50	161	101	50	162	102	50	163	102	50
7	166	50	166	100	50	169	102	50	168	101	50
8	170	50	172	102	50	175	103	50	174	103	50
9	174	50	175	101	50	178	102	50	178	102	50
10	177	50	180	102	50	182	103	50	181	102	50
11	182	50	184	101	50	186	102	50	186	102	50
12	184	50	187	102	50	190	103	50	189	103	50
13	187	50	189	101	50	194	104	50	192	103	50
17	197	50	199	101	50	202	103	50	199	101	50
21	209	50	212	102	49	213	102	50	208	99	50
25	211	50	215	102	49	217	103	50	211	100	50
29	215	50	222	103	49	222	103	50	218	101	50
33	226	50	229	101	49	227	100	50	223	98	49
37	234	50	237	101	49	234	100	50	228	97	48
41	244	50	246	101	49	240	98	50	234	96	47
45	250	50	252	101	49	246	98	50	238	95	46
49	258	50	260	101	49	253	98	50	246	96	46
53	266	49	272	102	49	259	98	50	254	96	45
57	278	49	282	102	48	269	97	50	262	94	45
61	288	49	293	102	48	273	95	50	264	92	45
65	294	49	301	103	48	281	96	50	273	93	45
69	303	49	307	101	48	287	95	50	279	92	44
73	309	49	315	102	48	292	95	49	283	92	44
77	318	48	322	101	48	293	92	48	289	91	42
81	321	47	325	101	48	301	94	44	292	91	41
85	328	46	328	100	45	303	92	43	294	90	39
89	331	46	333	101	44	304	92	41	291	88	39
93	335	42	336	101	40	304	91	40	295	88	39
97	336	37	340	101	39	307	92	39	297	88	37
101	336	36	342	102	33	305	91	37	294	88	36
Mean for weeks											
1-13	157		158	101		160	102		160	102	
14-52	227		230	102		228	101		223	98	
53-101	311		315	101		291	94		282	91	

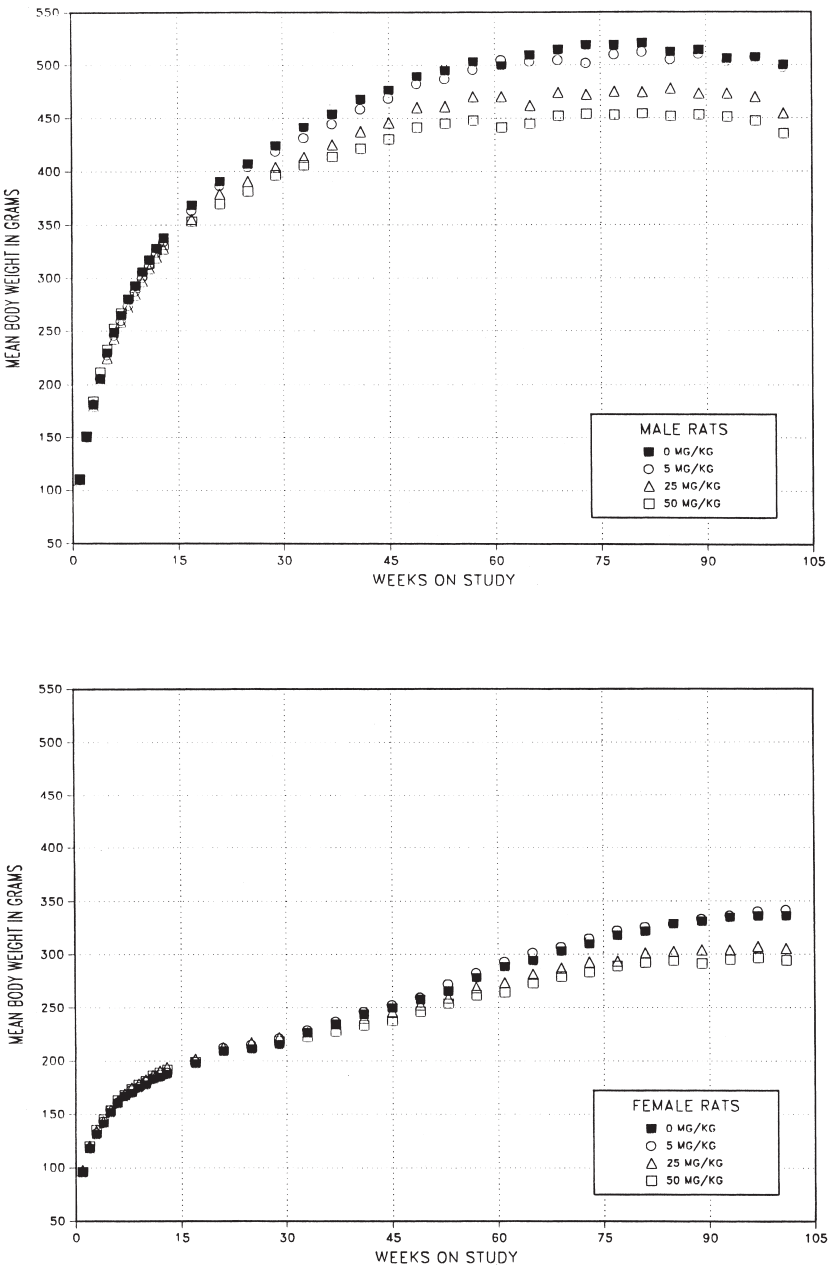


FIGURE 5
Growth Curves for Male and Female Rats
Administered Methylene Blue Trihydrate by Gavage for 2 Years

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of mononuclear cell leukemia and neoplasms and/or nonneoplastic lesions of the pancreatic islets, exocrine pancreas, spleen, mammary gland, and adrenal medulla. Summaries of the incidences of neoplasms and nonneoplastic lesions, 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.

Pancreatic Islets: The incidences of pancreatic islet cell adenoma and adenoma or carcinoma (combined) were increased in male rats. Although the increases in these incidences were only statistically significant in the 25 mg/kg group, the incidences in all dosed groups exceeded those for historical controls (all routes of administration and all vehicles) (Tables 11, A1, A2, and A3). Pancreatic islet cell adenomas were characterized by discrete, well-demarcated, single nodules, 1 to 10 mm in diameter, that often compressed the adjacent acinar tissue and were composed of a monomorphic population of cuboidal to polygonal cells with central round nuclei and vacuolated amphophilic cytoplasm. Islet cell carcinomas were similar to the adenomas with additional features of invasion, cellular anaplasia, and pleomorphism. The incidence of islet cell hyperplasia was significantly increased in the 50 mg/kg males. Affected hyperplastic islets were enlarged with round to oval outlines that could attain a diameter of 500 µm and consisted of either enlarged islet cells or retained

TABLE 11
Incidences of Neoplasms and Nonneoplastic Lesions of the Pancreatic Islets in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Number Examined Microscopically	50	50	50	50
Hyperplasia[a]	13 (1.2)[b]	13 (1.7)	17 (1.8)	26** (1.4)
Adenoma, multiple	0	1	0	1
Adenoma (includes multiple)[c]				
Overall rate[d]	4/50 (8%)	9/50 (18%)	12/50 (24%)	8/50 (16%)
Adjusted rate[e]	9.0%	19.9%	25.0%	18.8%
Terminal rate[f]	1/31 (3%)	5/33 (15%)	9/39 (23%)	4/31 (13%)
First incidence (days)	620	561	619	652
Poly-3 test[g]	P=0.201	P=0.121	P=0.037	P=0.155
Carcinoma[h]	0	0	2	0
Adenoma or Carcinoma[i]				
Overall rate	4/50 (8%)	9/50 (18%)	14/50 (28%)	8/50 (16%)
Adjusted rate	9.0%	19.9%	29.1%	18.8%
Terminal rate	1/31 (3%)	5/33 (15%)	10/39 (26%)	4/31 (13%)
First incidence (days)	620	561	619	652
Poly-3 test	P=0.174	P=0.121	P=0.013	P=0.155

** Significantly different (P≤0.01) from the vehicle control group by the Poly-3 test
[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, all routes, all vehicles (mean ± standard deviation): 66/1,448 (4.8% ± 3.1%), range 0%-10%
[d] Number of animals with neoplasm per number of animals with pancreatic islets examined microscopically
[e] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[f] Observed incidence at terminal kill
[g] Beneath the vehicle control incidence 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: 26/1,448 (2.0% ± 2.7%), range 0%-8%
[i] Historical incidence: 92/1,448 (6.8% ± 4.4%), range 0%-14%

normal cytologic appearances and arrangements. Islet cell hyperplasia, adenoma, and carcinoma are thought to constitute a morphological and biological continuum in the progression of islet cell proliferation. As in this study, islet cell proliferative lesions are observed to occur more frequently in males than females (Riley *et al.*, 1990).

Pancreas: The incidences of acinar cell focal hyperplasia were significantly increased in the 25 and 50 mg/kg males (Tables 12 and A4). Microscopically, focal acinar hyperplasia consisted of focal areas, less than 3 mm in overall diameter, of large, hypertrophic acini having increased cytoplasmic zymogen granules and slightly enlarged nuclei with prominent nucleoli. The increase in the incidences of acinar hyperplasia was not accompanied by significant increases in the incidence of acinar adenomas. Acinar atrophy occurred with significantly decreased incidences in all dosed groups of males and consisted of small, focal areas of acinar cell loss with relative increases in ducts and interstitial connective tissue often containing a mixed inflammatory cell infiltrate. Acinar atrophy is a common background lesion in older rats.

Spleen: The incidences of hematopoietic cell proliferation in dosed rats were greater than those in the vehicle controls, and the incidence in 50 mg/kg males was significantly increased (Tables 12, A4, and B3). The incidences of capsular fibrosis were significantly increased in all dosed groups of males and in 5 and 50 mg/kg females. Splenic capsule fibrosis consisted of one or more small areas of slight thickening of the capsule by mature fibrous connective tissue with collagen deposition that often extended outward from the surface of the spleen; occasionally, this area contained hematopoietic tissue or hemosiderin-laden macrophages. Increased incidences of capsule fibrosis have been previously observed in Fischer rats in studies of aniline and related compounds that damage the bone marrow and spleen (Hall, 1990; Stefanski *et al.*, 1990).

TABLE 12
Selected Nonneoplastic Lesions in Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control		5 mg/kg		25 mg/kg		50 mg/kg	
Male								
Pancreas[a]	50		50		50		50	
Acinus, Hyperplasia, Focal[b]	4	(2.0)[c]	6	(1.7)	15**	(1.9)	12*	(1.8)
Atrophy	43	(2.1)	31**	(1.8)	35*	(2.0)	32*	(1.9)
Spleen	50		50		50		50	
Hematopoietic Cell Proliferation	11	(1.5)	12	(2.0)	17	(1.5)	20*	(1.7)
Capsule, Fibrosis	1	(1.0)	7*	(1.3)	12**	(1.5)	30**	(1.8)
Female								
Spleen	49		48		49		49	
Hematopoietic Cell Proliferation	3	(4.0)	5	(2.4)	7	(2.7)	8	(1.6)
Capsule, Fibrosis	8	(1.0)	17*	(1.1)	12	(1.1)	20**	(1.0)

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

Mononuclear Cell Leukemia: Significantly decreased incidences of mononuclear cell leukemia occurred in all dosed groups of males and in 25 and 50 mg/kg females (Tables 13, A1, A2, B1, and B2). Mononuclear cell leukemia consisted of widely dispersed infiltrations of monomorphic populations of small, round to polygonal cells with central, round nuclei and scant cytoplasm in a variety of organs. Mononuclear cell leukemia was most often present in the bone marrow, spleen, and liver. Decreased incidences of mononuclear cell leukemia have been observed previously in Fischer rats in studies of aniline and related compounds that cause splenic toxicity (Stefanski *et al.*, 1990; Elwell *et al.*, 1996).

Mammary Gland: The incidence of fibroadenoma was significantly decreased in 50 mg/kg female rats; incidences of hyperplasia were significantly decreased in the 25 and 50 mg/kg females (Tables 14, B1, B2, and B3).

Adrenal Medulla: Significant decreases in the incidences of benign pheochromocytoma and benign, complex, or malignant pheochromocytoma (combined) occurred in 25 mg/kg males (Tables 15, A1, and A2).

TABLE 13
Incidences of Mononuclear Cell Leukemia in Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Male				
Overall rate[a,b]	23/50 (46%)	10/50 (20%)	2/50 (4%)	2/50 (4%)
Adjusted rate[c]	48.7%	22.5%	4.3%	4.8%
Terminal rate[d]	12/31 (39%)	7/33 (21%)	1/39 (3%)	2/31 (7%)
First incidence (days)	544	673	690	729 (T)
Poly-3 test[e]	P<0.001N	P=0.006N	P<0.001N	P<0.001N
Female				
Overall rate[f]	12/50 (24%)	6/49 (12%)	3/50 (6%)	2/50 (4%)
Adjusted rate	25.8%	13.4%	6.6%	4.9%
Terminal rate	6/35 (17%)	2/32 (6%)	1/36 (3%)	1/35 (3%)
First incidence (days)	591	585	521	725
Poly-3 test	P=0.004N	P=0.108N	P=0.012N	P=0.007N

(T) Terminal sacrifice
[a] Historical incidence for 2-year studies, all routes, all vehicles (mean ± standard deviation): 622/1,459 (41.4% ± 12.3%), range 22%-68%
[b] Number of animals with neoplasm per number of animals necropsied
[c] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[d] Observed incidence at terminal kill
[e] 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 dose group is indicated by **N**.
[f] Historical incidence: 383/1,459 (26.7% ± 10.5%), range 12%-52%

TABLE 14
Incidences of Neoplasms and Nonneoplastic Lesions of the Mammary Gland in Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Number Necropsied	50	49	50	50
Hyperplasia[a]	18 (2.3)[b]	19 (2.1)	9* (2.0)	7* (2.6)
Fibroadenoma[c]				
Overall rate[d]	28/50 (56%)	30/49 (61%)	28/50 (56%)	17/50 (34%)
Adjusted rate[e]	61.1%	64.9%	60.0%	40.2%
Terminal rate	23/35 (66%)	21/32 (66%)	20/36 (56%)	15/35 (43%)
First incidence (days)	652	568	492	509
Poly-3 test[f]	P=0.013N	P=0.432	P=0.543N	P=0.036N

* Significantly different (P≤0.05) from the vehicle control group by the Poly-3 test
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Number of animals with neoplasm per number of animals necropsied
[d] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[e] Observed incidence at terminal kill
[f] 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**.

TABLE 15
Incidences of Pheochromocytoma of the Adrenal Medulla in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Benign Pheochromocytoma				
Overall rate[a]	9/50 (18%)	13/50 (26%)	2/50 (4%)	3/50 (6%)
Adjusted rate[b]	20.5%	29.1%	4.3%	7.2%
Terminal rate[c]	7/31 (23%)	11/33 (33%)	2/39 (5%)	3/31 (10%)
First incidence (days)	702	576	729 (T)	729 (T)
Poly-3 test[d]	P=0.002N	P=0.244	P=0.018N	P=0.069N
Benign, Complex, or Malignant Pheochromocytoma				
Overall rate	11/50 (22%)	13/50 (26%)	2/50 (4%)	4/50 (8%)
Adjusted rate	24.8%	29.1%	4.3%	9.5%
Terminal rate	8/31 (26%)	11/33 (33%)	2/39 (5%)	4/31 (13%)
First incidence (days)	645	576	729 (T)	729 (T)
Poly-3 test	P=0.002N	P=0.417	P=0.005N	P=0.053N

(T) Terminal sacrifice
[a] Number of animals with neoplasm per number of animals with adrenal medulla examined microscopically
[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[c] Observed incidence at terminal kill
[d] Beneath the vehicle control incidence 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**.

MICE

1-MONTH STUDY

None of the mice in the 500, 1,000, or 2,000 mg/kg groups survived to the end of the study (Table 16). All mice in the 2,000 mg/kg groups died by study day 4; all mice in the 1,000 mg/kg groups died by day 8, and by day 9, all mice in the 500 mg/kg groups had died. In the 250 mg/kg groups, two females died on days 16 and 18 and two males died on days 6 and 13. All mice in the 125 mg/kg groups survived to the end of the study. Mean body weights of surviving dosed mice were similar to those of the vehicle controls. Blue staining of the urogenital area, tail, and fur resulting from excretion of test material in the urine and feces was observed in all dosed groups. Thinness, abnormal respiration, hypothermia, lethargy, ataxia, and ruffled fur were observed in a few animals in the 250 mg/kg groups.

The hematology data for mice in the 1-month study of methylene blue trihydrate are listed in Tables 17 and F4. As in rats, the primary responses to administration of methylene blue trihydrate were development of a methemoglobinemia, increased Heinz body formation, and development of a macrocytic, hyperchromic, responsive anemia. A treatment-related, but not dose-related, methemoglobinemia, evidenced by increased methemoglobin concentrations, occurred in the 125 and 250 mg/kg groups at the end of the study. Regardless of dose or sex, there was an approximate 2.5-fold increase in methemoglobin concentration. Heinz body formation responded similarly to methemoglobin. A dose-related increase in Heinz bodies occurred in the 125 and 250 mg/kg groups at the end of the study. Regardless of dose or sex, anywhere from 22% to 32% of the eryth-rocytes in the treated animals had Heinz bodies, compared to no Heinz bodies observed in erythrocytes of vehicle control animals.

At study termination, a dose-related anemia was apparent in dosed males and females. In the 250 mg/kg animals, the males demonstrated a slightly more severe

TABLE 16
Survival and Body Weights of Mice in the 1-Month Gavage Study of Methylene Blue Trihydrate

Dose Relative to Controls (mg/kg)	Final Weight	Survival[a]	Mean Body Weight[b] (g) Initial	Final	Change (%)
Male					
0	10/10	26.1 ± 0.2	29.4 ± 0.4	3.3 ± 0.4	
125	10/10	25.5 ± 0.3	29.2 ± 0.3	3.7 ± 0.3	99
250	8/10[c]	25.9 ± 0.2	30.1 ± 0.5	4.2 ± 0.5	102
500	0/10[c]	25.9 ± 0.3	—	—	—
1,000	0/10[d]	25.8 ± 0.3	—	—	—
2,000	0/10[d]	26.2 ± 0.3	—	—	—
Female					
0	10/10	20.2 ± 0.2	23.4 ± 0.4	3.2 ± 0.3	
125	10/10	20.2 ± 0.3	23.5 ± 0.3	3.2 ± 0.2	100
250	8/10[e]	20.7 ± 0.4	23.6 ± 0.4	2.9 ± 0.4	101
500	0/10[c]	20.9 ± 0.2	—	—	—
1,000	0/10[c]	20.7 ± 0.3	—	—	—
2,000	0/10[d]	20.7 ± 0.3	—	—	—

[a] Number of animals surviving at 1 month/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 deaths: 1, 2
[d] Week of deaths: 1
[e] Week of deaths: 3

TABLE 17
Selected Hematology Parameters for Mice in the 1-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	125 mg/kg	250 mg/kg
n	10	10	8
Male			
Hematocrit (%)	50.4 ± 0.7	35.1 ± 1.3**	24.5 ± 2.3**
Hemoglobin (g/dL)	17.0 ± 0.2	14.4 ± 0.3**	10.2 ± 1.2**
Erythrocytes (10^6/μL)	9.91 ± 0.13	6.00 ± 0.34**	3.62 ± 0.52**
Reticulocytes (10^6/μL)	0.20 ± 0.02	0.53 ± 0.05**	0.59 ± 0.12**
Nucleated erythrocytes (10^3/μL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00[b]
Mean cell volume (fL)	50.9 ± 0.1	59.1 ± 1.4**	72.0 ± 4.5**
Mean cell hemoglobin concentration (g/dL)	33.7 ± 0.3	41.4 ± 1.2**	41.2 ± 1.4**
Methemoglobin (g/dL)	0.13 ± 0.04	0.34 ± 0.05**	0.31 ± 0.04**
Heinz bodies (%)	0.0 ± 0.0	32.4 ± 2.8**	25.9 ± 3.3**
Female			
Hematocrit (%)	47.7 ± 0.7	39.0 ± 0.8**	30.9 ± 1.6**
Hemoglobin (g/dL)	16.5 ± 0.2	15.5 ± 0.2**	12.3 ± 0.5**
Erythrocytes (10^6/μL)	9.36 ± 0.16	6.79 ± 0.18**	4.34 ± 0.30**
Reticulocytes (10^6/μL)	0.21 ± 0.01	0.74 ± 0.08**	0.99 ± 0.11**
Nucleated erythrocytes (10^3/μL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	51.1 ± 0.3	57.6 ± 0.6**	71.9 ± 2.4**
Mean cell hemoglobin concentration (g/dL)	34.5 ± 0.3	39.8 ± 0.5**	39.9 ± 0.6**
Methemoglobin (g/dL)	0.11 ± 0.02	0.28 ± 0.03**	0.28 ± 0.05**
Heinz bodies (%)	0.0 ± 0.0	26.4 ± 2.1**	22.0 ± 2.8**

** Significantly different ($P \leq 0.01$) from the vehicle control group by Shirley's test
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data. No data presented for the 500, 1,000, and 2,000 mg/kg groups due to 100% mortality.
[b] n=7

erythron change than did the females, evidenced by decreases in the hematocrit (51% vs. 35%), hemoglobin (40% vs. 26%), and erythrocyte counts (64% vs. 54%) of males compared to females. Also, the mice in the 250 mg/kg groups had more severe erythron changes than the 500 mg/kg groups in the 1-month study. The macrocytosis was evidenced by increased mean cell volume, and the hyperchromia was indicated by increased mean cell hemoglobin concentration. A hematopoietic response was indicated by increased numbers of circulating reticulocytes.

Significant increases in absolute and relative spleen weights occurred in all surviving dosed groups of mice compared to vehicle controls (Table G3). Significant decreases occurred in the absolute and relative thymus weights of 250 mg/kg males and females and in the relative thymus weights of 125 mg/kg females. Significant decreases occurred in absolute lung weights of 125 and 250 mg/kg males, relative lung weights of 250 mg/kg males, and absolute lung weight of 250 mg/kg females. The absolute and relative heart weights of 125 and 250 mg/kg females were significantly increased, as was the relative heart weight of 250 mg/kg males. Finally, increases in the absolute and relative kidney weights of 250 mg/kg females and decreases in the absolute liver weight of 250 mg/kg males were also significant.

Splenic enlargement was observed in males and females at necropsy in all dosed groups except the 1,000 mg/kg males and 2,000 mg/kg males and females (all animals in the 2,000 mg/kg groups died by day 4). At necropsy, all animals had intense blue discoloration of tissues. Fluid-filled thoracic cavities were observed in five males and one female in the 2,000 mg/kg groups.

Dose-related lesions were observed in multiple organs. The nature and extent of the lesions and the number

of organs affected differed depending somewhat on the duration of survival. Histopathologic lesions were observed in the spleen, liver, thymus, lymph nodes, bone marrow, kidneys, forestomach, heart, and urinary bladder of males and females (Table 18).

Spleen lesions associated with methylene blue trihydrate administration included hematopoietic cell proliferation correlated with the enlarged spleens observed at necropsy (Table 18). This lesion was characterized by proliferation of hematopoietic cells, predominantly of the erythroid series, in the red pulp of the spleen. Pigment was observed in the spleen of most dosed mice. Pigment in the spleen was characterized by the presence of numerous macrophages containing golden-brown refractile granules in the red pulp and was confirmed as hemosiderin. The pigment accumulation was associated with erythrophagocytosis in all dosed groups and was characterized by the presence of one to multiple red blood cells within macrophages in the red pulp. Pigment granules and red blood cells were sometimes present within the same cell. Both erythrophagocytosis and pigment accumulation were the result of red blood cell destruction. The incidences of congestion of the spleen were significantly increased in males in the 1,000 and 2,000 mg/kg groups and females in the 2,000 mg/kg group. The congestion was characterized by enlargement of the spleen and dilated sinusoids packed with red blood cells in the red pulp. Lymphoid follicle depletion occurred in most dosed mice. This lesion consisted of necrosis and a loss of lymphocytes from the white pulp resulting in a reduced or indistinct mantle of lymphocytes around the splenic arteries.

Liver lesions associated with methylene blue trihydrate administration included periportal degeneration, necrosis (in animals that died early), hematopoietic cell proliferation, and Kupffer cell pigmentation with erythrophagocytosis (Table 18). Periportal degeneration was characterized by the presence of numerous small, clear vacuoles in the cytoplasm of the hepatocytes in the periportal region of the liver. Some vacuoles appeared to have a faint pink, finely granular material within them. The necrotic lesions included a spectrum of changes: from individual cell necrosis of hepatocytes that was diffusely present throughout the liver and had a tendency to be more severe in the periportal regions to vacuolar degeneration and coagulation necrosis of numerous hepatocytes in periportal tissues. Individual cell necrosis was characterized by swelling and rounding up of individual hepatocytes with separation from adjacent hepatocytes. The cytoplasm was homogenous, intensely eosinophilic, or slightly basophilic. Nuclei were often pyknotic. The necrosis was consistent with hypoxia resulting from the anemia. Hematopoietic cell proliferation was observed in all dosed groups except 2,000 mg/kg males that died during the first week of the study. This lesion consisted of small foci of hematopoietic cells, predominantly erythroid precursors, in the sinusoids scattered throughout the liver. Kupffer cell lesions of pigment accumulation and erythrophagocytosis were observed in mice in all dosed groups. The pigment consisted of golden to brown granules that were confirmed to be hemosiderin deposition. In addition to pigment, Kupffer cells often contained one to multiple red blood cells (erythrophagocytosis). Pigment granules and red blood cells were sometimes present within the same cell. These liver lesions are consistent with anemia and red blood cell destruction. A few dosed mice had periportal pigmentation of hepatocytes characterized by fine blue granules in multiple small cytoplasmic vacuoles. This blue pigment was distinctly different from the golden to brown granules of hemosiderin and could have been either methylene blue trihydrate or one of its metabolites.

The incidences of necrosis of the thymus were significantly increased in all dosed groups of mice (Table 18). This lesion consisted of a loss of lymphocytes in the cortex and medulla of the thymus. There was increase in the number of lymphocytes, as well as frank necrosis of the lymphocytes, and necrosis was indicated by the presence of numerous pyknotic and karyorrhectic lymphocyte nuclei and the presence of cellular fragments with macrophages.

The incidences of necrosis of the mandibular and mesenteric lymph nodes were significantly increased in 500 mg/kg or greater groups of mice (Table 18). The severity of these lesions tended to increase with increasing dose concentration. Necrosis consisted of individual lymphocytes in the cortex and was characterized by the presence of numerous macrophages in the lymphoid tissues of the cortex, resulting in a “starry sky” appearance. Pyknotic and karyorrhectic nuclear fragments of lymphocytes were present within the cytoplasm of the macrophages.

Pigment accumulation with erythrophagocytosis was diagnosed in the bone marrow of most mice in all dosed groups (Table 18). The pigment consisted of yellow to golden-brown granules within the cytoplasm of the

TABLE 18
Incidences of Selected Nonneoplastic Lesions in Mice in the 1-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg	1,000 mg/kg	2,000 mg/kg
Male						
Spleen[a]	10	10	10	10	10	10
Red Pulp, Hematopoietic Cell Proliferation[b]	1 (1.0)[c]	10** (2.8)	8** (3.4)	10** (3.4)	5 (2.2)	5 (1.6)
Pigmentation	0	10** (2.8)	9** (2.6)	10** (3.2)	9** (3.4)	10** (2.9)
Congestion	0	0	0	0	6** (1.8)	4* (2.0)
Lymphoid Follicle, Depletion Cellular	0	9** (2.1)	9** (3.1)	10** (3.0)	10** (2.6)	10** (2.1)
Liver	10	10	10	10	10	10
Periportal Degeneration	0	6** (1.0)	5* (1.0)	3 (1.0)	3 (1.7)	1 (2.0)
Necrosis	0	0	0	0	4* (1.0)	3 (3.0)
Hematopoietic Cell Proliferation	0	5* (1.0)	6** (1.0)	8** (1.6)	1 (1.0)	0
Kupffer Cell Pigmentation	0	9** (2.2)	10** (3.0)	10** (3.4)	10** (2.4)	9** (2.2)
Periportal Pigmentation	0	0	0	1 (1.0)	2 (1.5)	0
Thymus	10	10	10	10	9	10
Necrosis	0	7** (1.0)	9** (2.2)	10** (3.0)	8** (3.8)	8** (2.5)
Lymph Node, Mandibular	10	9	9	9	8	8
Necrosis	0	0	1 (1.0)	4* (1.3)	4* (1.8)	4* (1.5)
Lymph Node, Mesenteric	10	10	10	9	9	10
Necrosis	0	7** (1.1)	4* (1.0)	8** (1.6)	7** (1.6)	7** (2.3)
Bone Marrow	10	10	10	10	10	10
Pigmentation	0	10** (2.1)	10** (2.7)	10** (3.0)	10** (2.8)	7** (3.1)
Kidney	10	10	10	10	9	10
Renal Tubule, Pigmentation	0	0	4* (2.0)	5* (2.2)	5* (1.6)	3 (3.2)
Renal Tubule, Necrosis	0	0	2 (2.0)	1 (2.0)	2 (2.0)	2 (2.0)
Renal Tubule, Casts Protein	0	0	0	0	5* (1.2)	3 (1.7)
Stomach, Forestomach	10	10	10	10	10	10
Ulcer	0	0	0	2 (2.0)	6** (2.5)	0
Inflammation, Chronic Active	0	0	0	2 (2.0)	0	0
Hyperplasia, Focal, Squamous	0	0	1 (3.0)	2 (3.0)	1 (2.0)	0
Heart	10	10	10	10	10	10
Mineralization	0	2 (1.0)	4* (2.3)	0	0	0
Urinary Bladder	10	10	10	9	8	10
Muscularis Degeneration	0	0	0	7** (2.4)	0	1 (1.2)
Transitional Epithelium, Degeneration	0	0	5* (1.0)	9** (1.1)	8** (1.1)	9** (1.0)
Transitional Epithelium, Pigmentation	0	0	4* (2.0)	9** (2.4)	3 (2.7)	7** (2.6)

TABLE 18
Incidences of Selected Nonneoplastic Lesions in Mice in the 1-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg	1,000 mg/kg	2,000 mg/kg
Female						
Spleen	10	10	10	10	10	10
Red Pulp, Hematopoietic Cell Proliferation	0	10** (3.1)	10** (4.0)	10** (3.9)	10** (3.6)	0
Pigmentation	0	10** (2.1)	10** (2.1)	10** (3.2)	10** (3.0)	8** (2.5)
Congestion	0	0	0	0	0	9** (2.3)
Lymphoid Follicle, Depletion Cellular	0	10** (2.0)	10** (3.0)	10** (3.3)	10** (3.0)	10** (2.6)
Liver	10	10	10	10	10	10
Periportal Degeneration	0	6** (1.3)	0	6** (1.5)	1 (2.0)	0
Necrosis	0	0	0	0	3 (2.0)	6** (2.2)
Hematopoietic Cell Proliferation	2 (1.0)	7* (1.0)	10** (1.8)	10** (1.6)	9** (1.6)	2 (1.0)
Kupffer Cell Pigmentation	0	10** (1.6)	10** (3.7)	10** (3.7)	10** (2.8)	10** (2.1)
Periportal Pigmentation	0	0	0	1 (1.0)	1 (3.0)	0
Thymus	10	10	10	10	10	10
Necrosis	0	6** (1.2)	9** (2.0)	10** (3.6)	10** (3.3)	10** (3.0)
Lymph Node, Mandibular	10	10	9	8	8	10
Necrosis	0	1 (1.0)	1 (1.0)	4* (1.3)	5** (1.8)	8** (1.9)
Lymph Node, Mesenteric	10	10	9	10	8	9
Necrosis	0	5* (1.8)	2 (1.0)	7** (1.6)	7** (1.9)	9** (2.3)
Bone Marrow	10	10	10	10	10	10
Pigmentation	0	9** (1.8)	10** (2.8)	10** (2.5)	10** (2.8)	9** (1.8)
Kidney	10	10	10	10	10	10
Renal Tubule, Pigmentation	0	0	4* (1.5)	9** (2.2)	8** (2.5)	7** (2.3)
Renal Tubule, Necrosis	0	0	0	3 (1.7)	6** (2.0)	3 (1.7)
Renal Tubule, Casts, Protein	0	0	0	1 (2.0)	4* (1.3)	3 (2.7)
Stomach, Forestomach	10	10	10	10	9	10
Ulcer	0	1 (1.0)	0	3 (2.0)	4* (2.3)	2 (3.5)
Inflammation, Chronic, Active	0	0	0	5* (2.2)	3 (2.0)	0
Hyperplasia, Focal, Squamous	0	0	0	5* (3.0)	3 (3.3)	0
Heart	10	10	10	10	10	10
Myocardium, Necrosis, Acute	0	0	0	3 (1.0)	6** (1.2)	6** (1.2)
Urinary Bladder	9	10	9	10	10	10
Transitional Epithelium, Degeneration	0	0	6** (1.0)	9** (1.0)	8** (1.0)	8** (1.0)
Transitional Epithelium, Pigmentation	0	0	3 (1.3)	7** (2.0)	7** (1.7)	2 (1.5)

* 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

macrophages in the sinusoids of the bone marrow and was consistent with hemosiderin. The pigment accumulation was associated with erythrophagocytosis characterized by the presence of one to multiple red blood cells in macrophages. Erythrophagocytosis predominated in the higher dose animals that died early in the study, whereas pigment accumulation predominated in animals that survived to the end of the study. Pigment granules and red blood cells were sometimes present within the same cells. Pigment accumulation and erythrophagocytosis were the result of red blood cell destruction.

Kidney lesions consisted of renal tubule pigmentation, necrosis, and protein casts (Table 18). Pigmentation of renal tubules was observed in males and females in the 250 mg/kg or greater groups. Microscopically, the pigment in the renal tubules of the 500 mg/kg or greater groups was characterized by reddish-brown droplets and granules in the cytoplasm of proximal tubular epithelial cells and in the lumen of the proximal and distal convoluted tubules. The reddish-brown droplets and granules resembled hemoglobin, but this was not confirmed by special stains. In mice in the 250 mg/kg groups, pigment was more finely granular, dark brown to golden, and located within the cytoplasm of the proximal convoluted tubules. The fine, golden to brown granules were confirmed as hemosiderin. Renal tubule necrosis was diagnosed in early death animals and was characterized by sloughing of the epithelium in focal areas of the proximal convoluted tubules with cellular fragments and pyknotic nuclei present in the lumen. A few mice in the 500 mg/kg or greater groups had protein casts characterized by the presence of a homogenous pale pink to reddish material in the tubule lumens. The pigment, casts, and necrosis in the 500 mg/kg or greater groups are consistent with hemoglobin nephrosis resulting from red blood cell damage and hemolysis. The fine granules of hemosiderin in renal tubules in the 250 mg/kg mice that survived to the end of the study are consistent with a moderate hemolytic anemia.

Significantly increased incidences of forestomach lesions that were related to methylene blue trihydrate administration were observed in female mice administered 500 or 1,000 mg/kg and male mice administered 1,000 mg/kg; these lesions included focal ulcer, inflammation, and squamous hyperplasia (Table 18). Focal ulcer was diagnosed when there was focal loss of the squamous epithelium of the forestomach with an infiltrate of inflammatory cells in the underlying submucosa and usually hyperplasia of the epithelium at the margins of the defect. Chronic active inflammation consisted of focal inflammatory cell infiltrates of polymorphonuclear leukocytes and mononuclear cells in the submucosa, with an intact epithelium overlying the lesion. Squamous hyperplasia of the forestomach was characterized by thickening of the surface epithelium, a result of multiple cell layers that were often thrown into folds, and associated with chronic active inflammation.

Mineralization of the heart was diagnosed in a few dosed males in groups that survived to the end of the study, and acute myocardial necrosis occurred in females in the 500 mg/kg or greater groups (Table 18). Acute myocardial necrosis consisted of a focal lesion in the ventricular myocardium with swelling of the myocardium fibers, loss of striations, and an intensely eosinophilic cytoplasm. The nuclei were pyknotic and located at the periphery of the myocyte. Clear cytoplasmic vacuoles were present in a few cells.

Urinary bladder lesions consisted of pigmentation and/or degeneration of the transitional epithelium in groups of males and females administered 250 mg/kg or greater and degeneration in the muscularis of most 500 mg/kg males and one 2,000 mg/kg male (Table 18). Degeneration of transitional epithelium was characterized by the presence of one or multiple clear vacuoles within the cytoplasm of the superficial epithelial cells. Occasionally, these cells sloughed into the lumen. In these same groups, pigment was often present in the transitional epithelium associated with small cytoplasmic vacuoles and consisted of fine blue granules within the cytoplasm of superficial epithelial cells. The blue pigment was presumed to be either methylene blue trihydrate or a derivative. Degeneration of urinary bladder smooth muscle was characterized by swelling and eosinophilia of the muscularis. In more severe lesions, there was dropout of muscle fibers with fibrosis, infiltration by mononuclear cells, and mineralization.

Dose Selection Rationale for the 3-Month Study: Because of the effects on the hematopoietic system and the early deaths at doses of 250 mg/kg or greater, doses of 25, 50, 100, and 200 mg/kg were selected for the 3-month study in mice.

3-MONTH STUDY

One 200 mg/kg male and one 25 mg/kg female died early due to gavage accidents; all other mice survived to the end of the study (Table 19). Mean body weights of all dosed groups were similar to or only slightly less than those of the vehicle control groups. Blue staining of the urine, urogenital area, tail, and fur was observed in all dosed groups. The staining was not considered to be a toxic effect but resulted from test material in the urine and feces.

The hematology and clinical chemistry data for mice in the 3-month study of methylene blue trihydrate are listed in Tables 20 and F5. Similar to the 1-month mouse study, the responses to administration of methylene blue trihydrate were the development of a methemoglobinemia, increased Heinz body formation, and development of a macrocytic, hyperchromic, responsive anemia. In this study, the highest dose was 200 mg/kg. A dose-related methemoglobinemia, evidenced by increased methemoglobin concentrations, occurred in all dosed groups at week 1 and persisted throughout the study. Increased Heinz body formation followed the methemoglobin concentrations, demonstrating increases in all dosed groups at all time points.

A dose-related anemia occurred in all dosed groups starting at week 1 and persisting to month 3. The hematocrit, hemoglobin, and erythrocyte counts demonstrated the decreased erythron. The macrocytosis and hyperchromia were evidenced by increases in mean cell volume and mean cell hemoglobin concentration, respectively. A hematopoietic response was indicated by increased numbers of circulating reticulocytes. At month 3, minimal increases in bile salt concentrations occurred in the 100 and 200 mg/kg females and may represent some hepatocellular effect; there were no corresponding alterations in other markers of hepatic injury.

Absolute and relative spleen weights of 100 and 200 mg/kg males and 50 mg/kg or greater females were significantly greater than those of the vehicle control groups; the absolute spleen weight of 50 mg/kg males was also increased (Table G4). Relative kidney weight and absolute and relative heart weights were significantly increased in 200 mg/kg males. In females, there were

TABLE 19
Survival and Body Weights of Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	24.1 ± 0.4	35.4 ± 0.9	11.3 ± 0.7	
25	10/10	24.4 ± 0.4	36.2 ± 1.1	11.9 ± 1.2	102
50	10/10	24.3 ± 0.4	36.9 ± 1.3	12.6 ± 1.0	104
100	10/10	24.2 ± 0.3	35.1 ± 0.8	10.9 ± 0.6	99
200	9/10[c]	24.4 ± 0.4	33.2 ± 0.5	8.8 ± 0.7	94
Female					
0	10/10	19.8 ± 0.2	28.4 ± 0.8	8.7 ± 0.8	
25	9/10[d]	19.5 ± 0.4	29.3 ± 1.2	10.0 ± 0.9	103
50	10/10	19.2 ± 0.3	29.4 ± 0.7	10.2 ± 0.6	103
100	10/10	19.2 ± 0.3	28.1 ± 0.8	8.9 ± 0.7	99
200	10/10	19.4 ± 0.2	27.5 ± 0.5	8.1 ± 0.3	97

[a] Number of animals surviving at 1 month/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: 3
[d] Week of death: 2

TABLE 20
Selected Hematology Parameters for Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Week 1	10	10	10	10	10
Week 6	10	10	10	10	8
Month 3	9	10	9	10	9
Hematocrit (%)					
Week 1	50.8 ± 0.6	47.8 ± 0.5**	47.0 ± 0.9**	40.2 ± 0.6**	32.3 ± 0.7**
Week 6	46.6 ± 0.4	43.3 ± 0.9*	40.8 ± 0.4**	36.4 ± 1.0**	28.4 ± 1.7**
Month 3	48.1 ± 0.7	45.7 ± 0.9*	43.2 ± 0.7**	38.7 ± 0.5**	27.3 ± 0.9**
Hemoglobin (g/dL)					
Week 1	16.2 ± 0.2	15.4 ± 0.1**	15.3 ± 0.2**	15.3 ± 0.3**	14.8 ± 0.4**
Week 6	15.3 ± 0.1	14.7 ± 0.3	14.9 ± 0.2	14.8 ± 0.4	11.4 ± 0.6**
Month 3	15.9 ± 0.2	15.6 ± 0.2	16.5 ± 0.2	15.6 ± 0.2	11.4 ± 0.3**
Erythrocytes (10^6/μL)					
Week 1	10.06 ± 0.11	9.52 ± 0.11**	9.37 ± 0.19**	7.91 ± 0.11**	6.17 ± 0.15**
Week 6	9.47 ± 0.09	8.73 ± 0.21**	7.94 ± 0.07**	6.83 ± 0.17**	5.15 ± 0.31**
Month 3	9.83 ± 0.12	9.21 ± 0.17*	8.69 ± 0.14**	7.77 ± 0.11**	5.25 ± 0.21**
Reticulocytes (10^5/μL)					
Week 1	2.23 ± 0.19	2.29 ± 0.12	2.91 ± 0.18*	4.65 ± 0.61**	6.58 ± 0.49**
Week 6	2.19 ± 0.12	2.89 ± 0.23*	4.26 ± 0.34**	6.57 ± 0.50**	5.29 ± 0.82**
Month 3	2.37 ± 0.07	3.75 ± 0.33**	4.51 ± 0.29**	7.01 ± 0.42**	8.03 ± 1.43**
Nucleated erythrocytes/100 leukocytes					
Week 1	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.10
Week 6	0.00 ± 0.00	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Month 3	0.00 ± 0.00[b]	0.00 ± 0.00	0.11 ± 0.11	0.20 ± 0.13	3.00 ± 1.49**
Mean cell volume (fL)					
Week 1	50.4 ± 0.2	50.3 ± 0.2	50.3 ± 0.3	50.9 ± 0.2	52.5 ± 0.3**
Week 6	49.1 ± 0.2	49.6 ± 0.2	51.2 ± 0.1**	53.4 ± 0.3**	55.1 ± 0.6**
Month 3	48.8 ± 0.2	49.6 ± 0.2**	49.7 ± 0.2**	49.8 ± 0.3**	52.4 ± 1.1**
Mean cell hemoglobin concentration (g/dL)					
Week 1	32.0 ± 0.2	32.2 ± 0.2	32.6 ± 0.2*	38.1 ± 1.0**	46.0 ± 1.3**
Week 6	32.8 ± 0.3	34.0 ± 0.2**	36.7 ± 0.4**	40.7 ± 0.6**	40.3 ± 0.6**
Month 3	33.0 ± 0.1	34.2 ± 0.4*	38.2 ± 0.5**	40.5 ± 0.8**	41.9 ± 0.6**
Methemoglobin (g/dL)					
Week 1	0.24 ± 0.02	0.42 ± 0.03**	0.72 ± 0.04**	0.78 ± 0.04**[c]	0.86 ± 0.05**
Week 6	0.21 ± 0.01	0.44 ± 0.02**	0.54 ± 0.05**	0.60 ± 0.05**	0.61 ± 0.07**
Month 3	0.19 ± 0.01[b]	0.51 ± 0.02**	0.57 ± 0.04**[b]	0.60 ± 0.04**	0.60 ± 0.04**
Heinz bodies (%)					
Week 1	0.0 ± 0.0	6.2 ± 2.0**	37.6 ± 2.9**	89.0 ± 1.1**	88.6 ± 0.9**
Week 6	0.0 ± 0.0	20.5 ± 2.7**	37.4 ± 1.4**	89.3 ± 0.6**	89.3 ± 1.6**
Month 3	0.0 ± 0.0[b]	24.8 ± 2.0**	51.5 ± 3.5**	90.2 ± 0.6**	84.6 ± 2.3**

TABLE 20
Selected Hematology Parameters for Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female					
Week 1	9	10	10	10	8
Week 6	10	10	10	10	10
Month 3	10	8	10	10	10
Hematocrit (%)					
Week 1	48.1 ± 0.5	46.9 ± 0.8	44.3 ± 0.4**	39.9 ± 0.7**	31.5 ± 0.8**
Week 6	48.4 ± 0.8	43.8 ± 0.5**	42.4 ± 0.5**	40.8 ± 0.8**	34.0 ± 1.3**
Month 3	47.9 ± 0.6	44.7 ± 0.5**	41.5 ± 0.4**	38.4 ± 0.7**	30.7 ± 0.6**
Hemoglobin (g/dL)					
Week 1	15.7 ± 0.2	15.1 ± 0.2*	14.6 ± 0.1**	14.8 ± 0.3**	14.6 ± 0.3**
Week 6	16.2 ± 0.2	14.8 ± 0.2**	15.1 ± 0.1**	15.9 ± 0.3**	13.3 ± 0.4**
Month 3	15.9 ± 0.1	14.9 ± 0.2**	14.9 ± 0.2**	15.2 ± 0.2**	12.5 ± 0.3**
Erythrocytes (10^6/µL)					
Week 1	9.63 ± 0.10	9.42 ± 0.16	8.85 ± 0.09**	7.86 ± 0.09**	5.90 ± 0.17**
Week 6	9.72 ± 0.18	8.61 ± 0.10**	8.18 ± 0.11**	7.82 ± 0.16**	6.45 ± 0.27**
Month 3	9.73 ± 0.13	9.06 ± 0.09**	8.45 ± 0.08**	7.59 ± 0.15**	5.78 ± 0.12**
Reticulocytes (10^5/µL)					
Week 1	1.58 ± 0.07	1.92 ± 0.21	2.65 ± 0.17**	4.37 ± 0.79**	6.50 ± 0.44**[d]
Week 6	2.01 ± 0.25	2.99 ± 0.26**	4.01 ± 0.46**	5.73 ± 0.50**	9.86 ± 0.76**
Month 3	2.16 ± 0.17	2.70 ± 0.20	4.90 ± 0.17**	7.52 ± 0.77**	14.26 ± 1.31**
Nucleated erythrocytes/100 leukocytes					
Week 1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.16*
Week 6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Month 3	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.10	0.00 ± 0.00	0.30 ± 0.15
Mean cell volume (fL)					
Week 1	50.1 ± 0.2	49.8 ± 0.2	50.1 ± 0.1	50.8 ± 0.4	53.6 ± 0.5**
Week 6	49.8 ± 0.3	51.1 ± 0.3**	52.0 ± 0.2**	52.3 ± 0.3**	52.7 ± 0.5**
Month 3	49.3 ± 0.2	49.3 ± 0.3	49.2 ± 0.3	50.6 ± 0.3**	53.3 ± 0.7**
Mean cell hemoglobin concentration (g/dL)					
Week 1	32.8 ± 0.2	32.2 ± 0.2	33.0 ± 0.2	37.2 ± 0.4**	46.3 ± 0.8**
Week 6	33.6 ± 0.2	33.9 ± 0.1	35.6 ± 0.3**	39.0 ± 0.5**	39.2 ± 0.5**
Month 3	33.3 ± 0.3	33.3 ± 0.2	36.0 ± 0.5**	39.6 ± 0.4**	40.8 ± 0.8**
Methemoglobin (g/dL)					
Week 1	0.29 ± 0.01[b]	0.35 ± 0.05	0.56 ± 0.06**[c]	0.84 ± 0.07**[c]	0.98 ± 0.08**
Week 6	0.27 ± 0.02	0.50 ± 0.02**	0.46 ± 0.03**	0.64 ± 0.05**	0.56 ± 0.07**
Month 3	0.26 ± 0.03	0.54 ± 0.03**	0.64 ± 0.03**	0.67 ± 0.06**	0.42 ± 0.04
Heinz bodies (%)					
Week 1	0.0 ± 0.0	1.1 ± 0.6*	26.1 ± 5.0**	77.9 ± 5.3**	87.5 ± 0.6**[d]
Week 6	0.0 ± 0.0	13.8 ± 1.4**	26.8 ± 3.2**	68.0 ± 7.2**	84.4 ± 1.2**
Month 3	0.0 ± 0.0	18.7 ± 4.7**	51.9 ± 3.1**	80.1 ± 3.7**	75.2 ± 2.1**

* 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=10
[c] n=9
[d] n=7

significant decreases in the absolute and relative thymus weights at 50 mg/kg or greater and decreased relative thymus weight in the 25 mg/kg group. Except for the spleen, organ weight changes were not correlated with gross or microscopic findings.

Males had decreased sperm motility and increased epididymal sperm counts at 200 mg/kg; left testicular weights were significantly increased at 50 mg/kg (Table H3). No alterations were observed in the reproductive endpoints of females (Table H4).

In all dosed groups, the incidences of hematopoietic cell proliferation and pigmentation in the spleen were significantly greater than those in the vehicle controls (Table 21). Hematopoietic cell proliferation was characterized by proliferation of hematopoietic cells, foci of dense, basophilic, round nuclei consistent with red blood cell precursors in the red pulp of the spleen. The severities were minimal to mild in the 25 and 50 mg/kg groups and moderate to marked in the 100 and 200 mg/kg groups. The presence of hematopoietic cell proliferation correlated with enlarged spleens observed at necropsy. Pigment in the spleen was characterized by the presence of numerous macrophages containing golden-brown refractile granules in the red pulp and was confirmed to be hemosiderin. The pigment accumulation was associated with erythrophagocytosis in all dosed groups and was characterized by the presence of one to multiple red blood cells within macrophages in the red pulp. Pigment granules and red blood cells were sometimes present within the same cell. Erythrophagocytosis and pigment accumulation were the result of red blood cell destruction.

In the liver, incidences of hematopoietic cell proliferation were significantly increased in males and females in the 100 and 200 mg/kg groups and occurred in a few females in the 25 and 50 mg/kg groups (Table 21). The lesion consisted of small foci of hematopoietic cells, predominantly erythroid precursors, in the sinusoids scattered throughout the liver. The incidences of Kupffer cell pigmentation were significantly increased in males and females administered 50 mg/kg or greater. The pigment consisted of golden to brown granules within the sinusoidal lining macrophages and was confirmed to be hemosiderin deposition. Kupffer cells also often contained one to multiple red blood cells (erythrophagocytosis). Pigment granules and red blood cells were sometimes present within the same cell. These liver lesions were consistent with anemia and red blood cell destruction.

Renal tubule pigmentation was observed in males and females in the 200 mg/kg groups and in females in the 100 mg/kg group (Table 21). The pigment consisted of fine, dark, golden-brown granules in the cytoplasm of the proximal convoluted tubules. The fine granules were confirmed as hemosiderin. Hemosiderin in renal tubules is consistent with a hemolytic anemia.

The incidences of bone marrow pigmentation were significantly increased in all dosed groups of males and females except 25 mg/kg females (Table 21). The pigment consisted of yellow to golden-brown granules within the cytoplasm of the macrophages in the sinusoids of the bone marrow and was consistent with hemosiderin. Pigment accumulation was consistent with red blood cell destruction.

Dose Selection Rationale for the 2-Year Study: The dose concentrations selected for the 2-year study were 2.5, 12.5, and 25 mg/kg. In the 3-month study, the hematopoietic system was the major target of methylene blue trihydrate induced toxicity. Dose-related increased severity of regenerative anemia was observed in all groups administered methylene blue trihydrate. At the 25 mg/kg concentration, minimal regenerative anemia was observed, and it was considered that this concentration would not affect the longevity of mice or cause overt toxicity in a 2-year study. The lowest dose concentration selected was within the range of human therapeutic use of methylene blue trihydrate (HSDB, 2006).

TABLE 21
Incidences of Selected Nonneoplastic Lesions in Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Spleen[a]	10	10	10	10	10
Red Pulp, Hematopoietic Cell Proliferation[b]	0	10** (2.1)[c]	10** (2.0)	10** (2.9)	10** (4.0)
Pigmentation	0	10** (1.6)	10** (2.0)	10** (2.6)	9** (3.0)
Liver	10	10	10	10	10
Hematopoietic Cell Proliferation	0	0	0	10** (1.0)	10** (1.0)
Kupffer Cell Pigmentation	0	0	10** (1.0)	10** (2.0)	10** (2.9)
Kidney	10	0	0	1	10
Renal Tubule, Pigmentation	0			0	9** (2.8)
Bone Marrow	10	10	10	10	10
Pigmentation	0	10** (1.0)	10** (1.4)	10** (1.9)	9** (2.7)
Female					
Spleen	10	10	10	10	10
Red Pulp, Hematopoietic Cell Proliferation	0	9** (1.8)	10** (1.9)	10** (2.8)	10** (3.9)
Pigmentation	0	9** (2.0)	9** (2.0)	10** (2.1)	10** (3.0)
Liver	10	10	10	10	10
Hematopoietic Cell Proliferation	0	1 (1.0)	2 (1.0)	10** (1.0)	9** (1.7)
Kupffer Cell Pigmentation	0	0	9** (1.0)	10** (2.0)	10** (3.0)
Kidney	10	1	0	4	10
Renal Tubule, Pigmentation	0	0		4* (1.0)	10** (2.4)
Bone Marrow	10	1	4	10	10
Pigmentation	0	0	4* (1.0)	10** (1.5)	10** (1.8)

* 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

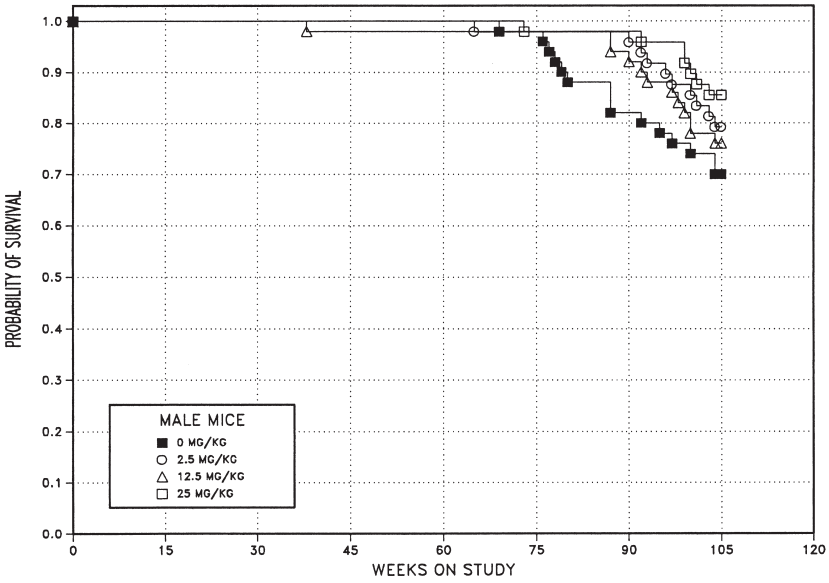
Survival

Estimates of 2-year survival probabilities for male and female mice are shown in Table 22 and in the Kaplan-Meier survival curves (Figure 6). Survival of dosed male and female groups exceeded that of the vehicle controls in a generally dose-related manner.

TABLE 22
Survival of Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Male				
Animals initially in study	50	50	50	50
Accidental deaths[a]	0	2	0	2
Moribund	4	4	7	4
Natural deaths	11	6	5	3
Animals surviving to study termination	35	38	38	41
Percent probability of survival at end of study[b]	70	79	76	86
Mean survival (days)[c]	693	698	705	715
Survival analysis[d]	P=0.131N	P=0.325N	P=0.547N	P=0.081N
Female				
Animals initially in study	50	50	50	50
Accidental deaths	1	1	1	0
Moribund	7	4	5	1
Natural deaths	9	5	2	6
Animals surviving to study termination	33[e]	40	42[f]	43[g]
Percent probability of survival at end of study	67	82	86	86
Mean survival (days)	693	696	712	710
Survival analysis	P=0.067N	P=0.179N	P=0.046N	P=0.055N

[a] Censored from survival analyses
[b] Kaplan-Meier determinations
[c] Mean of all deaths (uncensored, censored, and terminal sacrifice).
[d] The result of the life table trend test (Tarone, 1975) is in the vehicle control column, and the results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns. A negative trend or lower mortality in a dosed group is indicated by **N**.
[e] Includes four animals that died last week of study
[f] Includes one animal that died last week of study
[g] Includes two animals that died last week of study



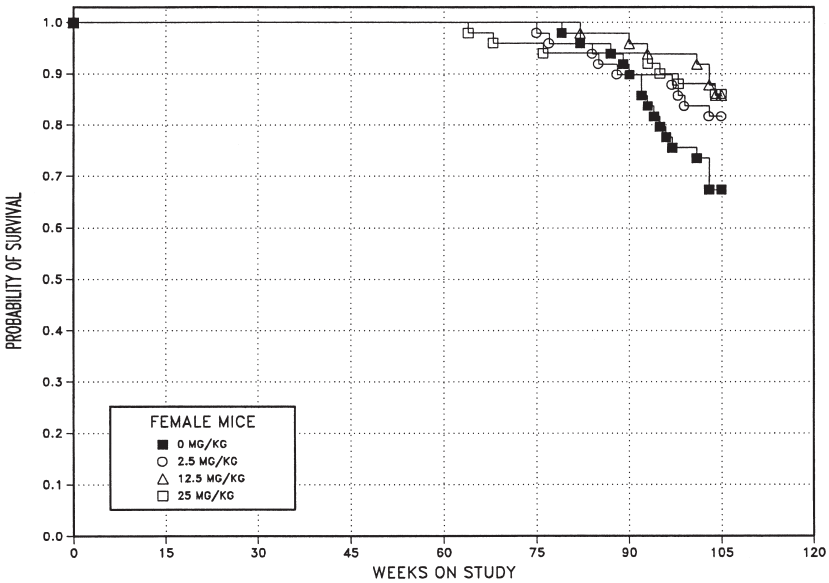


FIGURE 6
Kaplan-Meier Survival Curves for Male and Female Mice Administered Methylene Blue Trihydrate by Gavage for 2 Years

Body Weights and Clinical Findings

Mean body weights of male mice in all dosed groups were similar to those of the vehicle control group (Figure 7 and Table 23). Mean body weights of dosed female mice began to increase after weeks 29, 61, and 85, reaching final values that were 113%, 111%, and 106% of controls for the 2.5, 12.5, and 25 mg/kg groups, respectively (Figure 7 and Table 24). There were no chemical-related clinical findings in males or females.

Clinical Pathology

The hematology and urinalysis data for mice in the 2-year study of methylene blue trihydrate are listed in Table F6. The primary responses to methylene blue trihydrate administration were development of a methemoglobinemia and increased Heinz body formation. The highest dose in this study was 25 mg/kg, and the changes were not dramatic or consistent. Dose-related Heinz body formation occurred in the 12.5 and 25 mg/kg groups at essentially all time points, but the percentage of erythrocytes with Heinz bodies varied widely from less than 1% to greater than 20%, depending on the sex and time point. The increase in methemoglobin diminished, and increased concentrations occurred in the 12.5 and 25 mg/kg females at month 18; the 25 mg/kg males also may have been affected. It appeared that the macrocytic, hyperchromic, responsive anemia that had been apparent in the short-term studies was no longer macrocytic or hyperchromic and, at most, was minimal and transient.

Urinary Excretion

Urine was collected over a 24-hour period from five male and five female mice from the core study groups after 3, 12, and 18 months of dosing. Urine samples were sent to an analytical chemistry support contractor where they were analyzed for methylene blue and its metabolites using HPLC-PDA (600 nm). Methylene blue, leucomethylene blue, trimethylthionine, and leucotrimethylthionine were present in the urine. The 2.5 mg/kg male mice had methylene blue and trimethylthionine present in approximately equal amounts. The relative trimethylthionine metabolite concentrations increased with increasing dose and were approximately three times that of the unchanged methylene blue in the 25 mg/kg males. Concentrations of the leuco forms relative to their oxidized counterparts increased with dosing duration. At the 3-month time point, the concentrations of the leuco forms were approximately one-half that of the oxidized forms, while at the 18-month collection time point, the concentration of leucotrimethylthionine was approximately equal to that of the oxidized form and the leucomethylene blue concentration was twice that of oxidized methylene blue.

Trends in concentrations of methylene blue and trimethylthionine in female mice were similar to males, but the increases in trimethylthionine concentrations were only apparent at the 18-month collection time point. Concentrations of the leuco forms relative to their oxidized counterparts increased with dose and duration of dosing in female mice. At the 12-month collection time point, the concentrations of the leuco forms were about 50% that of the oxidized forms in the 2.5 mg/kg females but dropped to 25% of the oxidized form in the 12.5 mg/kg group before climbing to two and a half times the oxidized forms in the 25 mg/kg group. At the 18-month collection time point, the concentration of leucotrimethylthionine was approximately 65% of the oxidized form in the 2.5 mg/kg females but increased to twice the concentration of the oxidized form in the 25 mg/kg group. The leucomethylene blue concentration was equal to that of the oxidized form in the 2.5 mg/kg females but increased to four times the methylene blue concentration in the 25 mg/kg group.

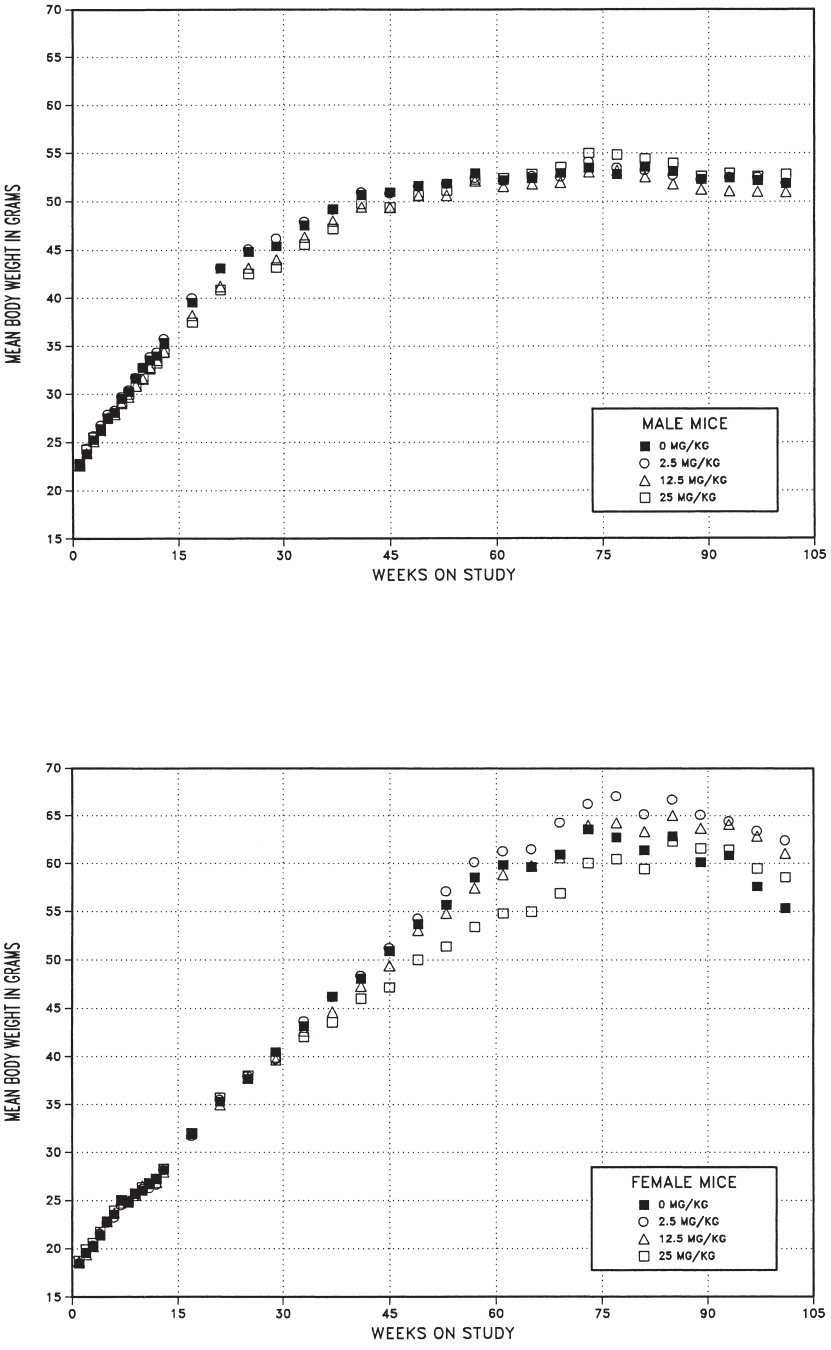


FIGURE 7
Growth Curves for Male and Female Mice
Administered Methylene Blue Trihydrate by Gavage for 2 Years

TABLE 23
Mean Body Weights and Survival of Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

Weeks on Study	Vehicle Control		2.5 mg/kg			12.5 mg/kg			25 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	22.6	50	22.6	100	50	22.5	100	50	22.7	100	50
2	23.8	50	24.4	103	50	23.9	100	50	24.3	102	50
3	25.3	50	25.6	101	49[a]	25.1	99	50[a]	25.5	101	50
4	26.3	50	26.7	102	49	26.2	100	50	26.4	100	50
5	27.5	50	27.9	102	49	27.6	100	50	27.4	100	50
6	28.1	50	28.3	101	49	27.9	99	50	27.8	99	50
7	29.5	50	29.7	101	49	29.0	98	50	29.1	99	50
8	30.2	50	30.4	101	49	29.7	98	50	29.9	99	50
9	31.6	50	31.7	100	49	30.9	98	50	30.8	98	50
10	32.8	50	32.8	100	49	31.6	96	50	31.6	96	50
11	33.5	50	33.9	101	49	32.8	98	50	32.6	97	50
12	34.0	50	34.3	101	49	33.5	99	50	33.2	98	50
13	35.3	50	35.8	101	49	34.4	98	50	34.3	97	50
17	39.6	50	40.0	101	49	38.2	97	50	37.5	95	50
21	43.1	50	43.1	100	49	41.2	96	50	40.9	95	50
25	44.8	50	45.0	100	49	43.1	96	50	42.5	95	50
29	45.4	50	46.2	102	49	44.0	97	50	43.2	95	50
33	47.5	50	47.9	101	49	46.3	98	50	45.6	96	50
37	49.2	50	49.1	100	49	48.0	98	50	47.2	96	50
41	50.7	50	51.0	101	49	49.4	97	49	49.7	98	50
45	51.0	50	50.8	100	49	49.4	97	49	49.4	97	50
49	51.6	50	51.6	100	49	50.6	98	49	50.6	98	50
53	51.9	50	51.8	100	49	50.6	98	49	51.1	99	50
57	52.9	50	52.4	99	49	52.1	99	49	52.3	99	50
61	52.2	50	52.1	100	49	51.5	99	49	52.4	100	50
65	52.4	50	52.6	100	48	51.8	99	49	52.8	101	50
69	52.9	49	52.6	99	48	52.0	98	49	53.5	101	50
73	53.5	49	54.1	101	48	53.0	99	49	55.0	103	49
77	52.8	47	53.4	101	48	53.2	101	49	54.8	104	49
81	53.6	44	53.2	99	48	52.5	98	49	54.4	102	49
85	53.1	44	52.7	99	48	51.8	98	49	54.0	102	49
89	52.3	41	52.3	100	47	51.2	98	47	52.6	101	47
93	52.5	40	52.5	100	44	51.1	97	44	52.9	101	46
97	52.2	38	52.5	101	42	51.0	98	43	52.6	101	46
101	51.8	37	51.9	100	40	50.9	98	39	52.8	102	43
Mean for weeks											
1-13	29.3		29.5	101		28.9	99		28.9	99	
14-52	47.0		47.2	100		45.6	97		45.2	96	
53-101	52.6		52.6	100		51.7	98		53.2	101	

[a] The number of animals weighed was less than the number of animals surviving.

TABLE 24
Mean Body Weights and Survival of Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

Weeks on Study	Vehicle Control		2.5 mg/kg			12.5 mg/kg			25 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	18.4	50	18.5	101	50	18.6	101	50	18.7	102	50
2	19.5	50	19.6	101	49	19.4	100	50	19.9	102	50
3	20.3	50	20.3	100	49	20.2	100	50	20.6	102	50
4	21.3	50	21.6	101	49	21.6	101	50	21.7	102	50
5	22.8	50	22.6	99	49	22.8	100	50	22.8	100	50
6	23.5	50	23.2	99	49	23.7	101	50	23.9	102	50
7	24.9	50	24.5	98	49	24.8	100	50	25.0	100	50
8	24.8	50	24.8	100	49	25.0	101	50	24.9	100	50
9	25.7	50	25.4	99	49	25.5	99	50	25.7	100	50
10	26.0	50	26.2	101	49	26.5	102	50	26.4	102	50
11	26.7	50	26.3	99	49	26.8	100	50	26.8	100	50
12	27.2	50	26.6	98	49	26.9	99	50	27.2	100	50
13	28.1	50	28.1	100	49	27.9	99	50	28.3	101	50
17	31.9	50	31.7	99	49	31.9	100	50	32.0	100	50
21	35.3	50	35.5	101	49	35.0	99	50	35.7	101	50
25	37.7	50	38.0	101	49	37.8	100	50	38.0	101	50
29	40.5	49	39.7	98	49	39.6	98	50	39.9	99	50
33	43.1	49	43.6	101	49	42.7	99	50	42.0	97	50
37	46.2	49	46.1	100	49	44.6	97	50	43.6	94	50
41	48.0	49	48.3	101	49	47.3	99	49	46.0	96	50
45	50.9	49	51.2	101	49	49.4	97	49	47.1	93	50
49	53.7	49	54.3	101	49	53.1	99	49	50.0	93	50
53	55.7	49	57.1	103	49	54.8	98	49	51.4	92	50
57	58.5	49	60.1	103	49	57.4	98	49	53.4	91	50
61	59.9	49	61.3	102	49	58.8	98	49	54.8	92	50
65	59.6	49	61.5	103	49	59.8	100	49	55.0	92	49
69	60.9	49	64.3	106	49	60.6	100	49	56.9	93	48
73	63.5	49	66.2	104	49	64.0	101	49	60.1	95	48
77	62.7	49	67.1	107	47	64.3	103	49	60.4	96	47
81	61.4	48	65.2	106	47	63.3	103	49	59.4	97	47
85	62.8	47	66.7	106	45	65.0	104	48	62.3	99	47
89	60.1	45	65.1	108	44	63.7	106	48	61.6	103	47
93	60.8	42	64.4	106	44	64.1	105	46	61.4	101	46
97	57.6	37	63.4	110	43	62.8	109	46	59.5	103	45
101	55.3	36	62.4	113	41	61.1	111	45	58.6	106	44
Mean for weeks											
1-13	23.8		23.7	100		23.8	100		24.0	101	
14-52	43.0		43.2	100		42.4	99		41.6	97	
53-101	59.9		63.4	106		61.5	103		58.1	97	

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of malignant lymphoma and in the incidences of neoplasms and/or nonneoplastic lesions of the small intestine, lung, spleen, and nose. Summaries of incidences of neoplasms and nonneoplastic lesions, 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 males and Appendix D for females.

Small Intestine: The incidences of carcinoma of the small intestine occurred with a positive trend in males (Tables 25, C1, and C2). The incidences were within the historical control range for all routes of administration; however, the incidence in the 25 mg/kg group was at the upper end of the historical control range for all gavage studies (Table C3a). In addition, the incidence of adenoma or carcinoma (combined) in the 25 mg/kg group exceeded the historical control range for all study routes. One vehicle control female had a carcinoma and one 2.5 mg/kg female had an adenoma (Table D1). The carcinomas were invasive tumors arising from the mucosal epithelium. The neoplastic epithelium had cellular atypia, basophilia, and frequent mitotic figures. They were usually pedunculated with papillary growth into the intestinal lumen.

Lung: There was a positive trend in the incidences of alveolar/bronchiolar carcinoma in males, and the incidence in 25 mg/kg males was significantly greater than that in the vehicle control group (Tables 26, C1, and C2). The incidences of alveolar/bronchiolar carcinoma in dosed groups of males were within the historical control range for all routes and the historical vehicle control range for all gavage studies; the incidence in vehicle controls in the current study was below both historical control ranges (Tables 26 and C3b). The incidences of alveolar/bronchiolar adenoma in dosed males were

TABLE 25
Incidences of Neoplasms of the Small Intestine (Site Unspecified) in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Number Necropsied	50	50	50	50
Adenoma[a]	1	1	2	2
Carcinoma[b]				
Overall rate[c]	0/50 (0%)	1/50 (2%)	2/50 (4%)	4/50 (8%)
Adjusted rate[d]	0.0%	2.2%	4.3%	8.4%
Terminal rate[e]	0/35 (0%)	1/38 (3%)	2/38 (5%)	3/41 (7%)
First incidence (days)	—[f]	729 (T)	729 (T)	705
Poly-3 test[g]	P=0.027	P=0.509	P=0.248	P=0.071
Adenoma or Carcinoma[h]				
Overall rate	1/50 (2%)	2/50 (4%)	4/50 (8%)	6/50 (12%)
Adjusted rate	2.3%	4.3%	8.6%	12.5%
Terminal rate	1/35 (3%)	2/38 (5%)	4/38 (11%)	4/41 (10%)
First incidence (days)	729 (T)	729 (T)	729 (T)	641
Poly-3 test	P=0.029	P=0.515	P=0.194	P=0.071

(T) Terminal sacrifice
[a] Number of animals with neoplasm
[b] Historical incidence for 2-year studies, all routes, all vehicles (mean ± standard deviation): 33/1,508 (2.2% ± 2.7%), range 0%-10%
[c] Number of animals with neoplasm per number of animals necropsied
[d] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[e] Observed incidence at terminal kill
[f] Not applicable, no neoplasms in animal group
[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: 39/1,508 (2.6% ± 2.8%), range 0%-10%

TABLE 26
Incidences of Neoplasms and Nonneoplastic Lesions of the Lung in Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Male				
Number Examined Microscopically	50	50	50	50
Alveolar Epithelium, Hyperplasia, Focal[a]	4 (1.5)[b]	3 (1.7)	2 (1.0)	4 (1.3)
Alveolar/bronchiolar Adenoma, Multiple	2	2	0	2
Alveolar/bronchiolar Adenoma (includes multiple)[c]	11	7	8	6
Alveolar/bronchiolar Carcinoma, Multiple	0	0	1	1
Alveolar/bronchiolar Carcinoma (includes multiple)[d]				
Overall rate[e]	1/50 (2%)	4/50 (8%)	5/50 (10%)	7/50 (14%)
Adjusted rate[f]	2.3%	8.7%	10.8%	14.7%
Terminal rate[g]	1/35 (3%)	4/38 (11%)	4/38 (11%)	6/41 (15%)
First incidence (days)	729 (T)	729 (T)	696	705
Poly-3 test[h]	P=0.043	P=0.192	P=0.114	P=0.039
Alveolar/bronchiolar Adenoma or Carcinoma[i]				
Overall rate	12/50 (24%)	10/50 (20%)	13/50 (26%)	13/50 (26%)
Adjusted rate	26.8%	21.7%	28.0%	26.9%
Terminal rate	10/35 (29%)	10/38 (26%)	12/38 (32%)	10/41 (24%)
First incidence (days)	605	729 (T)	696	610
Poly-3 test	P=0.406	P=0.373N	P=0.545	P=0.587

TABLE 26
Incidences of Neoplasms and Nonneoplastic Lesions of the Lung in Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Female				
Number Examined Microscopically	50	50	50	50
Alveolar Epithelium, Hyperplasia, Focal	2 (1.5)	0	1 (1.0)	1 (2.0)
Alveolar/bronchiolar Adenoma	1	2	3	2
Alveolar/bronchiolar Carcinoma, Multiple	1	0	0	0
Alveolar/bronchiolar Carcinoma (includes multiple)				
Overall rate	5/50 (10%)	0/50 (0%)	0/50 (0%)	1/50 (2%)
Adjusted rate	11.1%	0.0%	0.0%	2.1%
Terminal rate	3/33 (9%)	0/40 (0%)	0/42 (0%)	1/43 (2%)
First incidence (days)	638	—[j]	—	729 (T)
Poly-3 test	P=0.106N	P=0.029N	P=0.026N	P=0.092N
Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	6/50 (12%)	2/50 (4%)	3/50 (6%)	3/50 (6%)
Adjusted rate	13.2%	4.4%	6.3%	6.4%
Terminal rate	3/33 (9%)	2/40 (5%)	3/42 (7%)	3/43 (7%)
First incidence (days)	638	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.306N	P=0.130N	P=0.220N	P=0.225N

(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, all routes, all vehicles (mean ± standard deviation): 258/1,507 (16.7% ± 7.4%), range 4%-28%
[d] Historical incidence: 151/1,507 (9.9% ± 5.0%), range 4%-24%
[e] Number of animals with neoplasm per number of animals with lung examined microscopically
[f] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[g] Observed incidence at terminal kill
[h] 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**.
[i] Historical incidence: 385/1,507 (25.1% ± 9.4%), range 12%-44%
[j] Not applicable; no neoplasms in animal group

slightly decreased. Incidences of focal hyperplasia of the alveolar epithelium were similar among all groups of males. In females, the incidences of alveolar/bronchiolar carcinoma were decreased, and the decreases were significant in the 2.5 and 12.5 mg/kg groups (Tables 26, D1, and D2). The incidences of alveolar/bronchiolar adenoma and focal hyperplasia of the alveolar epithelium were similar among all female groups. Microscopically, alveolar/bronchiolar carcinomas usually consisted of large growths that were well demarcated from the surrounding lung tissues. However, the cellular margins were often irregular with invasion of adjacent tissues, lymphatics, blood vessels, the pleural cavity, and mediastinum. The tumor cells ranged from round to oval and from cuboidal to tall columnar. They were pleomorphic, had nuclear atypism, and were arranged in one to

multiple layers around prominent fibrovascular cores. They had heterogeneous growth patterns that included alveolar, papillary, and tubular structures or mixtures of these structures.

Malignant Lymphoma: The incidences of malignant lymphoma occurred with a positive trend in females, and the incidences in the 12.5 and 25 mg/kg groups were significantly greater than that in the vehicle controls (Tables 27, D1, and D2). The incidences, including that in the vehicle controls, were within the historical control ranges for all routes and gavage studies (Tables 27 and D3). In 25 mg/kg males, the incidence of malignant lymphoma was slightly increased and exceeded the historical control ranges for all routes and gavage studies (Tables 27, C1, C2, and C3c). Microscopically, lymphomas are a group of related neoplasms composed of relatively homogenous populations of lymphocytic cells that replace the normal structures of the spleen, thymus, various lymph nodes, and bone marrow and may infiltrate the portal areas of the liver.

Spleen: The incidences of hematopoietic cell proliferation were significantly increased in the 12.5 and 25 mg/kg males and in 25 mg/kg females (Tables 28, C4, and D4). Hematopoietic cell proliferation was characterized by the presence of random foci of dense, basophilic, round nuclei consistent with red blood cell precursors in the splenic parenchyma.

Nose: Dose-related increases in inflammation occurred in all dosed groups with significant increases in the 12.5 and 25 mg/kg females (Tables 28, C4, and D4). Inflammation consisted of proteinaceous fluid and inflammatory cells, primarily neutrophils, in the nasal cavities.

TABLE 27
Incidences of Malignant Lymphoma in Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Male				
Overall rate[a,b]	2/50 (4%)	2/50 (4%)	2/50 (4%)	5/50 (10%)
Adjusted rate[c]	4.5%	4.3%	4.3%	10.5%
Terminal rate[d]	2/35 (6%)	1/38 (3%)	2/38 (5%)	4/41 (10%)
First incidence (days)	729 (T)	717	729 (T)	687
Poly-3 test[e]	P=0.126	P=0.678N	P=0.676N	P=0.250
Female				
Overall rate[f]	6/50 (12%)	4/50 (8%)	9/50 (18%)	12/50 (24%)
Adjusted rate	13.2%	8.7%	18.6%	24.5%
Terminal rate	4/33 (12%)	2/40 (5%)	7/42 (17%)	8/43 (19%)
First incidence (days)	568	673	568	445
Poly-3 test	P=0.025	P=0.360N	P=0.334	P=0.126

(T) Terminal sacrifice
[a] Number of animals with malignant lymphoma per number of animals necropsied
[b] Historical incidence for 2-year studies, all routes, all vehicles (mean ± standard deviation): 70/1,508 (4.3% ± 2.3%), range 0%-8%
[c] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[d] Observed incidence at terminal kill
[e] 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 lower incidence in a dosed group is indicated by **N**.
[f] Historical incidence: 308/1,508 (19.7% ± 13.3%), range 6%-58%

TABLE 28
Incidences of Selected Nonneoplastic Lesions in Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control		2.5 mg/kg		12.5 mg/kg		25 mg/kg	
Male								
Spleen[a]	49		50		49		48	
Hematopoietic Cell Proliferation[b]	14	(2.6)[c]	16	(2.7)	25*	(2.8)	29**	(2.5)
Nose	50		50		50		50	
Inflammation	1	(2.0)	3	(1.3)	3	(2.3)	6	(1.8)
Female								
Spleen	47		47		49		50	
Hematopoietic Cell Proliferation	23	(2.7)	21	(2.5)	31	(2.4)	40**	(2.3)
Nose	50		50		50		50	
Inflammation	0		3	(2.0)	7*	(2.0)	11**	(1.9)

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

GENETIC TOXICOLOGY

Two different lots of methylene blue trihydrate were tested independently at two laboratories for mutagenicity in bacterial tester strains. In the first study, methylene blue trihydrate (1 to 200 µg/plate) was mutagenic in *Salmonella typhimurium* strains TA98 and TA100 when testing occurred in the presence of 30% rat or hamster liver S9 activation enzymes; without S9 enzymes, mutagenicity was seen only in strain TA98 (Table E1). In the second study, methylene blue trihydrate was mutagenic with and without 10% rat liver S9 in *S. typhimurium* strains TA98 and TA100 (0.25 to 150 µg/plate) and in *Escherichia coli* strain WP2 (0.25 to 1,500 µg/plate) (Table E2).

Three azure compounds (A, B, C) were tested in the same protocol as was used in the second bacterial mutagenicity study. All three compounds were positive in *S. typhimurium* TA100 and TA98 and *E. coli* WP2 with and without 10% rat liver S9 (Tables E3, E4, and E5). In cytogenetic tests with cultured Chinese hamster ovary cells, methylene blue trihydrate induced sister chromatid exchanges (0.17 to 2.5 µg/mL without S9; up to 5.0 µg/mL with S9) (Table E6) and chromosomal aberrations at 4.7 to 22 µg/mL (Table E7) with and without S9 activation enzymes.

In contrast to the clearly positive results in the *in vitro* studies, no increase in the frequency of micronucleated erythrocytes was observed in bone marrow or blood of male mice sampled 48 hours after a single intraperitoneal injection of 25, 50, or 150 mg/kg methylene blue trihydrate (Table E8). The negative response in mouse bone marrow may have been the result of suboptimal sampling time; bone marrow analysis is usually conducted 24 hours after dosing. Forty-eight hours after dosing, the young, exposed erythrocytes analyzed for the presence of micronuclei are typically found only in the peripheral blood. However, the peripheral blood micronucleus data from this short-term exposure study were also negative, and thus, they provide additional evidence of a lack of response by methylene blue in this *in vivo* assay for chromosomal damage. Furthermore, no increases in micronucleated erythrocytes were observed in peripheral blood samples taken from male and female mice at the end of the 3-month toxicity study (Table E9). However, a strong, dose-related increase in the percentage of polychromatic erythrocytes (reticulocytes) among total erythrocytes was seen in both male and female mice in the 3-month study; this observed increase in immature circulating erythrocytes indicates a stimulation of erythropoiesis consistent with other results, indicating a response to methylene blue trihydrate-induced anemia.

DISCUSSION AND CONCLUSIONS

Methylene blue trihydrate has a variety of therapeutic and diagnostic uses in human and veterinary medicine, including use as a bacteriology stain, a redox indicator dye, a food colorant, a targeting agent for melanoma, an antihemoglobinemic, and an antiseptic and disinfectant (*Merck*, 2001). One of the most common uses is in treatment of methemoglobinemia induced by drug treatment or exposure to environmental poisons such as excessive nitrates in well water or cyanide (Sills and Zinkkam, 1994; Christiansen *et al.*, 1996). Other medicinal uses of methylene blue include the management of chronic urolithiasis and treatment of cutaneous viral infections. Methylene blue is used in treatment of a number of psychiatric disorders because of its anxiolytic and antidepressant properties, which are attributed to its ability to block activation of guanyl cyclase by nitric oxide (Naylor *et al.*, 1986).

The current studies included 1-month, 3-month, and 2-year studies, in which male and female F344/N rats and B6C3F$_1$ mice were administered methylene blue trihydrate in 0.5% aqueous methylcellulose by gavage. The doses selected for the 1-month studies resulted in significant mortality, limiting the amount of information that could be used for 2-year study dose setting. Thus, 3-month studies were performed and used to select doses for the 2-year studies.

In the 3-month studies, groups of 10 male and 10 female rats and mice were administered methylene blue trihydrate at doses of 0, 25, 50, 100, or 200 mg/kg. There was no evidence of mortality related to toxicity; a minimal decrease (<10%) in body weight gain occurred only in 200 mg/kg male rats and mice. There was a small dose-related increase in liver weights of rats and a significant dose-related increase in spleen weights of rats and mice. The hematopoietic system was the major target of methylene blue trihydrate toxicity (Hejtmancik *et al.*, 2002). Regenerative Heinz body anemia was seen in all groups administered methylene blue trihydrate, and the severity increased with increasing dose concentration. Increases in methemoglobin and Heinz body formation, accompanied by decreases in the erythron and increased reticulocyte counts, were observed in rats and mice of both sexes. Mice were a bit more sensitive to methylene blue trihydrate toxicity than were rats. Splenomegaly occurred, and microscopic evaluation of the spleen revealed hematopoiesis in all dosed groups of mice and in rats administered 50 mg/kg or greater. Mice administered the higher dose concentrations also showed hematopoiesis in the liver and the accumulation of hemosiderin in Kupffer cells. Mice also showed a markedly higher accumulation of Heinz bodies in their blood than did rats at comparable gavage doses. Mice are thought to be more susceptible than rats to Heinz body formation from an equivalent level of oxidative stress to red blood cells, but the reasons for this are not fully understood (Smith, 1991). This underlying susceptibility of mice to form Heinz bodies may account for the greater overall evidence for hemolysis and stimulation of red blood cell regeneration in mice.

The results of the 3-month studies gave little evidence that there was a marked difference in methemoglobinemia in mice and rats receiving comparable doses of methylene blue trihydrate. Mammalian hemoglobins differ little in their rates of heme oxidation by various chemical oxidants (Bartels *et al.*, 1963), and presumably, this would also hold for methylene blue-induced oxidation of the heme iron. The "spontaneous" rate for the reduction of oxidized iron to the ferrous form in the red cell by an NADH-dependent reaction catalyzed by cytochrome b5 is at least as fast, if not faster, in mice than rats. However, a second mechanism involving an NADPH-dependent reduction of methylene blue to its leuco form, which in turn reduces methemoglobin to the functional ferrous form, has been reported to be faster in the rat than in the mouse (Smith, 1991). These differences in rates are relatively small and may offset each other. Another potential contributing factor is the kinetics that govern the blood levels of methylene blue that are actually achieved in the mice and rats in these gavage studies. Blood levels of methylene blue trihydrate were not determined in these studies, but analysis of urine data for parent and metabolite concentrations (and corrected for creatinine) suggested a greater bioavailability of methylene blue trihydrate in mice than rats. These estimates are approximate and need confirmation by

direct measures of methylene blue trihydrate in blood.

Dose concentrations of methylene blue trihydrate selected for the 2-year studies were 0, 5, 25, and 50 mg/kg for rats and 0, 2.5, 12.5, and 25 mg/kg for mice. The highest dose concentrations were based on the 3-month study results, where effects of regenerative anemia were minimal at 50 mg/kg in rats and 25 mg/kg in mice. The lowest dose concentrations were selected to approximate the human therapeutic dose used to treat methemoglobinemia (1 to 2 mg/kg intravenously). Additional animals were included for hematologic evaluation after receiving the same doses of methylene blue trihydrate for 2 weeks, 3, 12, or 18 months.

In the 2-year rat study, there were no differences in survival rates between dosed animals and vehicle controls. Mean body weights of 25 and 50 mg/kg male and female rats began to be less than vehicle controls after 9 and 8 months, respectively, and decreased steadily to about 91% and 88% that of vehicle controls, respectively, at 2 years.

For the 2-year rat study, a comparable decrease in the erythron with a concomitant increase in reticulocyte counts, methemoglobin concentration, and Heinz body formation, similar to that observed in male and female rats in the 25 and 50 mg/kg groups in the 3-month studies, was apparent at least to 18 months. The 2-year mouse study demonstrated a similar effect in the 25 mg/kg group (compared to the 3-month study), except the erythron and reticulocyte responses were not apparent at 18 months; the increased methemoglobin (females) and Heinz body formation (males and females) continued. This suggests that the oxidative effect of methylene blue on erythrocytes (Hejtmancik *et al*., 2002) was present and continued throughout the 2-year administration period in rats and mice.

The increased incidences of pancreatic islet cell neoplasms in male rats, accompanied by hyperplasia, were considered to be associated with methylene blue trihydrate administration. The incidences of islet cell adenoma, as well as adenoma or carcinoma (combined), were increased in all dosed groups, and the increase was statistically significant in 25 mg/kg males, which had an incidence double the highest rate observed in historical controls. Out of approximately 540 chemicals tested in rodent cancer studies by the NTP, pancreatic islet cell neoplasms have been observed in only eight other studies (Table 29). The incidence of this rather uncommon neoplasm peaked at an intermediate dose in five of the nine studies where it occurred. The reasons for this are unknown, but the high incidence of hyperplasia in the 50 mg/kg group of male rats in the present study suggests that proliferative lesions in these animals were somehow inhibited from progressing to adenomas.

In the nine studies with chemical-induced pancreatic islet cell neoplasia, there was no correlation with chemical structure (Table 29); indeed, the structures of the chemicals where it has been observed are quite different. Furthermore, while promethazine and methylene blue are built on the phenothiazine ring structure, the NTP study of promethazine provided no evidence of carcinogenic activity in male and female rats or mice (NTP, 1993).

Several proliferative lesions occurred at reduced incidences in dosed rats. These included mononuclear cell leukemia in males and females, mammary gland fibroadenoma in females, and adrenal medulla pheochromocytoma in males. Reduced incidences of mononuclear cell leukemia have been frequently observed in rats that also exhibit toxicity to the spleen (Hall, 1990; Stefanski *et al.*, 1990; Elwell *et al.*, 1996). Mononuclear cell leukemia is thought to have its origins in the spleen, and chemicals that are directly toxic to the spleen or damage the spleen secondary to hematotoxicity, as with methylene blue trihydrate, probably act to inhibit the spontaneous development of mononuclear cell leukemia through this mechanism.

The mechanism resulting in inhibition of mononuclear cell leukemia is not the same as that of hyperplasia in the bone marrow and hematopoietic cell proliferation in the spleen and liver. Bone marrow hyperplasia and hematopoietic cell proliferation are in response to anemia. Inhibition of mononuclear cell leukemia appears to be associated with toxicity of methylene blue metabolites or products from damaged red blood cells in the spleen. Capsular fibrosis is additional evidence of spleen injury caused by damaged red blood cells.

Mammary gland fibroadenoma development is known to be influenced by the body weight of female rats, and expected rates of fibroadenoma can be calculated for dosed groups that have reductions in body weight in relation to controls (Haseman *et al.,* 1997). In the methylene blue trihydrate study, the expected rates of mammary gland fibroadenoma were consistent with the observed body weights only in the 50 mg/kg group. The

TABLE 29
Chemical-related Pancreatic Islet Cell Neoplasia in 2-Year NTP Studies

Compound and Report	Molecular Formula	Uses	ROE[a]	Incidence of Pancreatic Islet Cell Neoplasia	STT[b] SAL	STT[b] MN
Methylene Blue Trihydrate (Current Study)	$C_{16}H_{24}ClN_3O_3S$	Nitrite poisoning antidote; bacteriology; hair colorant (former use); anti-hemoglobinemic; redox indicator; surgical skin marking; paper; photosensitizer		Male rat: Adenoma, multiple (0/50, 1/50, 0/50, 1/50) Adenoma (4/50, 9/50, 12/50, 8/50) Adenoma or carcinoma (4/50, 9/50, 14/50, 8/50)		–/–,–
Azinphosmethyl (NCI, 1978a)	$C_{10}H_{12}N_3O_3PS_2$	Agricultural insecticide		Male rat: Adenoma (2/92[c], 0/9, 1/47, 4/45) Adenoma or carcinoma (2/92[c], 0/9, 1/47, 4/45)		NT
Propyl Gallate (NTP, 1982)	$C_{10}H_{12}O_5$	Antioxidant in foods containing fats/oils, food packaging materials, and cosmetics		Male rat: Adenoma (0/50, 8/50, 2/50) Adenoma or carcinoma (2/50, 9/50, 4/50)		–,+
3-Nitropropionic Acid (NCI, 1978b)	$C_3H_5NO_4$	Naturally occurring; food contaminant		Male rat: Adenoma (4/49, 6/50, 11/50)		NT
2,4- and 2,6-Toluene Diisocyanate (NTP, 1986a)	$C_9H_6N_2O_2$	Manufacture of flexible polyurethane foams; lacquer and wood finish coatings		Male rat: Adenoma (1/47, 3/47, 7/49) Female rat: Adenoma (0/50, 6/49, 2/47)		NT
Cl Disperse Blue 1 (NTP, 1986b)	$C_{14}H_{12}N_4O_2$	Dye for sheepskins, furs, acetate, nylon, thermoplastic resins; semipermanent hair dye component		Male rat: Adenoma (1/49, 0/50, 4/50, 2/50) Adenoma or carcinoma (1/49, 2/50, 5/50, 3/50)		–
Malonaldehyde, Sodium Salt (NTP, 1988)	$C_3H_3O_2Na$	Food freshness indicator; no commercial use		Male rat: Adenoma (0/49, 9/50, 1/49)		NT
Sodium Chlorate (NTP, 2005)	$NaClO_3$	By-product of chlorine dioxide and hypochlorite disinfection; herbicide		Female mouse: Adenoma or carcinoma (0/46, 2/47, 2/49, 4/49)		–/–
Promethazine Hydrochloride (NTP, 1993)	$C_{17}H_{20}N_2S•HCl$	Antihistaminic		Absent[d]		NT

[a] F=feed, G=gavage, W=drinking water.
[b] STT=short-term test, SAL=*Salmonella typhimurium* (no indication of S9 requirement is shown); MN = micronucleus (blood vs. bone marrow is not indicated); + = positive result, +w = weak positive result, – = negative result, NT = not tested. The occurrence of two SAL results indicates multiple *S. typhimurium* tests; micronucleus results for both males and females are indicated by a slash (/) (strain not indicated); a single MN result indicates an acute study in male mice.
[c] Pooled controls from similar bioassays; matched control incidence: 0/9
[d] Promethazine hydrochloride was included because it is structurally similar to methylene blue trihydrate.

other dosed groups and vehicle controls had rates higher than anticipated. The reasons for this are not known, and consequently, the relationship between methylene blue trihydrate administration and reduced mammary gland fibroadenoma, if any, is uncertain.

In the 2-year study in mice, survival of dosed male and female groups exceeded that of vehicle controls in a generally dose-related manner. Hematological effects observed at 3, 12, and 18 months were similar in male and female mice. The incidences of hematopoietic cell proliferation in the spleen were significantly increased in both sexes.

The incidences of malignant lymphoma occurred with a positive trend in female mice. The incidence in the 25 mg/kg group (24%) was well within the historical control range (6% to 58%) for this highly variable neoplasm, and thus, the response in this study was considered equivocal. In males, the incidence in the 25 mg/kg group was numerically elevated, though not statistically significant, and exceeded the historical control range and was also considered an equivocal response.

Although not identified as a target organ in the 3-month study, male mice at 2 years exhibited a significant positive trend in the incidence of carcinoma and adenoma or carcinoma (combined) of the small intestine (site unspecified). Although the incidences in the dosed groups were not significant by pairwise comparison, the rate of adenoma or carcinoma (combined) in the 25 mg/kg group exceeded the historical control range for these combined neoplasms, and the rate in vehicle controls was consistent with the historical mean. Thus, the small intestine neoplasms observed in male mice were considered some evidence of carcinogenic activity of methylene blue trihydrate.

The incidence of alveolar/bronchiolar carcinoma of the lung in male mice was low but exhibited a positive trend and was also significant in the 25 mg/kg group. However, incidences of alveolar/bronchiolar adenoma alone were decreased in dosed groups, and the incidence of focal hyperplasia of the alveolar epithelium was low and similar across all groups. The incidences of alveolar/bronchiolar carcinoma in dosed groups were well within the range observed for historical controls. For these reasons, the observed alveolar/bronchiolar carcinomas were not considered related to methylene blue trihydrate administration.

Methylene blue trihydrate was mutagenic in a variety of bacterial tester strains, with and without rodent liver S9 activation enzymes, and it induced sister chromatid exchanges (indicative of DNA damage and repair) and chromosomal aberrations in cultured mammalian cells, with and without S9. In addition to these *in vitro* mutagenicity assays conducted with the parent compound, the NTP conducted additional bacterial mutagenicity tests with three azure compounds (A, B, C) that are metabolites of methylene blue trihydrate. All three azure compounds were positive in *Salmonella typhimurium* TA98, TA100, and *Escherichia coli* WP2, with and without 10% rat liver S9. In contrast to these positive results *in vitro*, no increase in the frequency of micronucleated erythrocytes (a biomarker of chromosomal damage) was observed in bone marrow or blood samples of male mice analyzed 48 hours after a single intraperitoneal injection of methylene blue trihydrate. The negative response in mouse bone marrow may have been due to suboptimal sampling time, since the immature erythrocytes analyzed for presence of micronuclei in this assay are typically found in the peripheral blood 48 hours after treatment; bone marrow analysis is usually conducted 24 hours after dosing. However, the peripheral blood micronucleus data from this short term exposure study, which were also negative, provide additional evidence of a lack of response by methylene blue trihydrate in this *in vivo* assay for chromosomal damage. Furthermore, micronucleated erythrocyte frequencies were unchanged in peripheral blood of male and female mice administered methylene blue trihydrate by gavage for 3 months. In the 3-month micronucleus studies, a marked, dose-related increase in the percentage of reticulocytes among total erythrocytes was observed in male and female mice, consistent with the hematopoietic stimulation seen in these mice. The negative results in the mouse micronucleus studies were somewhat surprising because stimulation of erythropoiesis has been shown to increase the baseline frequency of micronucleated erythrocytes in mice (Suzuki *et al*., 1989; Hirai *et al*., 1991) and because bone marrow was clearly a target of this *in vitro* clastogen.

CONCLUSIONS

Under the conditions of these 2-year gavage studies, there was *some evidence of carcinogenic activity** of methylene blue trihydrate in male F344/N rats based on increased incidences of pancreatic islet cell adenoma and adenoma or carcinoma (combined). There was *no evidence of carcinogenic activity* in female F344/N rats administered 5, 25, or 50 mg/kg. There was *some evidence of carcinogenic activity* in male B6C3F_1 mice based on increased incidences of carcinoma and of adenoma or carcinoma (combined) in the small intestine. The increased incidence of malignant lymphoma in males receiving 25 mg/kg may have been related to the administration of methylene blue trihydrate. There was *equivocal evidence of carcinogenic activity* in female B6C3F_1 mice based on marginally increased incidences of malignant lymphoma.

Methylene blue trihydrate administration caused methemoglobinemia and a regenerative Heinz body anemia with secondary injury to other organs in rats and mice.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 12. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 14.

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APPENDIX A
SUMMARY OF LESIONS IN MALE RATS IN THE 2-YEAR GAVAGE STUDY OF METHYLENE BLUE TRIHYDRATE

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths			1	2
Moribund	13	7	5	10
Natural deaths	6	10	5	7
Survivors				
Died last week of study	2			1
Terminal sacrifice	29	33	39	30
Animals examined microscopically	50	50	50	50
Alimentary System				
Intestine small, duodenum	(50)	(50)	(50)	(48)
Schwannoma malignant		1 (2%)		
Intestine small, jejunum	(47)	(47)	(47)	(48)
Intestine small, ileum	(48)	(50)	(49)	(48)
Liver	(50)	(50)	(50)	(50)
Hemangiosarcoma	1 (2%)			
Hepatocellular carcinoma			1 (2%)	
Hepatocellular adenoma	1 (2%)	1 (2%)		
Mesentery	(16)	(11)	(9)	(12)
Carcinoma, metastatic, Zymbal's gland				1 (8%)
Fibrous histiocytoma, metastatic, skin				1 (8%)
Hemangiosarcoma	1 (6%)			
Oral mucosa	(1)			(4)
Pharyngeal, squamous cell carcinoma	1 (100%)			1 (25%)
Pancreas	(50)	(50)	(50)	(50)
Carcinoma, metastatic, Zymbal's gland				1 (2%)
Mixed tumor benign	1 (2%)	1 (2%)		1 (2%)
Acinus, adenoma		2 (4%)	4 (8%)	3 (6%)
Stomach, forestomach	(50)	(50)	(50)	(50)
Stomach, glandular	(50)	(50)	(50)	(50)
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Carcinoma, metastatic, Zymbal's gland				1 (2%)
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Adenoma	1 (2%)		1 (2%)	2 (4%)
Osteosarcoma, metastatic, nose		1 (2%)		
Adrenal medulla	(50)	(50)	(50)	(50)
Pheochromocytoma malignant	1 (2%)			1 (2%)
Pheochromocytoma complex	1 (2%)			
Pheochromocytoma benign	7 (14%)	12 (24%)	2 (4%)	2 (4%)
Bilateral, pheochromocytoma benign	2 (4%)	1 (2%)		1 (2%)
Islets, pancreatic	(50)	(50)	(50)	(50)
Adenoma	4 (8%)	8 (16%)	12 (24%)	7 (14%)
Adenoma, multiple		1 (2%)		1 (2%)
Carcinoma			2 (4%)	

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Endocrine System (continued)				
Pituitary gland	(50)	(50)	(50)	(50)
Pars distalis, adenoma	25 (50%)	32 (64%)	26 (52%)	21 (42%)
Pars distalis, carcinoma			1 (2%)	
Thyroid gland	(50)	(50)	(50)	(50)
Bilateral, C-cell, adenoma	2 (4%)		1 (2%)	
C-cell, adenoma	4 (8%)	3 (6%)	6 (12%)	4 (8%)
C-cell, carcinoma	2 (4%)	2 (4%)		
Follicular cell, adenoma			2 (4%)	2 (4%)
General Body System				
Peritoneum	(1)	(1)	(1)	(2)
Carcinoma, metastatic, Zymbal's gland				1 (50%)
Genital System				
Epididymis	(50)	(50)	(50)	(50)
Preputial gland	(50)	(50)	(50)	(50)
Adenoma	1 (2%)	4 (8%)	5 (10%)	
Bilateral, adenoma			1 (2%)	
Prostate	(50)	(50)	(50)	(50)
Adenoma	1 (2%)			
Carcinoma, metastatic, Zymbal's gland				1 (2%)
Seminal vesicle	(50)	(50)	(50)	(50)
Testes	(50)	(50)	(50)	(50)
Bilateral, interstitial cell, adenoma	32 (64%)	28 (56%)	31 (62%)	31 (62%)
Interstitial cell, adenoma	9 (18%)	11 (22%)	14 (28%)	3 (6%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Lymph node	(21)	(17)	(10)	(18)
Mediastinal, carcinoma, metastatic, harderian gland				1 (6%)
Pancreatic, carcinoma, metastatic, Zymbal's gland				1 (6%)
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Carcinoma, metastatic, Zymbal's gland				1 (2%)
Spleen	(50)	(50)	(50)	(50)
Carcinoma, metastatic, Zymbal's gland				1 (2%)
Hemangiosarcoma	1 (2%)	1 (2%)	1 (2%)	
Thymus	(49)	(50)	(50)	(50)
Carcinoma, metastatic, Zymbal's gland				1 (2%)
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Fibroadenoma	2 (4%)	3 (6%)	1 (2%)	1 (2%)
Skin	(50)	(50)	(50)	(50)
Basal cell adenoma		2 (4%)	1 (2%)	
Keratoacanthoma	3 (6%)	1 (2%)	3 (6%)	
Squamous cell carcinoma			2 (4%)	
Squamous cell papilloma	3 (6%)	2 (4%)	5 (10%)	
Squamous cell papilloma, multiple	1 (2%)			1 (2%)
Lip, fibrosarcoma	1 (2%)			
Pinna, neural crest tumor	1 (2%)			

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Integumentary System (continued)				
Skin (continued)	(50)	(50)	(50)	(50)
Sebaceous gland, adenoma			1 (2%)	
Subcutaneous tissue, fibroma	5 (10%)	4 (8%)	1 (2%)	1 (2%)
Subcutaneous tissue, fibroma, multiple	1 (2%)			
Subcutaneous tissue, fibrosarcoma	2 (4%)	2 (4%)		
Subcutaneous tissue, fibrous histiocytoma				1 (2%)
Subcutaneous tissue, hemangiosarcoma			1 (2%)	
Subcutaneous tissue, lipoma				1 (2%)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Osteosarcoma			1 (2%)	1 (2%)
Skeletal muscle	(3)	(1)		(4)
Carcinoma, metastatic, Zymbal's gland				1 (25%)
Hemangioma	1 (33%)			
Nervous System				
Brain	(50)	(50)	(50)	(50)
Carcinoma, metastatic, pituitary gland			1 (2%)	
Glioma malignant		1 (2%)		
Peripheral nerve	(6)	(3)	(4)	(6)
Schwannoma benign		1 (33%)		
Schwannoma malignant	1 (17%)		1 (25%)	
Trigeminal, squamous cell carcinoma, metastatic, oral mucosa	1 (17%)			
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	1 (2%)		1 (2%)	2 (4%)
Alveolar/bronchiolar carcinoma				2 (4%)
Carcinoma, metastatic, thyroid gland		1 (2%)		
Carcinoma, metastatic, Zymbal's gland				1 (2%)
Fibrous histiocytoma, metastatic, skin				1 (2%)
Osteosarcoma, metastatic, nose		1 (2%)		
Pheochromocytoma malignant, metastatic, adrenal medulla	1 (2%)			
Schwannoma malignant, metastatic, peripheral nerve	1 (2%)			
Squamous cell carcinoma, metastatic, skin			1 (2%)	
Nose	(50)	(50)	(50)	(50)
Carcinoma, metastatic, harderian gland				1 (2%)
Osteosarcoma		1 (2%)		
Special Senses System				
Harderian gland	(50)	(50)	(50)	(50)
Adenoma			1 (2%)	
Carcinoma				1 (2%)
Zymbal's gland		(1)	(1)	(1)
Adenoma			1 (100%)	
Carcinoma		1 (100%)		1 (100%)

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Urinary System				
Kidney[a]	(50)	(50)	(50)	(50)
Carcinoma, metastatic, Zymbal's gland				1 (2%)
Renal tubule, adenoma				1 (2%)
Urinary bladder	(50)	(50)	(50)	(49)
Papilloma			1 (2%)	
Systemic Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Leukemia mononuclear	23 (46%)	10 (20%)	2 (4%)	2 (4%)
Mesothelioma malignant	3 (6%)	5 (10%)	2 (4%)	3 (6%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	49	50	50	48
Total primary neoplasms	146	141	135	98
Total animals with benign neoplasms	47	50	50	48
Total benign neoplasms	107	117	121	85
Total animals with malignant neoplasms	30	19	12	12
Total malignant neoplasms	38	24	14	13
Total animals with metastatic neoplasms	3	2	2	3
Total metastatic neoplasms	3	3	2	16
Total animals with uncertain neoplasms - benign or malignant	1			
Total uncertain neoplasms	1			

[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
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate[a]	9/50 (18%)	13/50 (26%)	2/50 (4%)	3/50 (6%)
Adjusted rate[b]	20.5%	29.1%	4.3%	7.2%
Terminal rate[c]	7/31 (23%)	11/33 (33%)	2/39 (5%)	3/31 (10%)
First incidence (days)	702	576	729 (T)	729 (T)
Poly-3 test[d]	P=0.002N	P=0.244	P=0.018N	P=0.069N
Adrenal Medulla: Benign, Complex, or Malignant Pheochromocytoma				
Overall rate	11/50 (22%)	13/50 (26%)	2/50 (4%)	4/50 (8%)
Adjusted rate	24.8%	29.1%	4.3%	9.5%
Terminal rate	8/31 (26%)	11/33 (33%)	2/39 (5%)	4/31 (13%)
First incidence (days)	645	576	729 (T)	729 (T)
Poly-3 test	P=0.002N	P=0.417	P=0.005N	P=0.053N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	1/50 (2%)	0/50 (0%)	1/50 (2%)	4/50 (8%)
Adjusted rate	2.3%	0.0%	2.1%	9.5%
Terminal rate	0/31 (0%)	0/33 (0%)	1/39 (3%)	3/31 (10%)
First incidence (days)	662	—[e]	729 (T)	715
Poly-3 test	P=0.023	P=0.499N	P=0.746N	P=0.164
Mammary Gland: Fibroadenoma				
Overall rate	2/50 (4%)	3/50 (6%)	1/50 (2%)	1/50 (2%)
Adjusted rate	4.6%	6.8%	2.1%	2.4%
Terminal rate	2/31 (7%)	3/33 (9%)	1/39 (3%)	1/31 (3%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.235N	P=0.504	P=0.476N	P=0.515N
Pancreas: Adenoma				
Overall rate	0/50 (0%)	2/50 (4%)	4/50 (8%)	3/50 (6%)
Adjusted rate	0.0%	4.5%	8.5%	7.2%
Terminal rate	0/31 (0%)	2/33 (6%)	4/39 (10%)	3/31 (10%)
First incidence (days)	—	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.106	P=0.239	P=0.070	P=0.111
Pancreatic Islets: Adenoma				
Overall rate	4/50 (8%)	9/50 (18%)	12/50 (24%)	8/50 (16%)
Adjusted rate	9.0%	19.9%	25.0%	18.8%
Terminal rate	1/31 (3%)	5/33 (15%)	9/39 (23%)	4/31 (13%)
First incidence (days)	620	561	619	652
Poly-3 test	P=0.201	P=0.121	P=0.037	P=0.155
Pancreatic Islets: Adenoma or Carcinoma				
Overall rate	4/50 (8%)	9/50 (18%)	14/50 (28%)	8/50 (16%)
Adjusted rate	9.0%	19.9%	29.1%	18.8%
Terminal rate	1/31 (3%)	5/33 (15%)	10/39 (26%)	4/31 (13%)
First incidence (days)	620	561	619	652
Poly-3 test	P=0.174	P=0.121	P=0.013	P=0.155
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	25/50 (50%)	32/50 (64%)	26/50 (52%)	21/50 (42%)
Adjusted rate	54.4%	65.7%	53.5%	46.1%
Terminal rate	16/31 (52%)	20/33 (61%)	19/39 (49%)	12/31 (39%)
First incidence (days)	641	473	586	504
Poly-3 test	P=0.078N	P=0.176	P=0.549N	P=0.278N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Pituitary Gland (Pars Distalis): Adenoma or Carcinoma				
Overall rate	25/50 (50%)	32/50 (64%)	27/50 (54%)	21/50 (42%)
Adjusted rate	54.4%	65.7%	55.5%	46.1%
Terminal rate	16/31 (52%)	20/33 (61%)	19/39 (49%)	12/31 (39%)
First incidence (days)	641	473	586	504
Poly-3 test	P=0.084N	P=0.176	P=0.537	P=0.278N
Preputial Gland: Adenoma				
Overall rate	1/50 (2%)	4/50 (8%)	6/50 (12%)	0/50 (0%)
Adjusted rate	2.3%	8.9%	12.8%	0.0%
Terminal rate	1/31 (3%)	2/33 (6%)	6/39 (15%)	0/31 (0%)
First incidence (days)	729 (T)	576	729 (T)	—
Poly-3 test	P=0.307N	P=0.185	P=0.068	P=0.509N
Skin: Keratoacanthoma				
Overall rate	3/50 (6%)	1/50 (2%)	3/50 (6%)	0/50 (0%)
Adjusted rate	6.8%	2.3%	6.4%	0.0%
Terminal rate	2/31 (7%)	1/33 (3%)	2/39 (5%)	0/31 (0%)
First incidence (days)	694	729 (T)	690	—
Poly-3 test	P=0.190N	P=0.303N	P=0.630N	P=0.127N
Skin: Squamous Cell Papilloma				
Overall rate	4/50 (8%)	2/50 (4%)	5/50 (10%)	1/50 (2%)
Adjusted rate	9.1%	4.5%	10.6%	2.4%
Terminal rate	3/31 (10%)	1/33 (3%)	4/39 (10%)	1/31 (3%)
First incidence (days)	662	682	649	729 (T)
Poly-3 test	P=0.285N	P=0.333N	P=0.544	P=0.193N
Skin: Squamous Cell Papilloma or Keratoacanthoma				
Overall rate	7/50 (14%)	3/50 (6%)	8/50 (16%)	1/50 (2%)
Adjusted rate	15.8%	6.8%	16.9%	2.4%
Terminal rate	5/31 (16%)	2/33 (6%)	6/39 (15%)	1/31 (3%)
First incidence (days)	662	682	649	729 (T)
Poly-3 test	P=0.122N	P=0.154N	P=0.559	P=0.035N
Skin: Squamous Cell Papilloma, Keratoacanthoma, or Squamous Cell Carcinoma				
Overall rate	7/50 (14%)	3/50 (6%)	9/50 (18%)	1/50 (2%)
Adjusted rate	15.8%	6.8%	19.0%	2.4%
Terminal rate	5/31 (16%)	2/33 (6%)	7/39 (18%)	1/31 (3%)
First incidence (days)	662	682	649	729 (T)
Poly-3 test	P=0.142N	P=0.154N	P=0.452	P=0.035N
Skin: Squamous Cell Papilloma, Keratoacanthoma, Basal Cell Adenoma, or Squamous Cell Carcinoma				
Overall rate	7/50 (14%)	5/50 (10%)	10/50 (20%)	1/50 (2%)
Adjusted rate	15.8%	11.3%	21.1%	2.4%
Terminal rate	5/31 (16%)	4/33 (12%)	8/39 (21%)	1/31 (3%)
First incidence (days)	662	682	649	729 (T)
Poly-3 test	P=0.101N	P=0.375N	P=0.353	P=0.035N
Skin (Subcutaneous Tissue): Fibroma				
Overall rate	6/50 (12%)	4/50 (8%)	1/50 (2%)	1/50 (2%)
Adjusted rate	13.6%	9.1%	2.1%	2.4%
Terminal rate	4/31 (13%)	4/33 (12%)	1/39 (3%)	1/31 (3%)
First incidence (days)	685	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.022N	P=0.367N	P=0.046N	P=0.063N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Skin (Subcutaneous Tissue): Fibrosarcoma				
Overall rate	3/50 (6%)	2/50 (4%)	0/50 (0%)	0/50 (0%)
Adjusted rate	6.8%	4.5%	0.0%	0.0%
Terminal rate	1/31 (3%)	1/33 (3%)	0/39 (0%)	0/31 (0%)
First incidence (days)	702	568	—	—
Poly-3 test	P=0.036N	P=0.492N	P=0.107N	P=0.127N
Skin (Subcutaneous Tissue): Fibrosarcoma or Fibrous Histiocytoma				
Overall rate	3/50 (6%)	2/50 (4%)	0/50 (0%)	1/50 (2%)
Adjusted rate	6.8%	4.5%	0.0%	2.4%
Terminal rate	1/31 (3%)	1/33 (3%)	0/39 (0%)	0/31 (0%)
First incidence (days)	702	568	—	687
Poly-3 test	P=0.157N	P=0.492N	P=0.107N	P=0.321N
Skin (Subcutaneous Tissue): Fibroma, Fibrous Histiocytoma, or Fibrosarcoma				
Overall rate	8/50 (16%)	6/50 (12%)	1/50 (2%)	2/50 (4%)
Adjusted rate	18.1%	13.4%	2.1%	4.8%
Terminal rate	5/31 (16%)	5/33 (15%)	1/39 (3%)	1/31 (3%)
First incidence (days)	685	568	729 (T)	687
Poly-3 test	P=0.012N	P=0.375N	P=0.012N	P=0.052N
Testes: Adenoma				
Overall rate	41/50 (82%)	39/50 (78%)	45/50 (90%)	34/50 (68%)
Adjusted rate	86.4%	83.2%	91.2%	76.1%
Terminal rate	29/31 (94%)	30/33 (91%)	36/39 (92%)	26/31 (84%)
First incidence (days)	563	540	489	401
Poly-3 test	P=0.161N	P=0.435N	P=0.326	P=0.138N
Thyroid Gland (C-cell): Adenoma				
Overall rate	6/50 (12%)	3/50 (6%)	7/50 (14%)	4/50 (8%)
Adjusted rate	13.6%	6.8%	14.9%	9.5%
Terminal rate	4/31 (13%)	3/33 (9%)	7/39 (18%)	3/31 (10%)
First incidence (days)	662	729 (T)	729 (T)	715
Poly-3 test	P=0.545N	P=0.241N	P=0.547	P=0.401N
Thyroid Gland (C-cell): Adenoma or Carcinoma				
Overall rate	8/50 (16%)	5/50 (10%)	7/50 (14%)	4/50 (8%)
Adjusted rate	18.1%	11.3%	14.9%	9.5%
Terminal rate	6/31 (19%)	5/33 (15%)	7/39 (18%)	3/31 (10%)
First incidence (days)	662	729 (T)	729 (T)	715
Poly-3 test	P=0.251N	P=0.274N	P=0.449N	P=0.200N
All Organs: Mononuclear Cell Leukemia				
Overall rate	23/50 (46%)	10/50 (20%)	2/50 (4%)	2/50 (4%)
Adjusted rate	48.7%	22.5%	4.3%	4.8%
Terminal rate	12/31 (39%)	7/33 (21%)	1/39 (3%)	2/31 (7%)
First incidence (days)	544	673	690	729 (T)
Poly-3 test	P<0.001N	P=0.006N	P<0.001N	P<0.001N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
All Organs: Malignant Mesothelioma				
Overall rate	3/50 (6%)	5/50 (10%)	2/50 (4%)	3/50 (6%)
Adjusted rate	6.8%	11.1%	4.2%	7.1%
Terminal rate	2/31 (7%)	2/33 (6%)	0/39 (0%)	2/31 (7%)
First incidence (days)	694	590	619	658
Poly-3 test	P=0.379N	P=0.371	P=0.465N	P=0.644
All Organs: Benign Neoplasms				
Overall rate	47/50 (94%)	50/50 (100%)	50/50 (100%)	48/50 (96%)
Adjusted rate	96.6%	100.0%	100.0%	99.4%
Terminal rate	30/31 (97%)	33/33 (100%)	39/39 (100%)	31/31 (100%)
First incidence (days)	563	473	489	401
Poly-3 test	P=0.294	P=0.269	P=0.269	P=0.372
All Organs: Malignant Neoplasms				
Overall rate	30/50 (60%)	19/50 (38%)	12/50 (24%)	12/50 (24%)
Adjusted rate	62.6%	40.8%	24.8%	27.5%
Terminal rate	16/31 (52%)	11/33 (33%)	6/39 (15%)	6/31 (19%)
First incidence (days)	544	540	489	563
Poly-3 test	P<0.001N	P=0.024N	P<0.001N	P<0.001N
All Organs: Benign or Malignant Neoplasms				
Overall rate	49/50 (98%)	50/50 (100%)	50/50 (100%)	48/50 (96%)
Adjusted rate	99.5%	100.0%	100.0%	99.4%
Terminal rate	31/31 (100%)	33/33 (100%)	39/39 (100%)	31/31 (100%)
First incidence (days)	544	473	489	401
Poly-3 test	P=0.892N	P=0.999	P=0.999	P=0.996N

(T) Terminal sacrifice
[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal medulla, lung, pancreas, pancreatic islets, pituitary gland, preputial gland, testes, 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 is the P value associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in dosed group is indicated by **N**.
[e] Not applicable; no neoplasms in animal group

TABLE A3
Historical Incidence of Pancreatic Islet Neoplasms in Control Male F/344N Rats[a]

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
Historical Incidence: Gavage Studies (all vehicles)			
Elmiron® (water)	1/50	0/50	1/50
2,4-Hexadienal (corn oil)	4/50	0/50	4/50
Methacrylonitrile (water)	0/50	0/50	0/50
Total (%)	5/150 (3.3%)	0/150	5/150 (3.3%)
Range	0%-8%		0%-8%
Overall Historical Incidence: All Routes			
Total (%)	66/1,448 (4.6%)	26/1,448 (1.8%)	92/1,448 (6.4%)
Mean ± standard deviation	4.8% ± 3.1%	2.0% ± 2.7%	6.8% ± 4.4%
Range	0%-10%	0%-8%	0%-14%

[a] Data as of January 28, 2005

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths			1	2
Moribund	13	7	5	10
Natural deaths	6	10	5	7
Survivors				
Died last week of study	2			1
Terminal sacrifice	29	33	39	30
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Perforation				2 (4%)
Intestine large, colon	(50)	(50)	(50)	(50)
Inflammation				1 (2%)
Intestine large, cecum	(49)	(50)	(49)	(47)
Inflammation, chronic				1 (2%)
Intestine small, duodenum	(50)	(50)	(50)	(48)
Ectopic tissue	1 (2%)	1 (2%)		
Ulcer			1 (2%)	
Intestine small, ileum	(48)	(50)	(49)	(48)
Hyperplasia, lymphoid				1 (2%)
Liver	(50)	(50)	(50)	(50)
Basophilic focus	5 (10%)	6 (12%)	8 (16%)	6 (12%)
Clear cell focus	14 (28%)	13 (26%)	16 (32%)	12 (24%)
Degeneration, cystic	5 (10%)	3 (6%)	2 (4%)	1 (2%)
Eosinophilic focus	5 (10%)	1 (2%)	4 (8%)	3 (6%)
Hepatodiaphragmatic nodule	9 (18%)	2 (4%)	3 (6%)	4 (8%)
Inflammation, chronic		1 (2%)		1 (2%)
Necrosis, focal	1 (2%)	1 (2%)		
Bile duct, hyperplasia	41 (82%)	35 (70%)	38 (76%)	37 (74%)
Centrilobular, necrosis	2 (4%)		1 (2%)	2 (4%)
Hepatocyte, vacuolization cytoplasmic	5 (10%)	1 (2%)	2 (4%)	3 (6%)
Serosa, inflammation				1 (2%)
Mesentery	(16)	(11)	(9)	(12)
Accessory spleen				1 (8%)
Fibrosis	1 (6%)			
Fat, necrosis	14 (88%)	9 (82%)	8 (89%)	7 (58%)
Oral mucosa	(1)			(4)
Pharyngeal, cyst				2 (50%)
Pharyngeal, inflammation				2 (50%)
Pancreas	(50)	(50)	(50)	(50)
Atrophy	43 (86%)	31 (62%)	35 (70%)	32 (64%)
Atrophy, focal				1 (2%)
Cyst				1 (2%)
Inflammation, chronic				1 (2%)
Acinus, hyperplasia, focal	4 (8%)	6 (12%)	15 (30%)	12 (24%)
Salivary glands	(50)	(50)	(50)	(50)
Hyperplasia				1 (2%)

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

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Alimentary System (continued)				
Stomach, forestomach	(50)	(50)	(50)	(50)
Edema	6 (12%)		1 (2%)	2 (4%)
Erosion	3 (6%)			1 (2%)
Ulcer	4 (8%)	1 (2%)	2 (4%)	2 (4%)
Epithelium, hyperplasia	4 (8%)		3 (6%)	3 (6%)
Stomach, glandular	(50)	(50)	(50)	(50)
Edema	1 (2%)			2 (4%)
Erosion	4 (8%)	2 (4%)	1 (2%)	1 (2%)
Hyperplasia	1 (2%)			
Inflammation	1 (2%)			
Mineralization		1 (2%)		
Ulcer	2 (4%)	1 (2%)	2 (4%)	1 (2%)
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	47 (94%)	46 (92%)	47 (94%)	45 (90%)
Mineralization		1 (2%)		
Thrombosis		1 (2%)	1 (2%)	
Pericardium, inflammation, granulomatous	1 (2%)			
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Accessory adrenal cortical nodule	23 (46%)	23 (46%)	28 (56%)	25 (50%)
Degeneration, fatty	7 (14%)	6 (12%)	7 (14%)	2 (4%)
Hyperplasia, focal	6 (12%)	5 (10%)	5 (10%)	5 (10%)
Hypertrophy, focal	6 (12%)	5 (10%)	9 (18%)	8 (16%)
Necrosis	1 (2%)			1 (2%)
Adrenal medulla	(50)	(50)	(50)	(50)
Hyperplasia	7 (14%)	12 (24%)	8 (16%)	2 (4%)
Thrombosis			1 (2%)	
Islets, pancreatic	(50)	(50)	(50)	(50)
Hyperplasia	13 (26%)	13 (26%)	17 (34%)	26 (52%)
Metaplasia, hepatocyte			1 (2%)	
Pituitary gland	(50)	(50)	(50)	(50)
Pars distalis, angiectasis	14 (28%)	30 (60%)	16 (32%)	15 (30%)
Pars distalis, cyst	6 (12%)	3 (6%)	6 (12%)	5 (10%)
Pars distalis, hyperplasia, focal	9 (18%)	8 (16%)	9 (18%)	13 (26%)
Pars distalis, hyperplasia, multifocal		1 (2%)		
Pars intermedia, cyst	1 (2%)			
Thyroid gland	(50)	(50)	(50)	(50)
Ultimobranchial cyst	3 (6%)	1 (2%)		2 (4%)
C-cell, hyperplasia	8 (16%)	2 (4%)	9 (18%)	4 (8%)
Follicle, cyst	1 (2%)	2 (4%)	4 (8%)	1 (2%)
Follicular cell, hyperplasia			1 (2%)	
General Body System				
None				

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Genital System				
Coagulating gland	(1)		(2)	
Inflammation			1 (50%)	
Penis	(1)			
Fibrosis	1 (100%)			
Preputial gland	(50)	(50)	(50)	(50)
Cyst	4 (8%)			3 (6%)
Inflammation, chronic	5 (10%)	5 (10%)	4 (8%)	4 (8%)
Prostate	(50)	(50)	(50)	(50)
Inflammation, chronic	31 (62%)	28 (56%)	27 (54%)	26 (52%)
Testes	(50)	(50)	(50)	(50)
Bilateral, germinal epithelium, atrophy	1 (2%)		2 (4%)	
Germinal epithelium, atrophy	1 (2%)			1 (2%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hyperplasia	7 (14%)	7 (14%)	4 (8%)	9 (18%)
Lymph node	(21)	(17)	(10)	(18)
Deep cervical, hyperplasia, lymphoid		1 (6%)	1 (10%)	
Deep cervical, inflammation, granulomatous	1 (5%)			
Mediastinal, hyperplasia, lymphoid	9 (43%)	9 (53%)	10 (100%)	15 (83%)
Mediastinal, inflammation, granulomatous	1 (5%)			
Pancreatic, hyperplasia, lymphoid	2 (10%)	4 (24%)	1 (10%)	1 (6%)
Lymph node, mesenteric	(50)	(50)	(50)	(50)
Hyperplasia, lymphoid	7 (14%)	3 (6%)	4 (8%)	9 (18%)
Spleen	(50)	(50)	(50)	(50)
Fibrosis	1 (2%)	2 (4%)	2 (4%)	2 (4%)
Hematopoietic cell proliferation	11 (22%)	12 (24%)	17 (34%)	20 (40%)
Hemorrhage				2 (4%)
Necrosis	2 (4%)			
Capsule, fibrosis	1 (2%)	7 (14%)	12 (24%)	30 (60%)
Lymphoid follicle, hyperplasia		1 (2%)	1 (2%)	
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Cyst	23 (46%)	18 (36%)	20 (40%)	20 (40%)
Hyperplasia	1 (2%)		1 (2%)	
Skin	(50)	(50)	(50)	(50)
Hyperkeratosis				1 (2%)
Inflammation, chronic			5 (10%)	1 (2%)
Lip, inflammation	1 (2%)			
Subcutaneous tissue, mineralization				1 (2%)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Cyst	1 (2%)			
Cranium, osteopetrosis		1 (2%)		

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Nervous System				
Brain	(50)	(50)	(50)	(50)
Compression	7 (14%)	10 (20%)	9 (18%)	6 (12%)
Hemorrhage	1 (2%)	2 (4%)		
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Congestion	3 (6%)	4 (8%)	3 (6%)	7 (14%)
Foreign body			1 (2%)	2 (4%)
Hemorrhage	1 (2%)			
Infiltration cellular, histiocyte	28 (56%)	29 (58%)	36 (72%)	26 (52%)
Inflammation, chronic	29 (58%)	23 (46%)	36 (72%)	30 (60%)
Inflammation, suppurative				1 (2%)
Necrosis			1 (2%)	1 (2%)
Alveolar epithelium, hyperplasia	7 (14%)	9 (18%)	4 (8%)	6 (12%)
Mediastinum, inflammation, granulomatous	1 (2%)			
Nose	(50)	(50)	(50)	(50)
Foreign body	7 (14%)	4 (8%)	12 (24%)	7 (14%)
Inflammation, chronic	12 (24%)	3 (6%)	20 (40%)	15 (30%)
Pleura	(1)			
Inflammation, granulomatous	1 (100%)			
Trachea	(50)	(50)	(50)	(50)
Foreign body				1 (2%)
Inflammation	1 (2%)		1 (2%)	
Necrosis				1 (2%)
Perforation			1 (2%)	
Special Senses System				
Eye	(50)	(49)	(48)	(48)
Cataract	1 (2%)	3 (6%)	4 (8%)	3 (6%)
Inflammation, chronic	1 (2%)		1 (2%)	1 (2%)
Phthisis bulbi				1 (2%)
Anterior chamber, inflammation, acute				1 (2%)
Bilateral, anterior chamber, inflammation, acute		1 (2%)		
Retina, degeneration	1 (2%)	2 (4%)	4 (8%)	3 (6%)
Harderian gland	(50)	(50)	(50)	(50)
Hyperplasia			1 (2%)	1 (2%)
Inflammation, chronic	2 (4%)	3 (6%)	1 (2%)	4 (8%)
Pigmentation	2 (4%)	1 (2%)	1 (2%)	3 (6%)
Urinary System				
Kidney	(50)	(50)	(50)	(50)
Cyst			1 (2%)	
Infarct	2 (4%)			
Nephropathy	35 (70%)	39 (78%)	33 (66%)	34 (68%)
Urethra				(1)
Inflammation				1 (100%)
Urinary bladder	(50)	(50)	(50)	(49)
Hemorrhage	1 (2%)			1 (2%)
Hyperplasia			1 (2%)	
Inflammation, chronic		2 (4%)		1 (2%)

APPENDIX B
SUMMARY OF LESIONS IN FEMALE RATS IN THE 2-YEAR GAVAGE STUDY OF METHYLENE BLUE TRIHYDRATE

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death				1
Moribund	9	13	5	3
Natural deaths	6	4	9	11
Survivors				
Died last week of study			2	
Terminal sacrifice	35	32	34	35
Other[b]		1		
Animals examined microscopically	50	49	50	50
Alimentary System				
Liver	(50)	(49)	(50)	(50)
Granulosa cell tumor malignant, metastatic, ovary			1 (2%)	
Mesentery	(17)	(14)	(12)	(8)
Granulosa cell tumor malignant, metastatic, ovary			1 (8%)	
Oral mucosa	(2)			(1)
Pharyngeal, squamous cell carcinoma	1 (50%)			
Pancreas	(49)	(48)	(48)	(49)
Acinus, adenoma	1 (2%)			
Salivary glands	(50)	(49)	(50)	(50)
Schwannoma malignant			1 (2%)	
Stomach, forestomach	(50)	(49)	(49)	(49)
Squamous cell papilloma	1 (2%)			
Tongue	(1)	(1)	(1)	(1)
Squamous cell papilloma			1 (100%)	
Cardiovascular System				
Heart	(50)	(49)	(50)	(50)
Schwannoma benign			1 (2%)	
Endocrine System				
Adrenal cortex	(50)	(49)	(50)	(50)
Adenoma	1 (2%)			
Adrenal medulla	(50)	(49)	(50)	(50)
Pheochromocytoma complex		1 (2%)		
Pheochromocytoma benign	2 (4%)		1 (2%)	1 (2%)
Islets, pancreatic	(49)	(48)	(48)	(49)
Adenoma	2 (4%)		1 (2%)	1 (2%)
Pituitary gland	(50)	(49)	(50)	(50)
Pars distalis, adenoma	36 (72%)	32 (65%)	31 (62%)	28 (56%)
Thyroid gland	(50)	(49)	(50)	(50)
Bilateral, C-cell, adenoma	1 (2%)			2 (4%)
C-cell, adenoma	6 (12%)	5 (10%)	8 (16%)	1 (2%)
C-cell, carcinoma		1 (2%)		
Follicular cell, adenoma			1 (2%)	
Follicular cell, carcinoma		1 (2%)		
General Body System				
None				

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Genital System				
Clitoral gland	(50)	(48)	(50)	(50)
Adenoma	7 (14%)	10 (21%)	8 (16%)	9 (18%)
Carcinoma	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Ovary	(50)	(49)	(50)	(50)
Granulosa cell tumor malignant			1 (2%)	
Uterus	(50)	(49)	(50)	(50)
Adenoma			1 (2%)	
Polyp stromal	13 (26%)	14 (29%)	13 (26%)	7 (14%)
Sarcoma stromal	2 (4%)		1 (2%)	1 (2%)
Hematopoietic System				
Bone marrow	(50)	(49)	(50)	(50)
Histiocytic sarcoma	1 (2%)			
Lymph node	(15)	(8)	(11)	(3)
Lymph node, mesenteric	(49)	(49)	(50)	(50)
Spleen	(49)	(48)	(49)	(49)
Granulosa cell tumor malignant, metastatic, ovary			1 (2%)	
Histiocytic sarcoma		1 (2%)		
Thymus	(50)	(49)	(50)	(50)
Thymoma benign	1 (2%)			
Integumentary System				
Mammary gland	(50)	(49)	(50)	(50)
Adenoma	1 (2%)	2 (4%)		
Carcinoma	1 (2%)			
Fibroadenoma	20 (40%)	17 (35%)	22 (44%)	13 (26%)
Fibroadenoma, multiple	8 (16%)	13 (27%)	6 (12%)	4 (8%)
Skin	(50)	(49)	(50)	(50)
Basal cell carcinoma	1 (2%)			
Squamous cell papilloma	1 (2%)	1 (2%)		
Trichoepithelioma	1 (2%)		1 (2%)	
Pinna, neural crest tumor				1 (2%)
Subcutaneous tissue, fibroma	1 (2%)	1 (2%)	2 (4%)	
Subcutaneous tissue, fibrosarcoma	1 (2%)	3 (6%)		
Subcutaneous tissue, hemangiosarcoma	1 (2%)			
Subcutaneous tissue, lipoma		1 (2%)		1 (2%)
Musculoskeletal System				
Bone	(50)	(49)	(50)	(50)
Osteosarcoma	1 (2%)			
Vertebra, chordoma				1 (2%)
Nervous System				
Brain	(50)	(49)	(50)	(50)
Astrocytoma malignant			1 (2%)	
Peripheral nerve	(3)	(4)	(4)	(2)
Schwannoma malignant		1 (25%)		

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Respiratory System				
Lung	(50)	(49)	(50)	(50)
Alveolar/bronchiolar adenoma		1 (2%)	1 (2%)	
Chordoma, metastatic, bone				1 (2%)
Pheochromocytoma complex, metastatic, adrenal medulla		1 (2%)		
Special Senses System				
Eye	(50)	(49)	(50)	(50)
Harderian gland	(50)	(49)	(50)	(50)
Zymbal's gland		(1)	(1)	
Carcinoma		1 (100%)	1 (100%)	
Urinary System				
Kidney	(50)	(49)	(50)	(50)
Renal tubule, adenoma				1 (2%)
Urinary bladder	(50)	(49)	(50)	(50)
Transitional epithelium, papilloma		1 (2%)		
Systemic Lesions				
Multiple organs[c]	(50)	(49)	(50)	(50)
Histiocytic sarcoma	1 (2%)	1 (2%)		
Leukemia mononuclear	12 (24%)	6 (12%)	3 (6%)	2 (4%)
Neoplasm Summary				
Total animals with primary neoplasms[d]	50	48	46	40
Total primary neoplasms	125	114	107	74
Total animals with benign neoplasms	47	45	45	40
Total benign neoplasms	103	98	98	68
Total animals with malignant neoplasms	19	16	8	5
Total malignant neoplasms	22	16	9	5
Total animals with metastatic neoplasms		1	1	1
Total metastatic neoplasms		1	3	1
Total animals with uncertain neoplasms-benign or malignant				1
Total uncertain neoplasms				1

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Animal escaped from cage and was removed from study without necropsy.
[c] Number of animals with any tissue examined microscopically
[d] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Clitoral Gland: Adenoma				
Overall rate[a]	7/50 (14%)	10/48 (21%)	8/50 (16%)	9/50 (18%)
Adjusted rate[b]	15.6%	23.0%	17.4%	21.9%
Terminal rate[c]	6/35 (17%)	8/32 (25%)	3/36 (8%)	8/35 (23%)
First incidence (days)	668	663	492	725
Poly-3 test[d]	P=0.408	P=0.269	P=0.519	P=0.318
Clitoral Gland: Adenoma or Carcinoma				
Overall rate	8/50 (16%)	11/48 (23%)	9/50 (18%)	10/50 (20%)
Adjusted rate	17.8%	25.3%	19.6%	24.3%
Terminal rate	7/35 (20%)	9/32 (28%)	4/36 (11%)	9/35 (26%)
First incidence (days)	668	663	492	725
Poly-3 test	P=0.405	P=0.274	P=0.521	P=0.317
Mammary Gland: Fibroadenoma				
Overall rate	28/50 (56%)	30/49 (61%)	28/50 (56%)	17/50 (34%)
Adjusted rate	61.1%	64.9%	60.0%	40.2%
Terminal rate	23/35 (66%)	21/32 (66%)	20/36 (56%)	15/35 (43%)
First incidence (days)	652	568	492	509
Poly-3 test	P=0.013N	P=0.432	P=0.543N	P=0.036N
Mammary Gland: Fibroadenoma or Adenoma				
Overall rate	29/50 (58%)	32/49 (65%)	28/50 (56%)	17/50 (34%)
Adjusted rate	63.3%	69.2%	60.0%	40.2%
Terminal rate	24/35 (69%)	23/32 (72%)	20/36 (56%)	15/35 (43%)
First incidence (days)	652	568	492	509
Poly-3 test	P=0.004N	P=0.345	P=0.456N	P=0.021N
Mammary Gland: Fibroadenoma, Adenoma, or Carcinoma				
Overall rate	30/50 (60%)	32/49 (65%)	28/50 (56%)	17/50 (34%)
Adjusted rate	65.4%	69.2%	60.0%	40.2%
Terminal rate	25/35 (71%)	23/32 (72%)	20/36 (56%)	15/35 (43%)
First incidence (days)	652	568	492	509
Poly-3 test	P=0.002N	P=0.432	P=0.369N	P=0.012N
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	36/50 (72%)	32/49 (65%)	31/50 (62%)	28/50 (56%)
Adjusted rate	77.3%	69.4%	64.6%	66.7%
Terminal rate	28/35 (80%)	24/32 (75%)	21/36 (58%)	25/35 (71%)
First incidence (days)	638	568	492	573
Poly-3 test	P=0.175N	P=0.260N	P=0.122N	P=0.182N
Skin: Squamous Cell Papilloma, Trichoepithelioma, or Basal Cell Carcinoma				
Overall rate	3/50 (6%)	1/49 (2%)	1/50 (2%)	0/50 (0%)
Adjusted rate	6.7%	2.3%	2.2%	0.0%
Terminal rate	2/35 (6%)	1/32 (3%)	0/36 (0%)	0/35 (0%)
First incidence (days)	638	729 (T)	563	—[e]
Poly-3 test	P=0.100N	P=0.315N	P=0.308N	P=0.136N
Skin (Subcutaneous Tissue): Fibrosarcoma				
Overall rate	1/50 (2%)	3/49 (6%)	0/50 (0%)	0/50 (0%)
Adjusted rate	2.2%	6.7%	0.0%	0.0%
Terminal rate	0/35 (0%)	2/32 (6%)	0/36 (0%)	0/35 (0%)
First incidence (days)	344	395	—	—
Poly-3 test	P=0.094N	P=0.298	P=0.506N	P=0.521N

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Skin (Subcutaneous Tissue): Fibroma or Fibrosarcoma				
Overall rate	2/50 (4%)	4/49 (8%)	2/50 (4%)	0/50 (0%)
Adjusted rate	4.4%	9.0%	4.5%	0.0%
Terminal rate	1/35 (3%)	3/32 (9%)	1/36 (3%)	0/35 (0%)
First incidence (days)	344	395	702	—
Poly-3 test	P=0.094N	P=0.327	P=0.683	P=0.261N
Thyroid Gland (C-cell): Adenoma				
Overall rate	7/50 (14%)	5/49 (10%)	8/50 (16%)	3/50 (6%)
Adjusted rate	15.5%	11.4%	18.1%	7.3%
Terminal rate	5/35 (14%)	5/32 (16%)	7/36 (19%)	3/35 (9%)
First incidence (days)	648	729 (T)	723	729 (T)
Poly-3 test	P=0.262N	P=0.402N	P=0.480	P=0.199N
Thyroid Gland (C-cell): Adenoma or Carcinoma				
Overall rate	7/50 (14%)	6/49 (12%)	8/50 (16%)	3/50 (6%)
Adjusted rate	15.5%	13.7%	18.1%	7.3%
Terminal rate	5/35 (14%)	6/32 (19%)	7/36 (19%)	3/35 (9%)
First incidence (days)	648	729 (T)	723	729 (T)
Poly-3 test	P=0.216N	P=0.525N	P=0.480	P=0.199N
Uterus: Stromal Polyp				
Overall rate	13/50 (26%)	14/49 (29%)	13/50 (26%)	7/50 (14%)
Adjusted rate	28.2%	30.5%	28.6%	16.3%
Terminal rate	9/35 (26%)	6/32 (19%)	10/36 (28%)	5/35 (14%)
First incidence (days)	591	576	540	312
Poly-3 test	P=0.088N	P=0.497	P=0.577	P=0.136N
Uterus: Stromal Polyp or Stromal Sarcoma				
Overall rate	15/50 (30%)	14/49 (29%)	13/50 (26%)	8/50 (16%)
Adjusted rate	32.0%	30.5%	28.6%	18.6%
Terminal rate	9/35 (26%)	6/32 (19%)	10/36 (28%)	6/35 (17%)
First incidence (days)	516	576	540	312
Poly-3 test	P=0.086N	P=0.527N	P=0.448N	P=0.113N
All Organs: Mononuclear Cell Leukemia				
Overall rate	12/50 (24%)	6/49 (12%)	3/50 (6%)	2/50 (4%)
Adjusted rate	25.8%	13.4%	6.6%	4.9%
Terminal rate	6/35 (17%)	2/32 (6%)	1/36 (3%)	1/35 (3%)
First incidence (days)	591	585	521	725
Poly-3 test	P=0.004N	P=0.108N	P=0.012N	P=0.007N
All Organs: Benign Neoplasms				
Overall rate	47/50 (94%)	45/49 (92%)	45/50 (90%)	40/50 (80%)
Adjusted rate	98.1%	94.6%	92.0%	88.9%
Terminal rate	35/35 (100%)	31/32 (97%)	33/36 (92%)	32/35 (91%)
First incidence (days)	591	568	492	312
Poly-3 test	P=0.039N	P=0.335N	P=0.155N	P=0.053N

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
All Organs: Malignant Neoplasms				
Overall rate	19/50 (38%)	16/49 (33%)	8/50 (16%)	5/50 (10%)
Adjusted rate	38.7%	34.4%	17.3%	12.2%
Terminal rate	8/35 (23%)	9/32 (28%)	3/36 (8%)	4/35 (11%)
First incidence (days)	344	395	521	725
Poly-3 test	P<0.001N	P=0.409N	P=0.016N	P=0.004N
All Organs: Benign or Malignant Neoplasms				
Overall rate	50/50 (100%)	48/49 (98%)	46/50 (92%)	40/50 (80%)
Adjusted rate	100.0%	98.0%	94.1%	88.9%
Terminal rate	35/35 (100%)	31/32 (97%)	34/36 (94%)	32/35 (91%)
First incidence (days)	344	395	492	312
Poly-3 test	P=0.003N	P=0.496N	P=0.102N	P=0.013N

(T) Terminal sacrifice
[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for clitoral gland, 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 is the P value associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in dosed group is indicated by **N.**
[e] Not applicable; no neoplasms in animal group

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental death				1
Moribund	9	13	5	3
Natural deaths	6	4	9	11
Survivors				
Died last week of study			2	
Terminal sacrifice	35	32	34	35
Other[b]		1		
Animals examined microscopically	50	49	50	50
Alimentary System				
Esophagus	(50)	(49)	(50)	(50)
Perforation			1 (2%)	
Intestine large, colon	(50)	(48)	(49)	(48)
Cyst				1 (2%)
Intestine small, duodenum	(49)	(48)	(49)	(48)
Ectopic tissue				1 (2%)
Liver	(50)	(49)	(50)	(50)
Basophilic focus	37 (74%)	34 (69%)	38 (76%)	40 (80%)
Clear cell focus	8 (16%)	5 (10%)	9 (18%)	8 (16%)
Degeneration, cystic	1 (2%)	1 (2%)		1 (2%)
Eosinophilic focus	3 (6%)	2 (4%)		
Hepatodiaphragmatic nodule	7 (14%)	4 (8%)	7 (14%)	4 (8%)
Inflammation, chronic	6 (12%)	4 (8%)	4 (8%)	5 (10%)
Necrosis, focal	1 (2%)		1 (2%)	
Bile duct, hyperplasia	7 (14%)	5 (10%)	6 (12%)	2 (4%)
Centrilobular, necrosis	2 (4%)		1 (2%)	
Hepatocyte, vacuolization cytoplasmic	4 (8%)	4 (8%)		
Mesentery	(17)	(14)	(12)	(8)
Accessory spleen		1 (7%)		
Fat, necrosis	17 (100%)	14 (100%)	11 (92%)	8 (100%)
Oral mucosa	(2)			(1)
Pharyngeal, cyst	1 (50%)			1 (100%)
Pancreas	(49)	(48)	(48)	(49)
Atrophy	26 (53%)	20 (42%)	23 (48%)	23 (47%)
Inflammation		1 (2%)		
Acinus, hyperplasia, focal	3 (6%)	1 (2%)	2 (4%)	1 (2%)
Salivary glands	(50)	(49)	(50)	(50)
Inflammation			1 (2%)	
Stomach, forestomach	(50)	(49)	(49)	(49)
Edema	2 (4%)	3 (6%)	1 (2%)	
Erosion				1 (2%)
Inflammation, chronic active	1 (2%)			2 (4%)
Ulcer	4 (8%)	3 (6%)	1 (2%)	1 (2%)
Epithelium, hyperplasia	2 (4%)	2 (4%)	2 (4%)	3 (6%)
Stomach, glandular	(50)	(49)	(48)	(49)
Edema	2 (4%)	1 (2%)	1 (2%)	
Erosion	2 (4%)	2 (4%)		
Ulcer	2 (4%)	1 (2%)	1 (2%)	

[a] Number of animals examined microscopically at the site and the number of animals with lesion
[b] Animal escaped from cage and was removed from study without necropsy.

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Alimentary System (continued)				
Tongue	(1)	(1)	(1)	(1)
Cyst		1 (100%)		
Hyperplasia	1 (100%)			1 (100%)
Inflammation		1 (100%)		
Cardiovascular System				
Heart	(50)	(49)	(50)	(50)
Cardiomyopathy	36 (72%)	41 (84%)	36 (72%)	32 (64%)
Thrombosis		1 (2%)		
Endocrine System				
Adrenal cortex	(50)	(49)	(50)	(50)
Accessory adrenal cortical nodule	9 (18%)	7 (14%)	8 (16%)	12 (24%)
Degeneration, fatty	11 (22%)	13 (27%)	13 (26%)	8 (16%)
Hyperplasia, focal	5 (10%)	8 (16%)	8 (16%)	6 (12%)
Hypertrophy, focal	12 (24%)	11 (22%)	13 (26%)	7 (14%)
Necrosis	2 (4%)	1 (2%)		
Adrenal medulla	(50)	(49)	(50)	(50)
Hyperplasia	1 (2%)	2 (4%)	1 (2%)	
Islets, pancreatic	(49)	(48)	(48)	(49)
Hyperplasia	13 (27%)	15 (31%)	15 (31%)	15 (31%)
Metaplasia, hepatocyte	2 (4%)			
Pituitary gland	(50)	(49)	(50)	(50)
Pars distalis, angiectasis	28 (56%)	27 (55%)	29 (58%)	21 (42%)
Pars distalis, cyst	21 (42%)	19 (39%)	16 (32%)	23 (46%)
Pars distalis, hyperplasia, focal	5 (10%)	8 (16%)	6 (12%)	6 (12%)
Pars intermedia, cyst	1 (2%)			
Thyroid gland	(50)	(49)	(50)	(50)
Ultimobranchial cyst	2 (4%)		1 (2%)	
C-cell, hyperplasia	10 (20%)	11 (22%)	7 (14%)	5 (10%)
Follicle, cyst	2 (4%)	1 (2%)	2 (4%)	4 (8%)
General Body System				
None				
Genital System				
Clitoral gland	(50)	(48)	(50)	(50)
Cyst	4 (8%)	7 (15%)	4 (8%)	6 (12%)
Inflammation, chronic	1 (2%)	5 (10%)	2 (4%)	4 (8%)
Ovary	(50)	(49)	(50)	(50)
Cyst	11 (22%)	4 (8%)	9 (18%)	10 (20%)
Uterus	(50)	(49)	(50)	(50)
Cyst	1 (2%)	3 (6%)	2 (4%)	3 (6%)
Hemorrhage	1 (2%)			1 (2%)
Hydrometra	1 (2%)	4 (8%)	2 (4%)	1 (2%)
Cervix, cyst	1 (2%)			
Endometrium, hyperplasia, cystic	4 (8%)	1 (2%)		2 (4%)
Vagina			(1)	
Cyst			1 (100%)	

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Hematopoietic System				
Bone marrow	(50)	(49)	(50)	(50)
Hyperplasia	8 (16%)	8 (16%)	9 (18%)	6 (12%)
Lymph node	(15)	(8)	(11)	(3)
Mediastinal, congestion	1 (7%)			
Mediastinal, hyperplasia, lymphoid	5 (33%)	7 (88%)	8 (73%)	2 (67%)
Pancreatic, hyperplasia, lymphoid	1 (7%)		1 (9%)	1 (33%)
Lymph node, mesenteric	(49)	(49)	(50)	(50)
Hyperplasia, lymphoid		1 (2%)	1 (2%)	
Spleen	(49)	(48)	(49)	(49)
Fibrosis	2 (4%)			
Hematopoietic cell proliferation	3 (6%)	5 (10%)	7 (14%)	8 (16%)
Necrosis	1 (2%)			
Capsule, fibrosis	8 (16%)	17 (35%)	12 (24%)	20 (41%)
Lymphoid follicle, atrophy			1 (2%)	
Lymphoid follicle, hyperplasia	1 (2%)			
Integumentary System				
Mammary gland	(50)	(49)	(50)	(50)
Cyst			2 (4%)	1 (2%)
Galactocele		1 (2%)	1 (2%)	1 (2%)
Hyperplasia	18 (36%)	19 (39%)	9 (18%)	7 (14%)
Hyperplasia, atypical	3 (6%)	3 (6%)	1 (2%)	
Duct, dilatation	31 (62%)	28 (57%)	29 (58%)	24 (48%)
Duct, hyperplasia	2 (4%)			1 (2%)
Skin	(50)	(49)	(50)	(50)
Inflammation, chronic		2 (4%)		
Inflammation, chronic active		1 (2%)		
Musculoskeletal System				
Bone	(50)	(49)	(50)	(50)
Inflammation			1 (2%)	
Cranium, osteopetrosis	1 (2%)			
Nervous System				
Brain	(50)	(49)	(50)	(50)
Compression	3 (6%)	8 (16%)	6 (12%)	5 (10%)
Hemorrhage	1 (2%)	1 (2%)	1 (2%)	
Respiratory System				
Lung	(50)	(49)	(50)	(50)
Congestion	5 (10%)	2 (4%)	5 (10%)	8 (16%)
Foreign body				1 (2%)
Infiltration cellular, histiocyte	41 (82%)	37 (76%)	39 (78%)	38 (76%)
Inflammation, chronic	29 (58%)	33 (67%)	29 (58%)	33 (66%)
Inflammation, suppurative			1 (2%)	
Necrosis			1 (2%)	
Alveolar epithelium, hyperplasia	1 (2%)	4 (8%)	3 (6%)	2 (4%)
Nose	(50)	(49)	(50)	(50)
Foreign body	4 (8%)	3 (6%)	1 (2%)	3 (6%)
Inflammation, chronic	8 (16%)	7 (14%)	9 (18%)	6 (12%)

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Special Senses System				
Eye	(50)	(49)	(50)	(50)
Cataract	1 (2%)	2 (4%)	5 (10%)	3 (6%)
Inflammation, chronic		2 (4%)		
Anterior chamber, inflammation			1 (2%)	
Cornea, inflammation, chronic	1 (2%)		1 (2%)	2 (4%)
Retina, degeneration	2 (4%)	2 (4%)	4 (8%)	2 (4%)
Harderian gland	(50)	(49)	(50)	(50)
Inflammation, chronic	3 (6%)	3 (6%)	3 (6%)	4 (8%)
Pigmentation	2 (4%)	2 (4%)	1 (2%)	1 (2%)
Urinary System				
Kidney	(50)	(49)	(50)	(50)
Cyst			1 (2%)	1 (2%)
Infarct	1 (2%)		2 (4%)	2 (4%)
Nephropathy	14 (28%)	15 (31%)	21 (42%)	16 (32%)

NOT FOR DISTRIBUTION OR ATTRIBUTION

APPENDIX C
SUMMARY OF LESIONS IN MALE MICE IN THE 2-YEAR GAVAGE STUDY OF METHYLENE BLUE TRIHYDRATE

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths		2		2
Moribund	4	4	7	4
Natural deaths	11	6	5	3
Survivors				
Terminal sacrifice	35	38	38	41
Animals examined microscopically	50	50	50	50
Alimentary System				
Gallbladder	(40)	(44)	(45)	(44)
Histiocytic sarcoma			1 (2%)	
Intestine large, cecum	(45)	(46)	(46)	(47)
Carcinoma	1 (2%)			
Intestine small, duodenum	(42)	(46)	(46)	(46)
Adenoma			1 (2%)	2 (4%)
Carcinoma		1 (2%)		1 (2%)
Histiocytic sarcoma			1 (2%)	
Intestine small, jejunum	(45)	(45)	(47)	(47)
Adenoma	1 (2%)	1 (2%)	1 (2%)	
Carcinoma			2 (4%)	2 (4%)
Hepatocholangiocarcinoma, metastatic, liver		1 (2%)		
Histiocytic sarcoma			1 (2%)	
Intestine small, ileum	(43)	(45)	(44)	(47)
Carcinoma				1 (2%)
Liver	(50)	(50)	(50)	(49)
Carcinoma, metastatic, pancreas				1 (2%)
Cholangiocarcinoma		1 (2%)		
Hemangiosarcoma	2 (4%)	5 (10%)	3 (6%)	1 (2%)
Hepatoblastoma	2 (4%)	2 (4%)	2 (4%)	
Hepatocellular carcinoma	11 (22%)	9 (18%)	8 (16%)	11 (22%)
Hepatocellular carcinoma, multiple	2 (4%)	1 (2%)	3 (6%)	3 (6%)
Hepatocellular adenoma	14 (28%)	20 (40%)	14 (28%)	14 (29%)
Hepatocellular adenoma, multiple	14 (28%)	13 (26%)	12 (24%)	13 (27%)
Hepatocholangiocarcinoma		1 (2%)		
Histiocytic sarcoma			2 (4%)	
Mesentery	(5)	(11)	(5)	(4)
Carcinoma, metastatic, pancreas				1 (25%)
Hemangiosarcoma		2 (18%)		
Hepatocellular carcinoma, metastatic, liver	1 (20%)			
Hepatocholangiocarcinoma, metastatic, liver		1 (9%)		
Histiocytic sarcoma			1 (20%)	
Pancreas	(48)	(50)	(49)	(48)
Hepatocholangiocarcinoma, metastatic, liver		1 (2%)		
Acinus, carcinoma				1 (2%)
Stomach, forestomach	(50)	(50)	(50)	(48)
Squamous cell papilloma		1 (2%)	2 (4%)	2 (4%)
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Hepatocholangiocarcinoma, metastatic, liver		1 (2%)		
Histiocytic sarcoma			1 (2%)	

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Endocrine System				
Adrenal cortex	(48)	(50)	(50)	(48)
Adenoma		1 (2%)		
Histiocytic sarcoma			1 (2%)	
Subcapsular, adenoma	5 (10%)	4 (8%)	2 (4%)	5 (10%)
Adrenal medulla	(48)	(50)	(50)	(48)
Histiocytic sarcoma			1 (2%)	
Pheochromocytoma benign			1 (2%)	2 (4%)
Islets, pancreatic	(50)	(50)	(49)	(48)
Adenoma		2 (4%)	2 (4%)	1 (2%)
Histiocytic sarcoma			1 (2%)	
Thyroid gland	(50)	(50)	(49)	(48)
Follicular cell, adenoma	3 (6%)	1 (2%)	3 (6%)	1 (2%)
General Body System				
Peritoneum			(1)	
Histiocytic sarcoma			1 (100%)	
Genital System				
Coagulating gland				(1)
Epididymis	(50)	(50)	(49)	(49)
Carcinoma, metastatic, pancreas				1 (2%)
Seminal vesicle	(49)	(50)	(50)	(49)
Carcinoma				1 (2%)
Testes	(50)	(50)	(50)	(50)
Interstitial cell, adenoma			1 (2%)	1 (2%)
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hemangiosarcoma	1 (2%)	2 (4%)		
Histiocytic sarcoma			1 (2%)	
Lymph node	(5)	(3)	(3)	(6)
Bronchial, carcinoma, metastatic, pancreas				1 (17%)
Iliac, histiocytic sarcoma			1 (33%)	
Mediastinal, carcinoma, metastatic, harderian gland	1 (20%)			
Mediastinal, carcinoma, metastatic, pancreas				1 (17%)
Mediastinal, histiocytic sarcoma			1 (33%)	
Pancreatic, histiocytic sarcoma			1 (33%)	
Renal, histiocytic sarcoma			1 (33%)	
Lymph node, mandibular	(43)	(46)	(48)	(47)
Carcinoma, metastatic, harderian gland	1 (2%)			
Lymph node, mesenteric	(47)	(50)	(48)	(48)
Hepatocholangiocarcinoma, metastatic, liver		1 (2%)		
Histiocytic sarcoma			2 (4%)	
Spleen	(49)	(50)	(49)	(48)
Hemangioma				1 (2%)
Hemangiosarcoma	2 (4%)	4 (8%)	1 (2%)	1 (2%)
Histiocytic sarcoma			2 (4%)	

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Integumentary System				
Skin	(50)	(50)	(50)	(50)
Fibroma		1 (2%)		
Squamous cell papilloma			1 (2%)	
Subcutaneous tissue, hemangiosarcoma	1 (2%)	1 (2%)	1 (2%)	
Subcutaneous tissue, histiocytic sarcoma			1 (2%)	
Subcutaneous tissue, sarcoma	1 (2%)			
Subcutaneous tissue, schwannoma malignant			1 (2%)	
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Hepatocholangiocarcinoma, metastatic, liver		1 (2%)		
Osteoma			1 (2%)	
Osteosarcoma				1 (2%)
Cranium, carcinoma, metastatic, harderian gland			1 (2%)	
Vertebra, hemangiosarcoma		1 (2%)		
Skeletal muscle		(4)	(1)	(1)
Carcinoma, metastatic, intestine small, ileum				1 (100%)
Hemangiosarcoma, multiple		1 (25%)		
Hepatocholangiocarcinoma, metastatic, liver		1 (25%)		
Nervous System				
None				
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	9 (18%)	5 (10%)	8 (16%)	4 (8%)
Alveolar/bronchiolar adenoma, multiple	2 (4%)	2 (4%)		2 (4%)
Alveolar/bronchiolar carcinoma	1 (2%)	4 (8%)	4 (8%)	6 (12%)
Alveolar/bronchiolar carcinoma, multiple			1 (2%)	1 (2%)
Carcinoma, metastatic, harderian gland			1 (2%)	
Carcinoma, metastatic, pancreas				1 (2%)
Carcinoma, metastatic, intestine small, ileum				1 (2%)
Hepatoblastoma, metastatic, liver		1 (2%)	1 (2%)	
Hepatocellular carcinoma, metastatic, liver	3 (6%)	4 (8%)	3 (6%)	4 (8%)
Hepatocholangiocarcinoma, metastatic, liver		1 (2%)		
Histiocytic sarcoma			1 (2%)	
Sarcoma, metastatic, skin	1 (2%)			
Mediastinum, hepatoblastoma, metastatic, liver		1 (2%)		
Mediastinum, hepatocholangiocarcinoma, metastatic, liver		1 (2%)		
Mediastinum, histiocytic sarcoma			1 (2%)	
Nose	(50)	(50)	(50)	(50)
Carcinoma, metastatic, harderian gland			1 (2%)	
Sarcoma, metastatic, skin	1 (2%)			
Special Senses System				
Eye	(45)	(49)	(49)	(47)
Carcinoma, metastatic, harderian gland	1 (2%)			
Sarcoma, metastatic, skin	1 (2%)			

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Special Senses System (continued)				
Harderian gland	(50)	(50)	(50)	(50)
Adenoma	7 (14%)	1 (2%)	11 (22%)	5 (10%)
Carcinoma	2 (4%)		4 (8%)	1 (2%)
Sarcoma, metastatic, skin	1 (2%)			
Bilateral, adenoma	1 (2%)			
Bilateral, carcinoma	1 (2%)			
Urinary System				
Kidney	(49)	(49)	(50)	(48)
Hepatocholangiocarcinoma, metastatic, liver		1 (2%)		
Histiocytic sarcoma			2 (4%)	
Renal tubule, adenoma		1 (2%)	1 (2%)	
Renal tubule, carcinoma	1 (2%)			
Urinary bladder	(48)	(50)	(50)	(48)
Hemangiosarcoma	1 (2%)			
Systemic Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Histiocytic sarcoma			3 (6%)	
Lymphoma malignant	2 (4%)	2 (4%)	2 (4%)	5 (10%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	46	47	46	47
Total primary neoplasms	87	90	96	89
Total animals with benign neoplasms	37	38	39	35
Total benign neoplasms	56	53	61	53
Total animals with malignant neoplasms	26	23	25	26
Total malignant neoplasms	31	37	35	36
Total animals with metastatic neoplasms	5	6	5	6
Total metastatic neoplasms	11	16	7	12

[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
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Adrenal Cortex: Adenoma				
Overall rate[a]	5/48 (10%)	5/50 (10%)	2/50 (4%)	5/48 (10%)
Adjusted rate[b]	11.7%	10.9%	4.3%	10.9%
Terminal rate[c]	4/34 (12%)	5/38 (13%)	2/38 (5%)	5/40 (13%)
First incidence (days)	723	729 (T)	729 (T)	729 (T)
Poly-3 test[d]	P=0.469N	P=0.582N	P=0.184N	P=0.587N
Harderian Gland: Adenoma				
Overall rate	8/50 (16%)	1/50 (2%)	11/50 (22%)	5/50 (10%)
Adjusted rate	17.7%	2.2%	23.3%	10.5%
Terminal rate	5/35 (14%)	1/38 (3%)	8/38 (21%)	5/41 (12%)
First incidence (days)	478	729 (T)	627	729 (T)
Poly-3 test	P=0.465	P=0.014N	P=0.341	P=0.244N
Harderian Gland: Carcinoma				
Overall rate	3/50 (6%)	0/50 (0%)	4/50 (8%)	1/50 (2%)
Adjusted rate	6.8%	0.0%	8.5%	2.1%
Terminal rate	2/35 (6%)	0/38 (0%)	2/38 (5%)	1/41 (2%)
First incidence (days)	723	—[e]	605	729 (T)
Poly-3 test	P=0.494N	P=0.111N	P=0.537	P=0.279N
Harderian Gland: Adenoma or Carcinoma				
Overall rate	11/50 (22%)	1/50 (2%)	15/50 (30%)	6/50 (12%)
Adjusted rate	24.3%	2.2%	31.2%	12.6%
Terminal rate	7/35 (20%)	1/38 (3%)	10/38 (26%)	6/41 (15%)
First incidence (days)	478	729 (T)	605	729 (T)
Poly-3 test	P=0.521	P=0.002N	P=0.304	P=0.117N
Small Intestine (Site Unspecified): Carcinoma				
Overall rate	0/50 (0%)	1/50 (2%)	2/50 (4%)	4/50 (8%)
Adjusted rate	0.0%	2.2%	4.3%	8.4%
Terminal rate	0/35 (0%)	1/38 (3%)	2/38 (5%)	3/41 (7%)
First incidence (days)	—	729 (T)	729 (T)	705
Poly-3 test	P=0.027	P=0.509	P=0.248	P=0.071
Small Intestine (Site Unspecified): Adenoma or Carcinoma				
Overall rate	1/50 (2%)	2/50 (4%)	4/50 (8%)	6/50 (12%)
Adjusted rate	2.3%	4.3%	8.6%	12.5%
Terminal rate	1/35 (3%)	2/38 (5%)	4/38 (11%)	4/41 (10%)
First incidence (days)	729 (T)	729 (T)	729 (T)	641
Poly-3 test	P=0.029	P=0.515	P=0.194	P=0.071
Liver: Hemangiosarcoma				
Overall rate	2/50 (4%)	5/50 (10%)	3/50 (6%)	1/49 (2%)
Adjusted rate	4.5%	10.8%	6.4%	2.1%
Terminal rate	1/35 (3%)	3/38 (8%)	0/38 (0%)	1/41 (2%)
First incidence (days)	665	717	605	729 (T)
Poly-3 test	P=0.162N	P=0.233	P=0.528	P=0.482N
Liver: Hepatocellular Adenoma				
Overall rate	28/50 (56%)	33/50 (66%)	26/50 (52%)	27/49 (55%)
Adjusted rate	61.4%	69.8%	55.4%	57.5%
Terminal rate	25/35 (71%)	28/38 (74%)	24/38 (63%)	25/41 (61%)
First incidence (days)	478	610	627	694
Poly-3 test	P=0.183N	P=0.256	P=0.353N	P=0.434N

TABLE C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Liver: Hepatocellular Carcinoma				
Overall rate	13/50 (26%)	10/50 (20%)	11/50 (22%)	14/49 (29%)
Adjusted rate	27.7%	20.8%	23.3%	29.3%
Terminal rate	5/35 (14%)	5/38 (13%)	7/38 (18%)	10/41 (24%)
First incidence (days)	536	452	638	506
Poly-3 test	P=0.332	P=0.295N	P=0.402N	P=0.520
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	37/50 (74%)	38/50 (76%)	33/50 (66%)	35/49 (71%)
Adjusted rate	77.5%	78.0%	69.3%	73.1%
Terminal rate	28/35 (80%)	30/38 (79%)	28/38 (74%)	30/41 (73%)
First incidence (days)	478	452	627	506
Poly-3 test	P=0.264N	P=0.576	P=0.244N	P=0.395N
Liver: Hepatocellular Carcinoma or Hepatoblastoma				
Overall rate	15/50 (30%)	12/50 (24%)	13/50 (26%)	14/49 (29%)
Adjusted rate	31.9%	24.9%	27.4%	29.3%
Terminal rate	7/35 (20%)	6/38 (16%)	8/38 (21%)	10/41 (24%)
First incidence (days)	536	452	638	506
Poly-3 test	P=0.528	P=0.297N	P=0.401N	P=0.478N
Liver: Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma				
Overall rate	38/50 (76%)	39/50 (78%)	35/50 (70%)	35/49 (71%)
Adjusted rate	79.6%	79.7%	73.3%	73.1%
Terminal rate	29/35 (83%)	30/38 (79%)	29/38 (76%)	30/41 (73%)
First incidence (days)	478	452	627	506
Poly-3 test	P=0.198N	P=0.598	P=0.308N	P=0.302N
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	11/50 (22%)	7/50 (14%)	8/50 (16%)	6/50 (12%)
Adjusted rate	24.6%	15.2%	17.3%	12.5%
Terminal rate	9/35 (26%)	7/38 (18%)	8/38 (21%)	4/41 (10%)
First incidence (days)	605	729 (T)	729 (T)	610
Poly-3 test	P=0.146N	P=0.194N	P=0.273N	P=0.106N
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	1/50 (2%)	4/50 (8%)	5/50 (10%)	7/50 (14%)
Adjusted rate	2.3%	8.7%	10.8%	14.7%
Terminal rate	1/35 (3%)	4/38 (11%)	4/38 (11%)	6/41 (15%)
First incidence (days)	729 (T)	729 (T)	696	705
Poly-3 test	P=0.043	P=0.192	P=0.114	P=0.039
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	12/50 (24%)	10/50 (20%)	13/50 (26%)	13/50 (26%)
Adjusted rate	26.8%	21.7%	28.0%	26.9%
Terminal rate	10/35 (29%)	10/38 (26%)	12/38 (32%)	10/41 (24%)
First incidence (days)	605	729 (T)	696	610
Poly-3 test	P=0.406	P=0.373N	P=0.545	P=0.587
Spleen: Hemangiosarcoma				
Overall rate	2/49 (4%)	4/50 (8%)	1/49 (2%)	1/48 (2%)
Adjusted rate	4.6%	8.6%	2.2%	2.2%
Terminal rate	1/35 (3%)	1/38 (3%)	0/38 (0%)	1/41 (2%)
First incidence (days)	723	676	690	729 (T)
Poly-3 test	P=0.156N	P=0.370	P=0.481N	P=0.477N

TABLE C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Thyroid Gland (Follicular Cell): Adenoma				
Overall rate	3/50 (6%)	1/50 (2%)	3/49 (6%)	1/48 (2%)
Adjusted rate	6.8%	2.2%	6.6%	2.2%
Terminal rate	3/35 (9%)	1/38 (3%)	3/38 (8%)	1/41 (2%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.366N	P=0.290N	P=0.647N	P=0.289N
All Organs: Hemangiosarcoma				
Overall rate	6/50 (12%)	7/50 (14%)	5/50 (10%)	1/50 (2%)
Adjusted rate	13.5%	15.1%	10.6%	2.1%
Terminal rate	4/35 (11%)	4/38 (11%)	1/38 (3%)	1/41 (2%)
First incidence (days)	665	676	605	729 (T)
Poly-3 test	P=0.020N	P=0.534	P=0.456N	P=0.045N
All Organs: Histiocytic Sarcoma				
Overall rate	0/50 (0%)	0/50 (0%)	3/50 (6%)	0/50 (0%)
Adjusted rate	0.0%	0.0%	6.4%	0.0%
Terminal rate	0/35 (0%)	0/38 (0%)	0/38 (0%)	0/41 (0%)
First incidence (days)	—	—[f]	645	—
Poly-3 test	P=0.507		P=0.130	—
All Organs: Malignant Lymphoma				
Overall rate	2/50 (4%)	2/50 (4%)	2/50 (4%)	5/50 (10%)
Adjusted rate	4.5%	4.3%	4.3%	10.5%
Terminal rate	2/35 (6%)	1/38 (3%)	2/38 (5%)	4/41 (10%)
First incidence (days)	729 (T)	717	729 (T)	687
Poly-3 test	P=0.126	P=0.678N	P=0.676N	P=0.250
All Organs: Benign Neoplasms				
Overall rate	37/50 (74%)	38/50 (76%)	39/50 (78%)	35/50 (70%)
Adjusted rate	79.5%	80.4%	81.9%	71.9%
Terminal rate	30/35 (86%)	33/38 (87%)	33/38 (87%)	30/41 (73%)
First incidence (days)	478	610	627	610
Poly-3 test	P=0.180N	P=0.563	P=0.483	P=0.260N
All Organs: Malignant Neoplasms				
Overall rate	26/50 (52%)	23/50 (46%)	25/50 (50%)	26/50 (52%)
Adjusted rate	54.1%	47.1%	51.4%	53.2%
Terminal rate	15/35 (43%)	13/38 (34%)	16/38 (42%)	20/41 (49%)
First incidence (days)	536	452	605	506
Poly-3 test	P=0.440	P=0.315N	P=0.476N	P=0.548N

TABLE C2
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
All Organs: Benign or Malignant Neoplasms				
Overall rate	46/50 (92%)	47/50 (94%)	46/50 (92%)	47/50 (94%)
Adjusted rate	94.0%	95.9%	93.8%	94.8%
Terminal rate	33/35 (94%)	36/38 (95%)	35/38 (92%)	39/41 (95%)
First incidence (days)	478	452	605	506
Poly-3 test	P=0.565N	P=0.508	P=0.655N	P=0.608

(T) Terminal sacrifice
[a] Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal cortex, liver lung, spleen, 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 is the P value associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in dosed group is indicated by **N**.
[e] Not applicable; no neoplasms in animal group
[f] Value of the statistic can not be computed

TABLE C3a
Historical Incidence of Small Intestine (Site Unspecified) Neoplasms in Control Male B6C3F$_1$ Mice[a]

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
Historical Incidence: Gavage Studies (all vehicles)			
Acrylonitrile (water)	0/50	2/50	2/50
Elmiron® (water)	0/50	4/50	4/50
2,4-Hexadienal (corn oil)	0/50	1/50	1/50
Methacrylonitrile (water)	2/49	1/49	3/49
Riddelliine (sodium phosphate)	0/50	0/50	0/50
Total (%)	2/249 (0.8%)	8/249 (3.2%)	10/249 (4.0%)
Range	0%-4%	0%-8%	0%-8%
Overall Historical Incidence: All Routes			
Total (%)	6/1,508 (0.4%)	33/1,508 (2.2%)	39/1,508 (2.6%)
Mean ± standard deviation	0.4% ± 1.0%	2.2% ± 2.7%	2.6% ± 2.8%
Range	0%-4%	0%-10%	0%-10%

[a] Data as of January 28, 2005

TABLE C3b
Historical Incidence of Alveolar/bronchiolar Neoplasms in Control Male B6C3F$_1$ Mice[a]

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
Historical Incidence: Gavage Studies (all vehicles)			
Acrylonitrile (water)	10/50	4/50	14/50
Elmiron® (water)	10/50	5/50	14/50
2,4-Hexadienal (corn oil)	11/50	9/50	19/50
Methacrylonitrile (water)	2/49	4/49	6/49
Riddelliine (sodium phosphate)	12/50	7/50	18/50
Total (%)	45/249 (18.1%)	29/249 (11.6%)	71/249 (28.5%)
Range	4%-24%	8%-18%	12%-38%
Overall Historical Incidence: All Routes			
Total (%)	258/1,507 (17.1%)	151/1,507 (10.0%)	385/1,507 (25.6%)
Mean ± standard deviation	16.7% ± 7.4%	9.9% ± 5.0%	25.1% ± 9.4%
Range	4%-28%	4%-24%	12%-44%

[a] Data as of January 28, 2005

TABLE C3c
Historical Incidence of Malignant Lymphoma in Control Male B6C3F$_1$ Mice[a]

Study	Incidence in Controls
Historical Incidence: Gavage Studies (all vehicles)	
Acrylonitrile (water)	3/50
Elmiron® (water)	3/50
2,4-Hexadienal (corn oil)	2/50
Methacrylonitrile (water)	2/49
Riddelliine (sodium phosphate)	3/50
Total (%)	13/249 (5.2%)
Range	4%-6%
Overall Historical Incidence: All Routes	
Total (%)	70/1,508 (4.6%)
Mean ± standard deviation	4.3% ± 2.3%
Range	0%-8%

[a] Data as of January 28, 2005. Includes data for histiocytic, lymphocytic, mixed, NOS, or undifferentiated lymphoma.

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths		2		2
Moribund	4	4	7	4
Natural deaths	11	6	5	3
Survivors				
Terminal sacrifice	35	38	38	41
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Foreign body		1 (2%)		1 (2%)
Inflammation	1 (2%)	1 (2%)		1 (2%)
Perforation		1 (2%)		1 (2%)
Intestine large, cecum	(45)	(46)	(46)	(47)
Edema		1 (2%)	1 (2%)	
Inflammation, chronic, focal				1 (2%)
Intestine small, duodenum	(42)	(46)	(46)	(46)
Epithelium, cyst		1 (2%)		
Intestine small, jejunum	(45)	(45)	(47)	(47)
Inflammation	1 (2%)			
Lymphoid tissue, hyperplasia	1 (2%)			
Peyer's patch, hyperplasia, lymphoid		1 (2%)		
Liver	(50)	(50)	(50)	(49)
Basophilic focus		1 (2%)		
Clear cell focus	20 (40%)	19 (38%)	22 (44%)	16 (33%)
Congestion				1 (2%)
Cyst		1 (2%)		1 (2%)
Eosinophilic focus		2 (4%)	2 (4%)	2 (4%)
Fibrosis, focal	1 (2%)			
Hematopoietic cell proliferation	1 (2%)	2 (4%)		
Hemorrhage		1 (2%)		
Hepatodiaphragmatic nodule		1 (2%)		1 (2%)
Infarct	1 (2%)			
Infiltration cellular, mixed cell	3 (6%)	1 (2%)	2 (4%)	6 (12%)
Mixed cell focus	3 (6%)	1 (2%)		
Thrombosis		2 (4%)	1 (2%)	1 (2%)
Bile duct, hyperplasia		1 (2%)		
Hepatocyte, necrosis, focal	2 (4%)	2 (4%)	2 (4%)	4 (8%)
Hepatocyte, syncytial alteration, focal				1 (2%)
Hepatocyte, vacuolization cytoplasmic	29 (58%)	19 (38%)	32 (64%)	23 (47%)
Hepatocyte, centrilobular, necrosis			1 (2%)	
Mesentery	(5)	(11)	(5)	(4)
Inflammation		1 (9%)		1 (25%)
Fat, necrosis, focal	4 (80%)	7 (64%)	4 (80%)	
Pancreas	(48)	(50)	(49)	(48)
Acinus, atrophy, diffuse	1 (2%)			
Acinus, atrophy, focal	1 (2%)	1 (2%)	2 (4%)	1 (2%)
Acinus, cytoplasmic alteration	1 (2%)	1 (2%)		
Acinus, inflammation			1 (2%)	
Duct, cyst			1 (2%)	

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

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Alimentary System (continued)				
Stomach, forestomach	(50)	(50)	(50)	(48)
Ulcer		3 (6%)		1 (2%)
Epithelium, hyperplasia	3 (6%)	2 (4%)	3 (6%)	2 (4%)
Stomach, glandular	(47)	(47)	(47)	(48)
Erosion	2 (4%)	1 (2%)	2 (4%)	1 (2%)
Ulcer	1 (2%)			
Tooth	(43)	(37)	(33)	(35)
Dysplasia	43 (100%)	37 (100%)	33 (100%)	35 (100%)
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Infiltration cellular, mixed cell	1 (2%)	1 (2%)	1 (2%)	
Mineralization, focal			1 (2%)	1 (2%)
Artery, inflammation, chronic		1 (2%)		
Endocrine System				
Adrenal cortex	(48)	(50)	(50)	(48)
Accessory adrenal cortical nodule	1 (2%)		1 (2%)	
Cytoplasmic alteration, focal	4 (8%)		5 (10%)	5 (10%)
Degeneration, cystic	1 (2%)		1 (2%)	
Hyperplasia, focal				1 (2%)
Hypertrophy	1 (2%)			
Hypertrophy, focal		1 (2%)		
Inflammation	1 (2%)			
Adrenal medulla	(48)	(50)	(50)	(48)
Hyperplasia, focal	2 (4%)	1 (2%)	1 (2%)	1 (2%)
Islets, pancreas	(50)	(50)	(49)	(48)
Hyperplasia	17 (34%)	17 (34%)	7 (14%)	13 (27%)
Parathyroid gland	(46)	(50)	(48)	(46)
Cyst		3 (6%)	3 (6%)	2 (4%)
Pituitary gland	(50)	(49)	(49)	(50)
Cyst	3 (6%)	1 (2%)	1 (2%)	3 (6%)
Pars distalis, hyperplasia, focal		1 (2%)		
Thyroid gland	(50)	(50)	(49)	(48)
Follicle, degeneration, cystic, focal	6 (12%)	12 (24%)	11 (22%)	7 (15%)
Follicular cell, hyperplasia, focal	14 (28%)	5 (10%)	11 (22%)	11 (23%)
General Body System				
Tissue NOS	(1)	(2)		(1)
Foreign body				1 (100%)
Abdominal, fibrosis		2 (100%)		
Abdominal, inflammation		1 (50%)		
Pelvic, inflammation	1 (100%)			

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Genital System				
Preputial gland	(50)	(50)	(50)	(48)
Degeneration, cystic	11 (22%)	11 (22%)	16 (32%)	11 (23%)
Inflammation, chronic	2 (4%)	1 (2%)	2 (4%)	3 (6%)
Prostate	(49)	(50)	(50)	(50)
Inflammation	1 (2%)	1 (2%)		1 (2%)
Epithelium, hyperplasia	1 (2%)	1 (2%)	1 (2%)	
Seminal vesicle	(49)	(50)	(50)	(49)
Dilatation	2 (4%)	2 (4%)		
Epithelium, hyperplasia			1 (2%)	
Testes	(50)	(50)	(50)	(50)
Angiectasis	1 (2%)			
Atrophy	1 (2%)		1 (2%)	
Thrombosis	1 (2%)			
Hematopoietic System				
Bone marrow	(50)	(50)	(50)	(50)
Hyperplasia	2 (4%)	3 (6%)	3 (6%)	2 (4%)
Lymph node	(5)	(3)	(3)	(6)
Inguinal, hyperplasia, lymphoid	1 (20%)			
Mediastinal, hemorrhage			1 (33%)	
Mediastinal, hyperplasia, lymphoid				1 (17%)
Lymph node, mandibular	(43)	(46)	(48)	(47)
Hemorrhage			1 (2%)	
Hyperplasia, lymphoid	2 (5%)	1 (2%)		2 (4%)
Lymph node, mesenteric	(47)	(50)	(48)	(48)
Hematopoietic cell proliferation				1 (2%)
Hemorrhage			3 (6%)	
Hyperplasia, lymphoid	2 (4%)	2 (4%)	2 (4%)	
Spleen	(49)	(50)	(49)	(48)
Atrophy			1 (2%)	
Fibrosis, focal				1 (2%)
Hematopoietic cell proliferation	14 (29%)	16 (32%)	25 (51%)	29 (60%)
Hyperplasia, lymphoid	7 (14%)	6 (12%)	2 (4%)	3 (6%)
Hyperplasia, mast cell	1 (2%)			
Lymphoid follicle, necrosis	1 (2%)		1 (2%)	
Thymus	(38)	(47)	(40)	(44)
Angiectasis		1 (2%)		
Hyperplasia, lymphoid		1 (2%)		
Epithelial cell, hyperplasia		1 (2%)		
Integumentary System				
Skin	(50)	(50)	(50)	(50)
Cyst epithelial inclusion	1 (2%)			
Ulcer				2 (4%)
Pinna, inflammation, focal				1 (2%)
Subcutaneous tissue, edema	1 (2%)	1 (2%)	2 (4%)	1 (2%)
Subcutaneous tissue, inflammation, focal, suppurative				1 (2%)

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Hyperostosis				2 (4%)
Vertebra, fracture		1 (2%)		
Skeletal muscle		(4)	(1)	(1)
Fibrosis, focal		1 (25%)		
Inflammation		1 (25%)		
Nervous System				
Brain	(50)	(50)	(50)	(50)
Compression, focal			1 (2%)	
Spinal cord	(4)	(4)	(1)	(2)
Hemorrhage		2 (50%)		
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Congestion	1 (2%)	1 (2%)		1 (2%)
Hemorrhage			1 (2%)	1 (2%)
Hyperplasia, histiocytic	1 (2%)		3 (6%)	2 (4%)
Inflammation	1 (2%)			
Thrombosis			1 (2%)	
Alveolar epithelium, hyperplasia, focal	4 (8%)	3 (6%)	2 (4%)	4 (8%)
Mediastinum, foreign body	1 (2%)			
Mediastinum, inflammation, suppurative	1 (2%)			
Mediastinum, thrombosis	1 (2%)			
Nose	(50)	(50)	(50)	(50)
Hyperplasia				1 (2%)
Inflammation	1 (2%)	3 (6%)	3 (6%)	6 (12%)
Nasolacrimal duct, hyperplasia, focal		1 (2%)		1 (2%)
Nasolacrimal duct, inflammation	3 (6%)		1 (2%)	1 (2%)
Special System				
Eye	(45)	(49)	(49)	(47)
Atrophy			1 (2%)	
Cataract				1 (2%)
Cornea, inflammation	2 (4%)			1 (2%)
Cornea, necrosis, focal	1 (2%)			
Retrobulbar, inflammation, chronic, focal				1 (2%)
Harderian gland	(50)	(50)	(50)	(50)
Cyst				1 (2%)
Inflammation	1 (2%)			1 (2%)
Epithelium, hyperplasia	2 (4%)		2 (4%)	

TABLE C4
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Urinary System				
Kidney	(49)	(49)	(50)	(48)
Hydronephrosis	2 (4%)			
Hyperplasia, lymphoid			1 (2%)	
Infarct		1 (2%)		1 (2%)
Metaplasia, focal, osseous	3 (6%)		3 (6%)	4 (8%)
Mineralization, focal			1 (2%)	
Nephropathy	40 (82%)	44 (90%)	44 (88%)	43 (90%)
Artery, inflammation, chronic			1 (2%)	
Renal tubule, accumulation, hyaline droplet			1 (2%)	
Renal tubule, cyst	4 (8%)	2 (4%)	3 (6%)	2 (4%)
Renal tubule, hyperplasia, atypical		1 (2%)		
Renal tubule, hyperplasia, focal	3 (6%)	2 (4%)	1 (2%)	2 (4%)
Renal tubule, pigmentation			1 (2%)	
Urethra	(1)			
Inflammation, chronic	1 (100%)			
Urinary bladder	(48)	(50)	(50)	(48)
Transitional epithelium, hyperplasia	1 (2%)			

APPENDIX D
SUMMARY OF LESIONS IN FEMALE MICE IN THE 2-YEAR GAVAGE STUDY OF METHYLENE BLUE TRIHYDRATE

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths	1	1	1	
Moribund	7	4	5	1
Natural deaths	9	5	2	6
Survivors				
Died last week of study	4		1	2
Terminal sacrifice	29	40	41	41
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Fibrosarcoma, metastatic, skin		1 (2%)		
Gallbladder	(42)	(45)	(47)	(44)
Intestine large, cecum	(43)	(46)	(47)	(44)
Intestine small, duodenum	(43)	(45)	(47)	(42)
Carcinoma	1 (2%)			
Intestine small, jejunum	(43)	(45)	(47)	(43)
Adenoma		1 (2%)		
Intestine small, ileum	(43)	(44)	(47)	(43)
Liver	(50)	(50)	(50)	(50)
Fibrosarcoma, metastatic, skin		1 (2%)		
Hemangiosarcoma			2 (4%)	
Hepatoblastoma			1 (2%)	
Hepatocellular carcinoma	5 (10%)	4 (8%)		3 (6%)
Hepatocellular adenoma	11 (22%)	15 (30%)	13 (26%)	9 (18%)
Hepatocellular adenoma, multiple	4 (8%)	4 (8%)	9 (18%)	4 (8%)
Histiocytic sarcoma	2 (4%)			
Serosa, fibrosarcoma, metastatic, skin				1 (2%)
Mesentery	(18)	(14)	(15)	(13)
Fibrosarcoma				1 (8%)
Fibrosarcoma, metastatic, skin		1 (7%)	1 (7%)	1 (8%)
Histiocytic sarcoma	1 (6%)			1 (8%)
Leiomyosarcoma, metastatic, uterus				1 (8%)
Rhabdomyosarcoma, metastatic, skin			1 (7%)	
Rhabdomyosarcoma, metastatic, uncertain, primary site	1 (6%)			
Pancreas	(46)	(46)	(49)	(49)
Fibrosarcoma, metastatic, skin			1 (2%)	
Histiocytic sarcoma				1 (2%)
Salivary glands	(50)	(50)	(50)	(49)
Stomach, forestomach	(50)	(50)	(50)	(49)
Squamous cell papilloma		1 (2%)	1 (2%)	1 (2%)
Stomach, glandular	(45)	(47)	(50)	(46)
Carcinoma			1 (2%)	
Tooth	(2)	(10)	(3)	(3)
Cardiovascular System				
Heart	(50)	(50)	(50)	(50)
Alveolar/bronchiolar carcinoma, metastatic, lung	1 (2%)			

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Adenoma			1 (2%)	
Histiocytic sarcoma	1 (2%)			
Capsule, fibrosarcoma, metastatic, skin		1 (2%)		
Adrenal medulla	(50)	(50)	(49)	(50)
Pheochromocytoma benign	1 (2%)	1 (2%)	2 (4%)	
Islets, pancreatic	(46)	(47)	(49)	(48)
Adenoma	1 (2%)			1 (2%)
Carcinoma				1 (2%)
Pituitary gland	(46)	(49)	(48)	(47)
Pars distalis, adenoma	1 (2%)	7 (14%)	5 (10%)	3 (6%)
Pars intermedia, adenoma	1 (2%)			
Thyroid gland	(46)	(50)	(49)	(47)
Follicular cell, adenoma	1 (2%)	1 (2%)		1 (2%)
Follicular cell, carcinoma				1 (2%)
General Body System				
Peritoneum		(1)		
Tissue NOS	(3)			(3)
Abdominal, histiocytic sarcoma				1 (33%)
Thoracic, histiocytic sarcoma	1 (33%)			
Genital System				
Clitoral gland	(48)	(50)	(46)	(48)
Ovary	(47)	(48)	(49)	(48)
Alveolar/bronchiolar carcinoma, metastatic, lung	1 (2%)			
Cystadenoma	2 (4%)	3 (6%)	3 (6%)	2 (4%)
Fibrosarcoma, metastatic, skin		1 (2%)		
Granulosa-theca tumor benign				1 (2%)
Hemangiosarcoma	1 (2%)			
Histiocytic sarcoma	1 (2%)			1 (2%)
Luteoma			1 (2%)	
Oviduct		(1)		
Uterus	(50)	(50)	(50)	(50)
Adenoma	1 (2%)	1 (2%)		
Hemangioma		1 (2%)		1 (2%)
Hemangiosarcoma	1 (2%)			
Histiocytic sarcoma	1 (2%)			
Leiomyoma	1 (2%)			
Leiomyosarcoma				1 (2%)
Endometrium, polyp stromal		2 (4%)	2 (4%)	
Vagina	(1)			
Squamous cell carcinoma	1 (100%)			
Hematopoietic System				
Bone marrow	(50)	(50)	(49)	(50)
Hemangiosarcoma			1 (2%)	
Histiocytic sarcoma	1 (2%)			

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Hematopoietic System (continued)				
Lymph node	(7)	(5)	(10)	(10)
Iliac, fibrosarcoma, metastatic, skin		1 (20%)		
Iliac, histiocytic sarcoma	1 (14%)			
Inguinal, fibrosarcoma, metastatic, skin	1 (14%)			
Inguinal, histiocytic sarcoma	1 (14%)			
Mediastinal, histiocytic sarcoma	1 (14%)			1 (10%)
Pancreatic, fibrosarcoma, metastatic, skin		1 (20%)		
Pancreatic, histiocytic sarcoma	1 (14%)			
Pancreatic, leiomyosarcoma, metastatic, uterus				1 (10%)
Popliteal, rhabdomyosarcoma, metastatic, skeletal muscle			1 (10%)	
Lymph node, mandibular	(48)	(49)	(48)	(46)
Histiocytic sarcoma	1 (2%)			
Lymph node, mesenteric	(46)	(49)	(49)	(48)
Histiocytic sarcoma	1 (2%)			1 (2%)
Spleen	(47)	(47)	(49)	(50)
Hemangiosarcoma			1 (2%)	
Histiocytic sarcoma	1 (2%)			
Thymus	(43)	(47)	(44)	(49)
Histiocytic sarcoma	1 (2%)			1 (2%)
Integumentary System				
Mammary gland	(50)	(50)	(50)	(50)
Carcinoma	2 (4%)	1 (2%)		2 (4%)
Skin	(50)	(50)	(50)	(50)
Basal cell carcinoma	1 (2%)			
Subcutaneous tissue, fibrosarcoma	3 (6%)	2 (4%)	2 (4%)	2 (4%)
Subcutaneous tissue, hemangioma	1 (2%)			
Subcutaneous tissue, hemangiosarcoma	1 (2%)	1 (2%)	2 (4%)	
Subcutaneous tissue, histiocytic sarcoma	1 (2%)			
Subcutaneous tissue, leiomyosarcoma, metastatic, uterus				1 (2%)
Subcutaneous tissue, lipoma			1 (2%)	
Subcutaneous tissue, rhabdomyosarcoma			1 (2%)	
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Cranium, osteosarcoma	1 (2%)			
Vertebra, osteosarcoma		1 (2%)		
Skeletal muscle	(1)	(4)	(3)	(3)
Alveolar/bronchiolar carcinoma, metastatic, lung	1 (100%)			
Fibrosarcoma, metastatic, skin		1 (25%)	1 (33%)	1 (33%)
Hemangioma				1 (33%)
Leiomyosarcoma, metastatic, uterus				1 (33%)
Rhabdomyosarcoma		2 (50%)	2 (67%)	
Nervous System				
Brain	(50)	(50)	(50)	(49)

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	1 (2%)	2 (4%)	3 (6%)	2 (4%)
Alveolar/bronchiolar carcinoma	4 (8%)			1 (2%)
Alveolar/bronchiolar carcinoma, multiple	1 (2%)			
Carcinoma, metastatic, harderian gland				1 (2%)
Fibrosarcoma, metastatic, mesentery				1 (2%)
Fibrosarcoma, metastatic, skin	1 (2%)		1 (2%)	1 (2%)
Hepatocellular carcinoma, metastatic, liver		2 (4%)		
Histiocytic sarcoma	1 (2%)			1 (2%)
Rhabdomyosarcoma, metastatic, skeletal muscle			1 (2%)	
Squamous cell carcinoma, metastatic, vagina	1 (2%)			
Mediastinum, alveolar/bronchiolar carcinoma, metastatic, lung	1 (2%)			
Mediastinum, fibrosarcoma, metastatic, skin			1 (2%)	
Mediastinum, histiocytic sarcoma				1 (2%)
Nose	(50)	(50)	(50)	(50)
Trachea	(49)	(50)	(50)	(49)
Carcinoma, metastatic, thyroid gland				1 (2%)
Special Senses System				
Eye	(43)	(48)	(47)	(47)
Harderian gland	(49)	(50)	(50)	(50)
Adenoma	4 (8%)	2 (4%)	7 (14%)	3 (6%)
Carcinoma		1 (2%)	2 (4%)	2 (4%)
Urinary System				
Kidney	(48)	(50)	(49)	(50)
Histiocytic sarcoma	1 (2%)			
Urinary bladder	(45)	(47)	(49)	(48)
Hemangiosarcoma				1 (2%)
Histiocytic sarcoma				1 (2%)
Systemic Lesions				
Multiple organs[b]	(50)	(50)	(50)	(50)
Histiocytic sarcoma	2 (4%)			1 (2%)
Lymphoma malignant	6 (12%)	4 (8%)	9 (18%)	12 (24%)
Neoplasm Summary				
Total animals with primary neoplasms[c]	38	36	40	37
Total primary neoplasms	60	57	72	57
Total animals with benign neoplasms	25	29	32	22
Total benign neoplasms	30	41	48	29
Total animals with malignant neoplasms	25	15	18	24
Total malignant neoplasms	30	16	24	28
Total animals with metastatic neoplasms	5	4	5	5
Total metastatic neoplasms	8	11	8	11
Total animals with malignant neoplasms of uncertain primary site	1	1		

[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
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Harderian Gland: Adenoma				
Overall rate[a]	4/50 (8%)	2/50 (4%)	7/50 (14%)	3/50 (6%)
Adjusted rate[b]	8.9%	4.4%	14.6%	6.4%
Terminal rate[c]	2/33 (6%)	1/40 (3%)	6/42 (14%)	3/43 (7%)
First incidence (days)	638	680	627	729 (T)
Poly-3 test[d]	P=0.516	P=0.329N	P=0.298	P=0.477N
Harderian Gland: Adenoma or Carcinoma				
Overall rate	4/50 (8%)	3/50 (6%)	9/50 (18%)	5/50 (10%)
Adjusted rate	8.9%	6.5%	18.7%	10.6%
Terminal rate	2/33 (6%)	1/40 (3%)	7/42 (17%)	4/43 (9%)
First incidence (days)	638	616	627	659
Poly-3 test	P=0.279	P=0.485N	P=0.140	P=0.530
Liver: Hepatocellular Adenoma				
Overall rate	15/50 (30%)	19/50 (38%)	22/50 (44%)	13/50 (26%)
Adjusted rate	32.8%	40.9%	45.7%	27.6%
Terminal rate	11/33 (33%)	17/40 (43%)	20/42 (48%)	13/43 (30%)
First incidence (days)	617	616	627	729 (T)
Poly-3 test	P=0.243N	P=0.276	P=0.141	P=0.374N
Liver: Hepatocellular Carcinoma				
Overall rate	5/50 (10%)	4/50 (8%)	0/50 (0%)	3/50 (6%)
Adjusted rate	11.1%	8.7%	0.0%	6.3%
Terminal rate	4/33 (12%)	3/40 (8%)	0/42 (0%)	2/43 (5%)
First incidence (days)	650	720	—[e]	680
Poly-3 test	P=0.178N	P=0.487N	P=0.026N	P=0.328N
Liver: Hepatocellular Carcinoma or Hepatoblastoma				
Overall rate	5/50 (10%)	4/50 (8%)	1/50 (2%)	3/50 (6%)
Adjusted rate	11.1%	8.7%	2.1%	6.3%
Terminal rate	4/33 (12%)	3/40 (8%)	1/42 (2%)	2/43 (5%)
First incidence (days)	650	720	729 (T)	680
Poly-3 test	P=0.206N	P=0.487N	P=0.088N	P=0.328N
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	19/50 (38%)	20/50 (40%)	22/50 (44%)[f]	16/50 (32%)
Adjusted rate	41.3%	43.1%	45.7%	33.8%
Terminal rate	14/33 (42%)	17/40 (43%)	20/42 (48%)	15/43 (35%)
First incidence (days)	617	616	627	680
Poly-3 test	P=0.236N	P=0.516	P=0.411	P=0.297N
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	1/50 (2%)	2/50 (4%)	3/50 (6%)	2/50 (4%)
Adjusted rate	2.2%	4.4%	6.3%	4.3%
Terminal rate	0/33 (0%)	2/40 (5%)	3/42 (7%)	2/43 (5%)
First incidence (days)	650	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.435	P=0.507	P=0.327	P=0.517
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	5/50 (10%)	0/50 (0%)	0/50 (0%)	1/50 (2%)
Adjusted rate	11.1%	0.0%	0.0%	2.1%
Terminal rate	3/33 (9%)	0/40 (0%)	0/42 (0%)	1/43 (2%)
First incidence (days)	638	—	—	729 (T)
Poly-3 test	P=0.106N	P=0.029N	P=0.026N	P=0.092N

TABLE D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	6/50 (12%)	2/50 (4%)	3/50 (6%)	3/50 (6%)
Adjusted rate	13.2%	4.4%	6.3%	6.4%
Terminal rate	3/33 (9%)	2/40 (5%)	3/42 (7%)	3/43 (7%)
First incidence (days)	638	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.306N	P=0.130N	P=0.220N	P=0.225N
Ovary: Cystadenoma				
Overall rate	2/47 (4%)	3/48 (6%)	3/49 (6%)	2/48 (4%)
Adjusted rate	4.8%	6.8%	6.4%	4.4%
Terminal rate	2/32 (6%)	3/39 (8%)	3/41 (7%)	2/41 (5%)
First incidence (days)	729 (T)	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.484N	P=0.521	P=0.547	P=0.668N
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	1/46 (2%)	7/49 (14%)	5/48 (10%)	3/47 (6%)
Adjusted rate	2.4%	15.4%	10.8%	6.8%
Terminal rate	1/31 (3%)	6/40 (15%)	4/40 (10%)	3/40 (8%)
First incidence (days)	729 (T)	680	568	729 (T)
Poly-3 test	P=0.482N	P=0.041	P=0.129	P=0.329
Skin (Subcutaneous Tissue): Fibrosarcoma				
Overall rate	3/50 (6%)	2/50 (4%)	2/50 (4%)	2/50 (4%)
Adjusted rate	6.6%	4.3%	4.2%	4.3%
Terminal rate	1/33 (3%)	1/40 (3%)	0/42 (0%)	2/43 (5%)
First incidence (days)	568	533	627	729 (T)
Poly-3 test	P=0.435N	P=0.489N	P=0.473N	P=0.483N
All Organs: Hemangiosarcoma				
Overall rate	3/50 (6%)	1/50 (2%)	3/50 (6%)	1/50 (2%)
Adjusted rate	6.7%	2.2%	6.3%	2.1%
Terminal rate	1/33 (3%)	1/40 (3%)	3/42 (7%)	1/43 (2%)
First incidence (days)	716	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.356N	P=0.296N	P=0.632N	P=0.287N
All Organs: Hemangiosarcoma or Hemangioma				
Overall rate	4/50 (8%)	2/50 (4%)	3/50 (6%)	3/50 (6%)
Adjusted rate	8.9%	4.4%	6.3%	6.4%
Terminal rate	1/33 (3%)	2/40 (5%)	3/42 (7%)	2/43 (5%)
First incidence (days)	604	729 (T)	729 (T)	722
Poly-3 test	P=0.524N	P=0.330N	P=0.470N	P=0.476N
All Organs: Malignant Lymphoma				
Overall rate	6/50 (12%)	4/50 (8%)	9/50 (18%)	12/50 (24%)
Adjusted rate	13.2%	8.7%	18.6%	24.5%
Terminal rate	4/33 (12%)	2/40 (5%)	7/42 (17%)	8/43 (19%)
First incidence (days)	568	673	568	445
Poly-3 test	P=0.025	P=0.360N	P=0.334	P=0.126
All Organs: Benign Neoplasms				
Overall rate	25/50 (50%)	29/50 (58%)	32/50 (64%)	22/50 (44%)
Adjusted rate	53.4%	62.0%	65.8%	46.7%
Terminal rate	17/33 (52%)	25/40 (63%)	29/42 (69%)	21/43 (49%)
First incidence (days)	604	616	568	722
Poly-3 test	P=0.197N	P=0.259	P=0.149	P=0.329N

TABLE D2
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
All Organs: Malignant Neoplasms				
Overall rate	26/50 (52%)	15/50 (30%)	18/50 (36%)	24/50 (48%)
Adjusted rate	55.2%	31.4%	36.7%	48.6%
Terminal rate	15/33 (46%)	9/40 (23%)	12/42 (29%)	18/43 (42%)
First incidence (days)	568	533	568	445
Poly-3 test	P=0.422	P=0.015N	P=0.052N	P=0.330N
All Organs: Benign or Malignant Neoplasms				
Overall rate	39/50 (78%)	36/50 (72%)	40/50 (80%)	37/50 (74%)
Adjusted rate	81.1%	75.2%	81.6%	74.9%
Terminal rate	25/33 (76%)	29/40 (73%)	34/42 (81%)	31/43 (72%)
First incidence (days)	568	533	568	445
Poly-3 test	P=0.381N	P=0.323N	P=0.578	P=0.310N

(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 is the P value associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in dosed group is indicated by **N**.
[e] Not applicable; no neoplasms in animal group
[f] One animal with an adenoma also had a hepatoblastoma.

TABLE D3
Historical Incidence of Malignant Lymphoma in Control Female B6C3F$_1$ Mice[a]

Study	Incidence in Controls
Overall Historical Incidence: Gavage Studies (all vehicles)	
Acrylonitrile (water)	4/50
Elmiron® (water)	7/50
2,4-Hexadienal (corn oil)	4/50
Methacrylonitrile (water)	9/50
Riddelliine (sodium phosphate)	7/50
Total (%)	31/250 (12.4%)
Range	8%-18%
Overall Historical Incidence: All Routes	
Total (%)	308/1,558 (19.8%)
Mean ± standard deviation	19.7% ± 13.3%
Range	6%-58%

[a] Data as of January 28, 2005. Includes data for histiocytic, lymphocytic, mixed, NOS, or undifferentiated lymphoma.

TABLE D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Disposition Summary				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths	1	1	1	
Moribund	7	4	5	1
Natural deaths	9	5	2	6
Survivors				
Died last week of study	4		1	2
Terminal sacrifice	29	40	41	41
Animals examined microscopically	50	50	50	50
Alimentary System				
Esophagus	(50)	(50)	(50)	(50)
Foreign body		1 (2%)		
Inflammation		1 (2%)		
Perforation		1 (2%)		
Gallbladder	(42)	(45)	(47)	(44)
Cyst	2 (5%)		1 (2%)	2 (5%)
Intestine large, rectum	(48)	(48)	(50)	(46)
Artery, inflammation, chronic, focal		1 (2%)		
Intestine large, cecum	(43)	(46)	(47)	(44)
Edema	1 (2%)			
Lymphoid tissue, hyperplasia, lymphoid			1 (2%)	
Intestine small, duodenum	(43)	(45)	(47)	(42)
Epithelium, hyperplasia, focal			1 (2%)	
Intestine small, jejunum	(43)	(45)	(47)	(43)
Epithelium, hyperplasia, focal		1 (2%)		
Lymphoid tissue, hyperplasia			1 (2%)	
Liver	(50)	(50)	(50)	(50)
Angiectasis, focal	2 (4%)			
Basophilic focus		1 (2%)		3 (6%)
Clear cell focus	3 (6%)	3 (6%)	4 (8%)	2 (4%)
Congestion	1 (2%)	2 (4%)	1 (2%)	1 (2%)
Eosinophilic focus	1 (2%)	1 (2%)	3 (6%)	1 (2%)
Fibrosis, focal	1 (2%)			
Hematopoietic cell proliferation	4 (8%)	1 (2%)	6 (12%)	2 (4%)
Hemorrhage	1 (2%)			1 (2%)
Hepatodiaphragmatic nodule	1 (2%)			
Infarct			1 (2%)	
Infiltration cellular, mixed cell	11 (22%)	12 (24%)	12 (24%)	15 (30%)
Mixed cell focus	2 (4%)	1 (2%)		1 (2%)
Bile duct, cyst	1 (2%)			
Hepatocyte, necrosis, focal	3 (6%)	3 (6%)	1 (2%)	
Hepatocyte, vacuolization cytoplasmic	12 (24%)	11 (22%)	8 (16%)	15 (30%)
Kupffer cell, pigmentation	1 (2%)		1 (2%)	
Mesentery	(18)	(14)	(15)	(13)
Hemorrhage			1 (7%)	1 (8%)
Infiltration cellular, lymphoid		1 (7%)		
Artery, inflammation, chronic, focal	1 (6%)	1 (7%)	1 (7%)	
Fat, necrosis, focal	16 (89%)	12 (86%)	13 (87%)	4 (31%)

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

TABLE D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Alimentary System (continued)				
Pancreas	(46)	(46)	(49)	(49)
Acinus, atrophy, diffuse				1 (2%)
Acinus, atrophy, focal	1 (2%)	1 (2%)		1 (2%)
Acinus, cytoplasmic alteration			1 (2%)	
Duct, cyst	2 (4%)		2 (4%)	2 (4%)
Salivary glands	(50)	(50)	(50)	(49)
Hyperplasia, lymphoid		1 (2%)		
Stomach, forestomach	(50)	(50)	(50)	(49)
Diverticulum	1 (2%)		1 (2%)	3 (6%)
Inflammation, focal	1 (2%)		1 (2%)	
Ulcer	2 (4%)		3 (6%)	1 (2%)
Epithelium, hyperplasia	2 (4%)	2 (4%)	2 (4%)	1 (2%)
Stomach, glandular	(45)	(47)	(50)	(46)
Cyst	1 (2%)			1 (2%)
Epithelium, cytoplasmic alteration, focal	1 (2%)			
Epithelium, hyperplasia	1 (2%)			
Tooth	(2)	(10)	(3)	(3)
Dysplasia	2 (100%)	10 (100%)	3 (100%)	3 (100%)
Cardiovascular System				
Blood vessel		(1)		
Aneurysm		1 (100%)		
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	1 (2%)			1 (2%)
Infiltration cellular, mixed cell			1 (2%)	
Mineralization, focal				1 (2%)
Thrombosis	1 (2%)			
Artery, inflammation, chronic			1 (2%)	
Endocrine System				
Adrenal cortex	(50)	(50)	(50)	(50)
Accessory adrenal cortical nodule	2 (4%)	2 (4%)		
Angiectasis			1 (2%)	
Cytoplasmic alteration, focal	1 (2%)			
Degeneration, cystic	1 (2%)			
Hyperplasia, focal			1 (2%)	
Infiltration cellular, mixed cell	1 (2%)			
Adrenal medulla	(50)	(50)	(49)	(50)
Hyperplasia, focal			1 (2%)	
Parathyroid gland	(48)	(48)	(46)	(49)
Cyst	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Pituitary gland	(46)	(49)	(48)	(47)
Angiectasis	2 (4%)	1 (2%)		
Cyst			1 (2%)	1 (2%)
Pars distalis, cytoplasmic alteration, focal	1 (2%)	4 (8%)	1 (2%)	2 (4%)
Pars distalis, hyperplasia, focal	1 (2%)	1 (2%)	1 (2%)	1 (2%)

TABLE D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Endocrine System (continued)				
Thyroid gland	(46)	(50)	(49)	(47)
Cyst	1 (2%)		1 (2%)	
Inflammation, chronic, focal	1 (2%)	1 (2%)	1 (2%)	
C-cell, hyperplasia		1 (2%)		1 (2%)
Follicle, degeneration, cystic, focal	17 (37%)	15 (30%)	28 (57%)	23 (49%)
Follicular cell, hyperplasia, focal	5 (11%)	2 (4%)	4 (8%)	5 (11%)
General Body System				
Tissue NOS	(3)			(3)
Pelvic, fibrosis	1 (33%)			
Genital System				
Clitoral gland	(48)	(50)	(46)	(48)
Degeneration, cystic		1 (2%)	1 (2%)	
Ovary	(47)	(48)	(49)	(48)
Angiectasis	3 (6%)	2 (4%)		2 (4%)
Cyst	11 (23%)	12 (25%)	16 (33%)	13 (27%)
Hemorrhage			1 (2%)	1 (2%)
Pigmentation, focal				1 (2%)
Thrombosis	3 (6%)			3 (6%)
Bilateral, cyst	1 (2%)			
Interstitial cell, hyperplasia		1 (2%)		
Uterus	(50)	(50)	(50)	(50)
Angiectasis	1 (2%)	1 (2%)	1 (2%)	
Hemorrhage		1 (2%)		
Inflammation, chronic		1 (2%)		
Inflammation, suppurative	1 (2%)			
Endometrium, hyperplasia, cystic	48 (96%)	45 (90%)	49 (98%)	48 (96%)
Hematopoietic System				
Bone marrow	(50)	(50)	(49)	(50)
Hyperplasia	10 (20%)	4 (8%)	9 (18%)	4 (8%)
Lymph node	(7)	(5)	(10)	(10)
Iliac, angiectasis	1 (14%)			
1 (14%)		2 (20%)	2 (20%)	
Mediastinal, hyperplasia, lymphoid	2 (29%)			1 (10%)
Mediastinal, inflammation				1 (10%)
Pancreatic, hyperplasia, lymphoid		1 (20%)	1 (10%)	1 (10%)
Renal, hyperplasia, lymphoid	1 (14%)		1 (10%)	1 (10%)
Lymph node, mandibular	(48)	(49)	(48)	(46)
Hemorrhage	1 (2%)			
Hyperplasia, lymphoid	9 (19%)	11 (22%)	12 (25%)	16 (35%)
Hyperplasia, mast cell			1 (2%)	
Lymph node, mesenteric	(46)	(49)	(49)	(48)
Ectasia		1 (2%)	2 (4%)	
Hemorrhage	1 (2%)	2 (4%)	2 (4%)	1 (2%)
Hyperplasia, histiocytic			1 (2%)	
Hyperplasia, lymphoid	6 (13%)	7 (14%)	10 (20%)	8 (17%)

TABLE D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Hematopoietic System (continued)				
Spleen	(47)	(47)	(49)	(50)
Angiectasis, focal		1 (2%)		
Congestion		2 (4%)		
Fibrosis, focal		1 (2%)	1 (2%)	
Hematopoietic cell proliferation	23 (49%)	21 (45%)	31 (63%)	40 (80%)
Hyperplasia, lymphoid	17 (36%)	15 (32%)	17 (35%)	11 (22%)
Thymus	(43)	(47)	(44)	(49)
Hyperplasia, lymphoid	8 (19%)	13 (28%)	11 (25%)	14 (29%)
Inflammation		1 (2%)		
Integumentary System				
Skin	(50)	(50)	(50)	(50)
Subcutaneous tissue, edema				1 (2%)
Musculoskeletal System				
Bone	(50)	(50)	(50)	(50)
Hyperostosis	5 (10%)	5 (10%)	4 (8%)	5 (10%)
Vertebra, hypertrophy, focal	3 (6%)	2 (4%)	1 (2%)	1 (2%)
Nervous System				
Brain	(50)	(50)	(50)	(49)
Compression, focal	1 (2%)			1 (2%)
Artery, meninges, inflammation, chronic	1 (2%)			
Spinal cord	(1)	(2)	(1)	(1)
Demyelination, focal		1 (50%)		
Respiratory System				
Lung	(50)	(50)	(50)	(50)
Congestion	1 (2%)			
Foreign body	1 (2%)		1 (2%)	
Hemorrhage	1 (2%)	1 (2%)		
Hyperplasia, histiocytic	1 (2%)			1 (2%)
Hyperplasia, lymphoid				1 (2%)
Infiltration cellular, polymorphonuclear	1 (2%)			
Infiltration cellular, mixed cell	1 (2%)			
Inflammation	1 (2%)		1 (2%)	
Metaplasia, focal, osseous	1 (2%)			
Alveolar epithelium, hyperplasia, focal	2 (4%)		1 (2%)	1 (2%)
Mediastinum, foreign body		1 (2%)	1 (2%)	
Mediastinum, inflammation		1 (2%)	1 (2%)	
Serosa, fibrosis			1 (2%)	
Nose	(50)	(50)	(50)	(50)
Inflammation		3 (6%)	7 (14%)	11 (22%)
Nasolacrimal duct, hyperplasia, focal				1 (2%)
Nasolacrimal duct, inflammation		1 (2%)		

TABLE D4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Special Senses System				
Eye	(43)	(48)	(47)	(47)
Atrophy				1 (2%)
Cataract				1 (2%)
Anterior chamber, inflammation	1 (2%)			
Artery, retrobulbar, inflammation, chronic, focal		1 (2%)		
Cornea, inflammation	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Cornea, necrosis, focal		1 (2%)		
Harderian gland	(49)	(50)	(50)	(50)
Epithelium, hyperplasia	2 (4%)	1 (2%)		3 (6%)
Urinary System				
Kidney	(48)	(50)	(49)	(50)
Hemorrhage				1 (2%)
Hyperplasia, lymphoid	1 (2%)			
Infarct		1 (2%)	2 (4%)	2 (4%)
Metaplasia, focal, osseous	2 (4%)	1 (2%)	1 (2%)	
Nephropathy	6 (13%)	5 (10%)	9 (18%)	5 (10%)
Bilateral, infarct				1 (2%)
Capsule, fibrosis, focal		1 (2%)		
Renal tubule, accumulation, hyaline droplet	2 (4%)			2 (4%)
Renal tubule, cyst	1 (2%)		3 (6%)	
Urinary bladder	(45)	(47)	(49)	(48)
Hyperplasia, lymphoid		1 (2%)		
Inflammation	1 (2%)			
Transitional epithelium, hyperplasia	1 (2%)			1 (2%)

APPENDIX E
GENETIC TOXICOLOGY

GENETIC TOXICOLOGY

SALMONELLA TYPHIMURIUM MUTAGENICITY TEST PROTOCOL

Two independent mutagenicity assays were conducted with methylene blue trihydrate. Testing for the first assay was performed as reported by Zeiger *et al.* (1988). The second assay, conducted with the same lot of methylene blue trihydrate tested in the 2-year study, used a slightly modified protocol (activation only with rat liver S9) and also employed *Escherichia coli* strain WP2 *uvrA*/pKM101 as a bacterial tester strain in addition to *Salmonella typhimurium* strains. Methylene blue trihydrate was sent to the laboratories as a coded aliquot. It was incubated with the bacterial tester strains (TA98, TA100, and *E. coli* WP2) 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.

Adjunct studies were conducted with Azure A, Azure B, and Azure C. All three compounds were tested in the protocol used in the second assay described above that used the *E. coli* strain and 10% rat liver S9 only. The three azure compounds were supplied to the testing laboratory as coded aliquots. Incubation and colony scoring was conducted as described above.

Each trial consisted of triplicate plates of concurrent positive and negative controls and at least five doses of methylene blue trihydrate. The high dose was limited by toxicity. All positive trials were repeated.

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.

CHINESE HAMSTER OVARY CELL CYTOGENETICS PROTOCOLS

Testing was performed as reported by Galloway *et al.* (1987). Methylene blue trihydrate was sent to the laboratory as a coded aliquot by Radian Corporation (Austin, TX). It was tested in cultured Chinese hamster ovary (CHO) cells for induction of sister chromatid exchanges (SCEs) and chromosomal aberrations (Abs), both in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 and cofactor mix. Cultures were handled under gold lights to prevent photolysis of bromodeoxyuridine-substituted DNA. Each test consisted of concurrent solvent and positive controls and of at least three doses of methylene blue trihydrate; the high dose was limited by toxicity. A single flask per dose was used, and the tests without S9 were repeated.

Sister Chromatid Exchange Test: In the SCE test without S9, CHO cells were incubated for 26 hours with methylene blue trihydrate in supplemented McCoy's 5A medium. Bromodeoxyuridine (BrdU) was added 2 hours after culture initiation. After 26 hours, the medium containing methylene blue trihydrate was removed and replaced with fresh medium plus BrdU and Colcemid, and incubation was continued for 2 hours. Cells were then harvested by mitotic shake-off, fixed, and stained with Hoechst 33258 and Giemsa. In the SCE test with S9, cells were incubated with methylene blue trihydrate, serum-free medium, and S9 for 2 hours. The medium was then removed and replaced with medium containing serum and BrdU and no methylene blue trihydrate. Incubation proceeded for an additional 26 hours, with Colcemid present for the final 2 hours. Harvesting and staining were the same as for cells treated without S9. All slides were scored blind, and those from a single test were read by the same person. Fifty second-division metaphase cells were scored for frequency of SCEs/cell from each dose

level. When significant chemical-induced cell cycle delay was seen, incubation time was lengthened to ensure a sufficient number of scorable (second-division metaphase) cells.

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

Chromosomal Aberrations Test: In the Abs test without S9, cells were incubated in McCoy's 5A medium with methylene blue trihydrate for 11.7 hours; Colcemid was added, and incubation continued for 2 hours. The cells were then harvested by mitotic shake-off, fixed, and stained with Giemsa. For the Abs test with S9, cells were treated with methylene blue trihydrate and S9 for 2 hours, after which the treatment medium was removed and the cells were incubated for 11.5 hours in fresh medium, with Colcemid present for the final 2 hours. Cells were harvested in the same manner as for the treatment without S9. The harvest time for the Abs test was based on the cell cycle information obtained in the SCE test.

Cells were selected for scoring on the basis of good morphology and completeness of karyotype (21 ± 2 chromosomes). All slides were scored blind, and those from a single test were read by the same person. Two hundred first-division metaphase cells were scored at each dose level. Classes of aberrations included simple (breaks and terminal deletions), complex (rearrangements and translocations), and other (pulverized cells, despiralized chromosomes, and cells containing 10 or more aberrations).

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

Mouse Bone Marrow and Peripheral Blood Micronucleus Test Protocols (Single Dose)

Preliminary range-finding studies were performed. Factors affecting dose selection included chemical solubility and toxicity and the extent of cell cycle delay induced by methylene blue trihydrate exposure. Male $B6C3F_1$ mice were administered a single intraperitoneal injection with methylene blue trihydrate dissolved in corn oil (Tice *et. al*, 1990). Solvent control animals were injected with corn oil only. The positive control animals received injections of cyclophosphamide. The animals were killed 48 hours after injection, and smears of bone marrow cells obtained from the femurs along with peripheral blood slides were prepared. Air-dried smears were fixed and stained; 2,000 polychromatic erythrocytes (PCEs) were scored per animal in each tissue for frequency of micronucleated cells. In addition, the percentage of PCEs among the total erythrocyte population in both tissues was scored for each group as a measure of bone marrow toxicity.

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 (3-MONTH STUDIES)

A detailed discussion of this assay is presented by MacGregor *et al.* (1990). At the end of the 3-month toxicity study, peripheral blood samples were obtained from male and female B6C3F$_1$ mice. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange and coded. Slides were scanned to determine the frequency of micronucleated cells in 2,000 normochromatic erythrocytes (NCEs) per mouse. In addition, the percentage of PCBs (reticulocytes) in a population of 1,000 erythrocytes was determined as a measure of chemical-related bone marrow toxicity.

The results for NCEs were tabulated as described for PCEs in the single dose bone marrow and peripheral blood micronucleus tests. Results of the 3-month study were accepted without repeat tests because additional 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

Two different lots of methylene blue trihydrate were tested independently at two laboratories for mutagenicity in bacterial tester strains. In the first study, methylene blue trihydrate (1 to 200 µg/plate) was mutagenic in *S. typhimurium* strains TA98 and TA100 when testing occurred in the presence of 30% rat or hamster liver S9 activation enzymes; without S9 enzymes, mutagenicity was seen only in strain TA98 (Table E1). In the second study, methylene blue trihydrate was mutagenic with and without 10% rat liver S9 in *S. typhimurium* strains TA98 and TA100 (0.25 to 150 µg/plate) and in *E. coli* strain WP2 (0.25 to 1,500 µg/plate) (Table E2).

Three azure compounds (A, B, C) were tested in the same protocol as was used in the second bacterial mutagenicity study. All three compounds were positive in *S. typhimurium* TA100 and TA98 and *E. coli* WP2

with and without 10% rat liver S9 (Tables E3, E4, and E5). In cytogenetic tests with cultured CHO cells, methylene blue trihydrate induced SCEs (0.17 to 2.5 µg/mL without S9; up to 5.0 µg/mL with S9) (Table E6) and chromosomal aberrations at 4.7 to 22 µg/mL (Table E7) with and without S9 activation enzymes.

In contrast to the clearly positive results in the *in vitro* studies, no increase in the frequency of micronucleated erythrocytes was observed in bone marrow or blood of male mice sampled 48 hours after a single intraperitoneal injection of 25, 50, or 150 mg/kg methylene blue trihydrate (Table E8). The negative response in mouse bone marrow may have been the result of suboptimal sampling time; bone marrow analysis is usually conducted 24 hours after dosing. Forty-eight hours after dosing, the young, exposed erythrocytes analyzed for the presence of micronuclei are typically found only in the peripheral blood. However, the peripheral blood micronucleus data from this short-term exposure study were also negative, and thus, they provide additional evidence of a lack of response by methylene blue in this *in vivo* assay for chromosomal damage. Furthermore, no increases in micronucleated erythrocytes were observed in peripheral blood samples taken from male and female mice at the end of the 3-month toxicity study (Table E9). However, a strong, dose-related increase in the percentage of polychromatic erythrocytes (reticulocytes) among total erythrocytes was seen in both male and female mice in the 3-month study; this observed increase in immature circulating erythrocytes indicates a stimulation of erythropoiesis consistent with other results, indicating a response to methylene blue trihydrate-induced anemia.

TABLE E1
Mutagenicity of Methylene Blue Trihydrate in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Revertants/Plate[b]					
		–S9		+30% hamster S9		+30% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
TA100	0	119 ± 6.8		136 ± 15.2	137 ± 2.0	147 ± 3.1	156 ± 3.2
	1	97 ± 5.9					
	3.3	132 ± 6.3			157 ± 2.4		155 ± 2.0
	10	143 ± 2.2		130 ± 11.5	213 ± 12.6	179 ± 1.5	176 ± 1.5
	33	159 ± 5.6		246 ± 25.7	792 ± 31.6	339 ± 9.7	603 ± 41.0
	67			407 ± 14.5	351 ± 35.7	478 ± 26.0	703 ± 41.4
	100	45 ± 5.8[c]		475 ± 16.5	146 ± 29.1[c]	509 ± 29.2	192 ± 41.6[c]
	200			180 ± 30.8[c]	341 ± 78.2[c]	146 ± 21.8[c]	153 ± 31.5[c]
Trial summary		Equivocal		Positive	Positive	Positive	Positive
Positive control[d]		543 ± 11.6		623 ± 23.7	881 ± 16.5	1,412 ± 33.5	506 ± 45.3
TA98	0	16 ± 1.5	18 ± 4.2	22 ± 0.3	28 ± 3.4	26 ± 4.9	33 ± 2.6
	1	22 ± 2.0	20 ± 0.7				
	3.3	19 ± 4.3	27 ± 3.9		79 ± 6.0		78 ± 3.8
	10	25 ± 3.8	33 ± 1.2	54 ± 8.1	85 ± 3.2	77 ± 4.2	107 ± 11.9
	33	37 ± 1.5	50 ± 5.0	287 ± 26.0	155 ± 6.2	119 ± 9.0	175 ± 17.4
	50		21 ± 1.5				
	67			191 ± 6.1	58 ± 10.3	176 ± 13.0	149 ± 6.4
	100	6 ± 1.8[c]	9 ± 4.7[c]	161 ± 11.7	40 ± 8.9[c]	214 ± 17.4	31 ± 7.5[c]
	200			61 ± 9.6[c]	47 ± 1.2[c]	43 ± 10.7[c]	23 ± 6.1[c]
Trial summary		Weakly Positive	Positive	Positive	Positive	Positive	Positive
Positive control		342 ± 7.1	298 ± 48.8	560 ± 12.2	806 ± 64.7	356 ± 8.1	137 ± 4.4

[a] Study was performed at BioReliance Corporation. The detailed protocol is presented by Zeiger *et al.* (1988). 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] Slight toxicity
[d] The positive controls in the absence of metabolic activation were sodium azide (TA100) and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with both strains was 2-aminoanthracene.

TABLE E2
Mutagenicity of Methylene Blue Trihydrate (Lot No. 68H3728) in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+ 10% rat S9 Trial 1	+ 10% rat S9 Trial 2
TA100	0	52 ± 3	55 ± 2	67 ± 5	66 ± 8
	0.25		107 ± 5		
	0.5		133 ± 2		
	1	105 ± 10	193 ± 5		
	2.5		271 ± 20		
	5	124 ± 8	115 ± 9	95 ± 2	87 ± 4
	10	42 ± 5		194 ± 2	111 ± 15
	15	Toxic			
	25	Toxic		238 ± 11	187 ± 9
	50			265 ± 7	253 ± 6
	100			342 ± 5	313 ± 1
	150				Toxic
Trial summary		Positive	Positive	Positive	Positive
Positive control[c]		365 ± 13	396 ± 10	649 ± 22	798 ± 14
TA98	0	22 ± 2	29 ± 6	34 ± 2	46 ± 1
	0.25		33 ± 2		
	0.5		64 ± 5		
	1	151 ± 27	155 ± 3		
	2.5		124 ± 6		
	5	44 ± 2	73 ± 1	59 ± 3	73 ± 9
	10	Toxic		122 ± 2	109 ± 10
	15	Toxic			
	25	Toxic		201 ± 8	146 ± 6
	50			203 ± 14	234 ± 4
	100			87 ± 6	110 ± 2
	150			40 ± 2	
Trial summary		Positive	Positive	Positive	Positive
Positive control		615 ± 34	476 ± 32	1,314 ± 23	1,078 ± 20
***Escherichia coli* WPM *uvrA*/pKM101 (Analogous to TA102)**					
	0	141 ± 18	105 ± 1	220 ± 8	178 ± 12
	0.25	158 ± 9			
	0.5	255 ± 11			
	1	376 ± 2	570 ± 14		
	2.5	426 ± 6			
	5		491 ± 39		
	10		166 ± 21	243 ± 16	198 ± 31
	15		124 ± 6		
	25		Toxic	275 ± 5	338 ± 38
	100			310 ± 18	437 ± 31
	500			462 ± 15	368 ± 34
	1,500			193 ± 17	186 ± 8
Trial summary		Positive	Positive	Positive	Positive
Positive control		2,149 ± 59	2,035 ± 15	1,162 ± 19	1,069 ± 7

[a] Study was performed at SITEK Research Laboratories. 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (WPM *uvrA*/pKM101). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE E3
Mutagenicity of Azure A in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+ 10% rat S9 Trial 1	+ 10% rat S9 Trial 2
TA100	0	100 ± 4	79 ± 11	69 ± 9	67 ± 3
	5	140 ± 6			
	10	252 ± 5			
	25	520 ± 46	557 ± 27	87 ± 8	93 ± 8
	50	482 ± 24	191 ± 53	112 ± 2	125 ± 6
	100	144 ± 47	123 ± 21	110 ± 9	245 ± 38
	250		Toxic	144 ± 5	131 ± 3
	500			Toxic	79 ± 14[c]
	1,000				
Trial summary		Positive	Positive	Positive	Positive
Positive control[d]		438 ± 27	636 ± 34	1,114 ± 95	1,053 ± 35
TA98	0	31 ± 4	30 ± 1	26 ± 2	30 ± 1
	5		47 ± 7		
	10		121 ± 16		
	25	180 ± 12	322 ± 3	31 ± 3	33 ± 2
	50	143 ± 29	350 ± 21	44 ± 4	48 ± 5
	100	131 ± 44	111 ± 40	39 ± 2	95 ± 16
	200	35 ± 11		67 ± 4	62 ± 4
	500	3 ± 2[c]		66 ± 6	69 ± 8
	1,000				64 ± 7[e]
Trial summary		Positive	Positive	Positive	Positive
Positive control		418 ± 49	257 ± 31	1,059 ± 53	818 ± 101
***Escherichia coli* WPM *uvrA*/pKM101 (Analogous to TA102)**					
	0	178 ± 10	164 ± 2	194 ± 10	203 ± 13
	50	646 ± 5		211 ± 13	
	100	552 ± 15		297 ± 23	
	250	577 ± 82	540 ± 119	543 ± 72	311 ± 8
	500	540 ± 5	531 ± 28	344 ± 31	414 ± 32
	1,500	249 ± 13	161 ± 23[c]	488 ± 38	381 ± 19
	2,500		113 ± 7[e]		392 ± 26
	3,000	7 ± 3[c]		129 ± 13	
	3,500		8 ± 7[c]		178 ± 10
Trial summary		Positive	Positive	Positive	Positive
Positive control		1,714 ± 58	1,895 ± 117	737 ± 79	1,148 ± 40

[a] Study was performed at SITEK Research Laboratories. 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] Slight toxicity and precipitate on plate
[d] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (WPM *uvrA*/pKM101). The positive control for metabolic activation with all strains was 2-aminoanthracene.
[e] Precipitate on plate

TABLE E4
Mutagenicity of Azure B in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+ 10% rat S9 Trial 1	+ 10% rat S9 Trial 2
TA100	0	95 ± 1	79 ± 11	69 ± 9	78 ± 13
	1	102 ± 8			
	5	134 ± 3			
	10	196 ± 4	229 ± 3	164 ± 6	
	25	232 ± 3	317 ± 16	175 ± 18	124 ± 11
	50	314 ± 4	250 ± 28	165 ± 19	202 ± 35
	100	359 ± 24	47 ± 7	94 ± 11	99 ± 6
	250		Toxic	51 ± 11	49 ± 5
	500				7 ± 3[c]
Trial summary		Positive	Positive	Positive	Positive
Positive control[d]		322 ± 29	636 ± 34	1,114 ± 95	743 ± 40
TA98	0	31 ± 4	23 ± 1	28 ± 1	32 ± 1
	1		29 ± 3		
	5		45 ± 7		
	10	104 ± 4	41 ± 4	57 ± 7	70 ± 1
	25	107 ± 15	79 ± 4	59 ± 4	75 ± 2
	50	86 ± 13	82 ± 9	46 ± 5	81 ± 3
	100	27 ± 6	85 ± 1	44 ± 6	49 ± 5
	250	Toxic		45 ± 5	44 ± 3
	500				50 ± 11
Trial summary		Positive	Positive	Positive	Positive
Positive control		451 ± 16	250 ± 3	1,059 ± 53	592 ± 24
***Escherichia coli* WPM *uvrA*/pKM101 (Analogous to TA102)**					
	0	174 ± 7	164 ± 2	218 ± 2	203 ± 13
	1	174 ± 14			
	5	232 ± 10			
	10	297 ± 28			
	25	357 ± 6			
	50	464 ± 20	675 ± 36		279 ± 20
	100	460 ± 21	650 ± 67	292 ± 8	356 ± 16
	250		436 ± 26	392 ± 37	288 ± 23
	500		228 ± 21	369 ± 33	378 ± 18
	750		169 ± 31	420 ± 29	543 ± 28
	1,000			425 ± 31	
Trial summary		Positive	Positive	Positive	Positive
Positive control		1,333 ± 110	1,895 ± 117	759 ± 22	1,148 ± 40

[a] Study was performed at SITEK Research Laboratories. 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] Slight toxicity and precipitate on plate
[d] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (WPM *uvrA*/pKM101). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE E5
Mutagenicity of Azure C in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+ 10% rat S9 Trial 1	+ 10% rat S9 Trial 2
TA100	0	100 ± 4	79 ± 11	69 ± 9	67 ± 3
	1	94 ± 4			
	5	129 ± 5			
	10	153 ± 4			
	25	229 ± 37	270 ± 23	85 ± 6	
	50	260 ± 11	320 ± 14	84 ± 6	
	100		311 ± 57	124 ± 6	112 ± 4
	250		198 ± 24	113 ± 6	127 ± 2
	500		58 ± 9	100 ± 7	66 ± 5
	750		Toxic	84 ± 4	55 ± 4[c]
	1,000				71 ± 4[c]
Trial summary		Positive	Positive	Positive	Positive
Positive control[d]		438 ± 27	636 ± 34	1,114 ± 95	1,053 ± 35
TA98	0	31 ± 4	23 ± 5	28 ± 1	30 ± 1
	1		22 ± 2		
	5		40 ± 5		
	10		62 ± 5		
	25	67 ± 2	94 ± 8	31 ± 3	
	50	93 ± 7	100 ± 7	35 ± 3	
	100	69 ± 10		46 ± 7	48 ± 1
	250	73 ± 13		62 ± 0	88 ± 15
	500	39 ± 4		69 ± 1	66 ± 5
	750	32 ± 5		63 ± 4	68 ± 2
	1,000				35 ± 2[c]
Trial summary		Positive	Positive	Positive	Positive
Positive control		418 ± 49	257 ± 31	1,059 ± 53	818 ± 101
***Escherichia coli* WPM *uvrA*/pKM101 (Analogous to TA102)**					
	0	178 ± 10	164 ± 2	194 ± 10	203 ± 13
	5	207 ± 18			
	10	266 ± 31			
	25	320 ± 9			
	50	384 ± 6		266 ± 11	
	100	496 ± 49	616 ± 31	315 ± 8	292 ± 4
	250		588 ± 20		312 ± 19
	500		398 ± 52	354 ± 30	393 ± 29
	1,000		156 ± 9	597 ± 29[c]	441 ± 17
	1,500			500 ± 78[c]	
	2,500		130 ± 12		346 ± 52[c]
	3,500		58 ± 36		212 ± 20[c]
	5,000		1 ± 1[d]		134 ± 11[c]
Trial summary		Positive	Positive	Positive	Positive
Positive control		1,714 ± 58	1,895 ± 117	737 ± 79	1,148 ± 40

[a] Study was performed at SITEK Research Laboratories. 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] Precipitate on plate
[d] The positive controls in the absence of metabolic activation were sodium azide (TA100), 4-nitro-*o*-phenylenediamine (TA98), and methyl methanesulfonate (WPM *uvrA*/pKM101). The positive control for metabolic activation with all strains was 2-aminoanthracene.
[e] Slight toxicity and precipitate on plate

TABLE E6
Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Methylene Blue Trihydrate[a]

Compound	Dose (µg/mL)	Total Cells Scored	No. of Chromo-somes	No. of SCEs	SCEs/ Chromo-some	SCEs/ Cell	Hrs in BrdU	Relative Change of SCEs/ Chromosome[b] (%)
–S9								
Trial 1								
Summary: Weakly positive								
Water[c]		50	1,048	365	0.35	7.3	26.0	
Methylene blue trihydrate	0.17	50	1,047	385	0.37	7.7	26.0	5.58
	0.5	50	1,050	406	0.39	8.1	26.0	11.02
	1.7	50	1,047	445	0.43	8.9	26.0	22.03*
	5	0					31.0[d]	
						P=0.002[e]		
Mitomycin-C[f]	0.001	50	1,050	514	0.49	10.3	26.0	40.55*
	0.004	10	210	203	0.97	20.3	26.0	177.55*
Trial 2								
Summary: Positive								
Water		50	1,049	344	0.33	6.88	26.0	
Methylene blue trihydrate	0.63	50	1,048	470	0.45	9.40	26.0	36.76*
	1.3	50	1,048	497	0.47	9.94	26.0	44.61*
	2.5	50	1,046	559	0.53	11.18	26.0	62.97*
	5	Toxic					31.0[d]	
						P≤0.001		
Mitomycin-C	0.001	50	1,050	549	0.52	10.98	26.0	59.44*
	0.004	10	210	243	1.16	24.30	26.0	252.86*

TABLE E6
Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Methylene Blue Trihydrate

Compound	Dose (µg/mL)	Total Cells Scored	No. of Chromo-somes	No. of SCEs	SCEs/ Chromo-some	SCEs/ Cell	Hrs in BrdU	Relative Change of SCEs/ Chromosome (%)
+S9								
Trial 1								
Summary: Weakly positive								
Water		50	1,048	395	0.38	7.90	26.0	
		50	1,050	412	0.39	8.24	31.0[d]	
Methylene blue trihydrate	0.5	50	1,047	399	0.38	7.98	26.0	1.11
	1.7	50	1,050	452	0.43	9.04	26.0	14.21
	5[d]	50	1,048	770	0.73	15.40	31.0[d]	94.94*
	17	Toxic					31.0[d]	
						P≤0.001		
Cyclophosphamide[f]	0.125	50	1,050	479	0.46	9.58	26.0	21.03*
	0.5	10	210	199	0.95	19.90	26.0	151.42*

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

TABLE E7
Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Methylene Blue Trihydrate[a]

Compound	Dose (µg/mL)	Total Cells Scored	Number of Aberrations	Aberrations/ Cell	Cells with Aberrations (%)
–S9					
Trial 1					
Harvest time: 13.7 hours					
Summary: Positive					
Water[b]		200	0	0.00	0.0
Methylene blue trihydrate	7.5	200	6	0.03	3.0*
	10	200	8	0.04	4.0*
	15	200	14	0.07	7.0*
	25	50	30	0.60	34.0*
	35	Toxic			0.0
				P≤0.001[c]	
Mitomycin-C[d]	0.4	25	17	0.68	52.0
Trial 2					
Harvest time: 13.7 hours					
Summary: Positive					
Water		200	0	0.00	0.0
Methylene blue trihydrate	4.7	200	3	0.02	0.5
	10	200	5	0.03	2.5*
	22	200	50	0.25	18.5*
	47	Toxic			
	100	Toxic			
				P≤0.001	
Mitomycin-C	0.4	25	18	0.72	48.0*

TABLE E7
Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Methylene Blue Trihydrate

Compound	Dose (µg/mL)	Total Cells Scored	Number of Aberrations	Aberrations/ Cell	Cells with Aberrations (%)
+S9					
Trial 1					
Harvest time: 13.5 hours					
Summary: Weakly positive					
Water		200	3	0.02	1.5
Methylene blue trihydrate	1.0	200	3	0.02	1.5
	2.2	200	5	0.03	2.5
	4.7	200	42	0.21	12.0*
	10	0			
					P≤0.001
Cyclophosphamide[d]	20	25	12	0.48	24.0

* Positive response (P≤0.05) versus the solvent control
[a] Study was performed at SITEK Research Laboratories. The detailed protocol is presented by Galloway *et al.* (1987).
[b] Solvent control
[c] Significance of percent cells with aberrations tested by the linear regression trend test versus log of the dose
[d] Positive control

TABLE E8
Induction of Micronuclei in Polychromatic Erythrocytes of Male Mice Treated with a Single Intraperitoneal Injection of Methylene Blue Trihydrate[a]

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs[b]	P Value[c]	PCEs[b] (%)
Bone Marrow (48 hours)					
Corn oil[d]	0	5	1.1 ± 0.40		41.7 ± 4.71
Methylene blue trihydrate	25	5	1.9 ± 0.53	0.0719	30.1 ± 4.77
	50	4	1.4 ± 0.24	0.2999	25.5 ± 3.20
	150	4	1.5 ± 0.20	0.2277	33.4 ± 3.22
			P=0.401[e]		
Cyclophosphamide[f]	25	5	2.8 ± 0.58	0.0032	26.7 ± 5.01
Peripheral Blood (48 hours)					
Corn oil	0	5	2.8 ± 0.51		2.7 ± 0.15
Methylene blue trihydrate	25	5	4.3 ± 0.75	0.0373	4.5 ± 0.34
	50	4	2.6 ± 0.97	0.5886	3.8 ± 0.51
	150	4	1.5 ± 0.35	0.9672	2.8 ± 0.81
			P=0.994		
Cyclophosphamide	25	5	8.4 ± 1.21		3.9 ± 0.46

[a] Study was performed at ILS, Inc. The detailed protocol is presented by Tice *et al*. (1990). PCE=polychromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the vehicle control; dosed group values are significant at P≤0.008; 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 E9
Frequency of Micronuclei in Mouse Peripheral Blood Erythrocytes Following Treatment with Methylene Blue Trihydrate by Gavage for 3 Months[a]

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs[b] (%)
Male					
Methylcellulose[d]	0	5	0.0 ± 0.0		3.6 ± 0.43
Methylene blue trihydrate	25	5	0.3 ± 0.20	0.0416	5.0 ± 0.41
	50	5	0.0 ± 0.00	0.5000	10.0 ± 1.20
	100	5	0.1 ± 0.10	0.1586	11.0 ± 0.76
	200	5	0.2 ± 0.12	0.0786	36.9 ± 2.10
			P=0.235[e]		
Female					
Methylcellulose	0	5	0.6 ± 0.19		1.9 ± 0.11
Methylene blue trihydrate	25	5	0.1 ± 0.10	0.9706	3.4 ± 0.20
	50	5	0.3 ± 0.12	0.8414	6.8 ± 0.97
	100	5	0.1 ± 0.10	0.9706	13.6 ± 1.22
	200	5	0.1 ± 0.10	0.9706	19.7 ± 1.64
			P=0.959		

[a] Study was performed at SITEK Research Laboratories. The detailed protocol is presented by MacGregor *et al*. (1999).
[b] NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte
[c] Mean ± standard error
[d] Pairwise comparison with the vehicle control; dosed group values are significant at P≤0.006 (ILS, 1990)
[e] Vehicle control
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 1-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg	1,000 mg/kg	2,000 mg/kg
Male						
Hematology						
Day 4	9	7	10	8	10	6
Day 30	10	10	10	8	0	0
Hematocrit (%)						
Day 4	43.0 ± 0.5	42.0 ± 0.4	44.2 ± 0.5	43.9 ± 0.7	43.5 ± 0.7	42.2 ± 2.2
Day 30	46.1 ± 0.3	43.6 ± 0.5**	42.6 ± 0.5**	40.9 ± 0.9**		
Hemoglobin (g/dL)						
Day 4	14.3 ± 0.1	14.2 ± 0.1	14.5 ± 0.1	14.3 ± 0.3	14.2 ± 0.2	14.6 ± 0.8
Day 30	15.7 ± 0.2	14.9 ± 0.1**	14.7 ± 0.2**	14.6 ± 0.2**		
Erythrocytes (10^6/μL)						
Day 4	6.87 ± 0.07	6.83 ± 0.05	7.15 ± 0.09	7.15 ± 0.13	7.02 ± 0.12	6.80 ± 0.37
Day 30	8.26 ± 0.08	7.35 ± 0.08**	6.46 ± 0.10**	5.82 ± 0.21**		
Reticulocytes (10^6/μL)						
Day 4	0.41 ± 0.03	0.40 ± 0.03	0.37 ± 0.02	0.40 ± 0.03	0.35 ± 0.03	0.33 ± 0.06
Day 30	0.24 ± 0.03	0.48 ± 0.04**	0.69 ± 0.09**	0.99 ± 0.05**		
Nucleated erythrocytes (10^3/μL)						
Day 4	0.09 ± 0.04	0.08 ± 0.03	0.07 ± 0.06	0.09 ± 0.05	0.08 ± 0.03	0.27 ± 0.08
Day 30	0.02 ± 0.01	0.18 ± 0.06**	0.96 ± 0.16**	1.07 ± 0.21**		
Mean cell volume (fL)						
Day 4	62.6 ± 0.3	61.5 ± 0.3	61.9 ± 0.3	61.3 ± 0.3	62.0 ± 0.3	62.1 ± 0.3
Day 30	55.8 ± 0.2	59.3 ± 0.2**	66.1 ± 0.8**	70.5 ± 1.4**		
Mean cell hemoglobin (pg)						
Day 4	20.8 ± 0.1	20.7 ± 0.2	20.3 ± 0.2	20.0 ± 0.1**	20.3 ± 0.1	21.4 ± 0.2
Day 30	19.0 ± 0.1	20.2 ± 0.1**	22.8 ± 0.3**	25.3 ± 0.6**		
Mean cell hemoglobin concentration (g/dL)						
Day 4	33.3 ± 0.3	33.8 ± 0.4	32.7 ± 0.2	32.6 ± 0.2	32.7 ± 0.2	34.5 ± 0.4
Day 30	34.1 ± 0.1	34.1 ± 0.2	34.4 ± 0.2	35.9 ± 0.3**		
Platelets (10^3/μL)						
Day 4	1018.9 ± 15.2	1,024.3 ± 27.7	1,032.4 ± 16.0	1,179.4 ± 24.7**	1,144.1 ± 24.1*	701.2 ± 145.4
Day 30	789.2 ± 15.3	942.9 ± 14.5**	865.8 ± 19.7	877.4 ± 23.8*		
Leukocytes (10^3/μL)						
Day 4	10.26 ± 0.74	10.13 ± 0.54	11.06 ± 0.47	11.10 ± 0.57	9.48 ± 0.51	4.78 ± 0.48**
Day 30	8.13 ± 0.28	9.28 ± 0.46*	11.14 ± 0.50**	12.05 ± 0.60**		
Segmented neutrophils (10^3/μL)						
Day 4	2.48 ± 0.24	2.92 ± 0.47	3.10 ± 0.29	2.06 ± 0.24	1.65 ± 0.31	0.49 ± 0.23**
Day 30	1.21 ± 0.10	1.56 ± 0.15	2.05 ± 0.26**	2.17 ± 0.44*		
Lymphocytes (10^3/μL)						
Day 4	7.51 ± 0.54	7.03 ± 0.55	7.81 ± 0.37	8.86 ± 0.35	7.64 ± 0.45	4.25 ± 0.48*
Day 30	6.80 ± 0.29	7.56 ± 0.35	8.84 ± 0.36**	9.59 ± 0.41**		
Monocytes (10^3/μL)						
Day 4	0.21 ± 0.06	0.13 ± 0.03	0.10 ± 0.05	0.18 ± 0.06	0.14 ± 0.03	0.04 ± 0.02
Day 30	0.07 ± 0.03	0.13 ± 0.02	0.19 ± 0.05*	0.24 ± 0.05**		
Eosinophils (10^3/μL)						
Day 4	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.03	0.00 ± 0.00	0.05 ± 0.02	0.01 ± 0.01
Day 30	0.05 ± 0.02	0.04 ± 0.02	0.06 ± 0.03	0.05 ± 0.03		
Methemoglobin (g/dL)						
Day 4	0.25 ± 0.02[b]	0.42 ± 0.03**[b]	0.62 ± 0.04**	1.00 ± 0.06**[b]	1.18 ± 0.08**	1.13 ± 0.03**
Day 30	0.36 ± 0.03	0.59 ± 0.02**	0.63 ± 0.05**	0.68 ± 0.05**		
Heinz bodies (%)						
Day 4	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0	57.7 ± 15.2**[b]	95.0 ± 0.4**	95.2 ± 0.8**
Day 30	0.0 ± 0.0	1.7 ± 0.8**	20.2 ± 1.9**	26.6 ± 1.2**[c]		

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 1-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg	1,000 mg/kg	2,000 mg/kg
Male (continued)						
Clinical Chemistry						
Day 4	10	10	10	10	10	6
Day 30	10	10	10	8	0	0
Urea nitrogen (mg/dL)						
Day 4	21.5 ± 0.6	22.4 ± 0.6	22.0 ± 0.7	21.0 ± 1.0	17.4 ± 0.8*	44.7 ± 10.0
Day 30	24.9 ± 0.4	23.3 ± 0.2*	23.4 ± 0.6*	23.8 ± 0.6		
Creatinine (mg/dL)						
Day 4	0.67 ± 0.02	0.68 ± 0.02	0.65 ± 0.02	0.64 ± 0.07	0.64 ± 0.02	0.73 ± 0.07
Day 30	0.70 ± 0.00	0.68 ± 0.01	0.70 ± 0.00	0.69 ± 0.01		
Total protein (g/dL)						
Day 4	6.3 ± 0.1	6.3 ± 0.1	6.3 ± 0.1	6.0 ± 0.1**	5.8 ± 0.1**	4.7 ± 0.2**
Day 30	6.7 ± 0.1	6.7 ± 0.0	6.8 ± 0.1	6.8 ± 0.1		
Albumin (g/dL)						
Day 4	4.4 ± 0.0	4.4 ± 0.0	4.3 ± 0.0	4.2 ± 0.1**	4.0 ± 0.1**	3.0 ± 0.1**
Day 30	4.6 ± 0.0	4.7 ± 0.0*	4.9 ± 0.1**	4.8 ± 0.0**		
Alanine aminotransferase (IU/L)						
Day 4	43 ± 1	41 ± 1	43 ± 2	45 ± 3	38 ± 2*	28 ± 4**[d]
Day 30	47 ± 2	40 ± 1*	46 ± 3	45 ± 1		
Alkaline phosphatase (IU/L)						
Day 4	1,050 ± 16	1,015 ± 17	1,060 ± 24	818 ± 29**	587 ± 26**	303 ± 17**
Day 30	605 ± 13	542 ± 9**	505 ± 13**	531 ± 3**		
Creatine kinase (IU/L)						
Day 4	587 ± 83	837 ± 110	628 ± 94	427 ± 79	410 ± 27	943 ± 185
Day 30	356 ± 64	424 ± 54[e]	300 ± 35	357 ± 79		
Sorbitol dehydrogenase (IU/L)						
Day 4	25 ± 1	25 ± 1	28 ± 1	26 ± 2	26 ± 2	25 ± 4[d]
Day 30	21 ± 1	20 ± 1	24 ± 3	24 ± 1		
Bile salts (μmol/L)						
Day 4	34.7 ± 2.9	36.2 ± 3.7	37.1 ± 4.1	38.4 ± 2.9	29.2 ± 3.0	110.0 ± 53.3
Day 30	20.5 ± 2.7	25.9 ± 3.9	37.5 ± 3.8**	55.3 ± 6.8**		
Female						
Hematology						
Day 4	10	10	10	10	10	5
Day 30	10	10	10	6	0	0
Hematocrit (%)						
Day 4	44.0 ± 0.5	44.0 ± 0.4	45.0 ± 0.3	45.8 ± 0.3	43.4 ± 1.3	37.4 ± 2.3
Day 30	46.6 ± 0.4	43.7 ± 0.4**	41.8 ± 0.6**	42.3 ± 0.2**		
Hemoglobin (g/dL)						
Day 4	14.5 ± 0.2	14.6 ± 0.1	14.7 ± 0.1	14.9 ± 0.1	14.7 ± 0.3	13.5 ± 0.8
Day 30	15.5 ± 0.1	14.5 ± 0.1**	14.2 ± 0.1**	14.8 ± 0.1**		
Erythrocytes (10^6/μL)						
Day 4	6.86 ± 0.10	6.88 ± 0.08	7.10 ± 0.05	7.24 ± 0.07*	6.87 ± 0.20	5.90 ± 0.34
Day 30	7.74 ± 0.06	6.70 ± 0.05**	6.11 ± 0.10**	5.61 ± 0.13**		

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 1-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg	1,000 mg/kg	2,000 mg/kg
Female (continued)						
Hematology (continued)						
Day 4	10	10	10	10	10	5
Day 30	10	10	10	6	0	0
Reticulocytes (10^6/μL)						
Day 4	0.36 ± 0.01	0.37 ± 0.02	0.39 ± 0.02	0.39 ± 0.03	0.28 ± 0.02	0.27 ± 0.04
Day 30	0.12 ± 0.01	0.23 ± 0.02**	0.35 ± 0.02**	0.55 ± 0.04**		
Nucleated erythrocytes (10^3/μL)						
Day 4	0.02 ± 0.01	0.05 ± 0.02	0.05 ± 0.02	0.07 ± 0.06	0.17 ± 0.08	0.15 ± 0.08
Day 30	0.00 ± 0.00	0.06 ± 0.03*	0.24 ± 0.10**	3.17 ± 1.13**		
Mean cell volume (fL)						
Day 4	64.2 ± 0.3	63.9 ± 0.2	63.4 ± 0.2	63.2 ± 0.3	63.2 ± 0.3	63.3 ± 0.3
Day 30	60.3 ± 0.2	65.3 ± 0.3**	68.4 ± 0.4**	75.6 ± 1.6**		
Mean cell hemoglobin (pg)						
Day 4	21.2 ± 0.1	21.2 ± 0.2	20.7 ± 0.1	20.6 ± 0.1	21.5 ± 0.3	22.8 ± 0.2
Day 30	20.1 ± 0.1	21.7 ± 0.1**	23.3 ± 0.3**	26.4 ± 0.7**		
Mean cell hemoglobin concentration (g/dL)						
Day 4	33.0 ± 0.2	33.2 ± 0.2	32.6 ± 0.1	32.5 ± 0.2	34.1 ± 0.5	36.1 ± 0.4*
Day 30	33.3 ± 0.1	33.3 ± 0.2	34.1 ± 0.3	34.9 ± 0.2**		
Platelets (10^3/μL)						
Day 4	996.1 ± 8.9	1,023.0 ± 16.6	1,081.4 ± 14.6**	1,167.9 ± 11.6**	1,141.4 ± 54.2**	484.0 ± 114.9*
Day 30	786.0 ± 14.4	901.5 ± 10.1**	895.3 ± 15.9**	859.3 ± 24.5		
Leukocytes (10^3/μL)						
Day 4	9.70 ± 0.66	9.75 ± 0.29	10.90 ± 0.58	11.80 ± 0.68	9.18 ± 0.75	3.38 ± 0.28*
Day 30	7.76 ± 0.31	7.66 ± 0.46	9.55 ± 0.48*	13.70 ± 1.30**		
Segmented neutrophils (10^3/μL)						
Day 4	2.09 ± 0.24	2.57 ± 0.29	3.39 ± 0.32	2.77 ± 0.22	0.81 ± 0.23*	0.07 ± 0.01**
Day 30	1.25 ± 0.10	1.95 ± 0.22**	2.34 ± 0.22**	3.03 ± 0.84**		
Lymphocytes (10^3/μL)						
Day 4	7.37 ± 0.57	6.93 ± 0.14	7.26 ± 0.40	8.63 ± 0.53	8.09 ± 0.61	3.18 ± 0.26*
Day 30	6.28 ± 0.23	5.63 ± 0.42	7.10 ± 0.29	10.52 ± 0.94**		
Monocytes (10^3/μL)						
Day 4	0.16 ± 0.04	0.19 ± 0.05	0.19 ± 0.05	0.28 ± 0.05	0.25 ± 0.07	0.14 ± 0.03
Day 30	0.14 ± 0.02	0.05 ± 0.02*	0.08 ± 0.03	0.11 ± 0.06		
Eosinophils (10^3/μL)						
Day 4	0.08 ± 0.02	0.06 ± 0.03	0.06 ± 0.02	0.12 ± 0.05	0.03 ± 0.02	0.00 ± 0.00
Day 30	0.11 ± 0.03	0.04 ± 0.02	0.04 ± 0.03*	0.05 ± 0.03		
Methemoglobin (g/dL)						
Day 4	0.23 ± 0.03	0.77 ± 0.03**	1.05 ± 0.06**	1.48 ± 0.06**	1.31 ± 0.03**	1.60 ± 0.23**
Day 30	0.35 ± 0.04	0.62 ± 0.03**	0.70 ± 0.04**	0.72 ± 0.11**		
Heinz bodies (%)						
Day 4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	7.2 ± 2.1**	77.6 ± 11.9**	95.6 ± 0.5**
Day 30	0.0 ± 0.0	0.1 ± 0.1	10.8 ± 2.4**	20.1 ± 1.4**		

Female (continued)

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 1-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg	1,000 mg/kg	2,000 mg/kg
Clinical Chemistry						
Day 4	10	10	10	10	10	5
Day 30	10	10	10	6	0	0
Urea nitrogen (mg/dL)						
Day 4	20.5 ± 0.5	19.9 ± 0.7	20.7 ± 0.5	19.1 ± 0.6	19.4 ± 0.8	53.2 ± 11.3*
Day 30	23.4 ± 0.6	21.8 ± 0.7	21.2 ± 0.7	21.7 ± 0.8		
Creatinine (mg/dL)						
Day 4	0.63 ± 0.02	0.63 ± 0.02	0.69 ± 0.01	0.69 ± 0.01	0.65 ± 0.02	0.72 ± 0.08
Day 30	0.62 ± 0.01	0.60 ± 0.02	0.60 ± 0.00	0.62 ± 0.02		
Total protein (g/dL)						
Day 4	5.8 ± 0.1	5.9 ± 0.1	5.9 ± 0.1	5.9 ± 0.1	5.8 ± 0.2	4.4 ± 0.2*
Day 30	6.1 ± 0.1	6.2 ± 0.1	6.3 ± 0.1*	6.4 ± 0.1*		
Albumin (g/dL)						
Day 4	4.2 ± 0.0	4.2 ± 0.1	4.2 ± 0.0	4.1 ± 0.0	4.0 ± 0.1*	2.9 ± 0.1**
Day 30	4.4 ± 0.0	4.4 ± 0.1	4.5 ± 0.0**	4.6 ± 0.1**		
Alanine aminotransferase (IU/L)						
Day 4	32 ± 1	33 ± 1	35 ± 1*	44 ± 2**	56 ± 5**	1,204 ± 525**
Day 30	37 ± 1	44 ± 2*	47 ± 2**	58 ± 3**		
Alkaline phosphatase (IU/L)						
Day 4	810 ± 21	802 ± 17	859 ± 12	714 ± 15**	546 ± 46**	296 ± 14**
Day 30	516 ± 9	516 ± 8	498 ± 13	631 ± 33*		
Creatine kinase (IU/L)						
Day 4	530 ± 59	546 ± 31	502 ± 51	408 ± 34	576 ± 132	1,062 ± 287
Day 30	469 ± 141	293 ± 33	382 ± 67	384 ± 59		
Sorbitol dehydrogenase (IU/L)						
Day 4	21 ± 1	25 ± 1**	27 ± 1**	28 ± 1**	32 ± 7**	867 ± 301**
Day 30	17 ± 1	19 ± 2	20 ± 1*	31 ± 4**		
Bile salts (µmol/L)						
Day 4	35.4 ± 6.2	37.2 ± 2.8	39.1 ± 4.6	36.1 ± 3.7	21.5 ± 1.3	131.2 ± 52.7
Day 30	22.5 ± 3.6	38.9 ± 6.2*	65.7 ± 8.7**	76.4 ± 12.7**		

* 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. No data presented for the 1,000 and 2,000 mg/kg groups on day 30 due to 100% mortality.
[b] n=10
[c] n=7
[d] n=3
[e] n=9

TABLE F2
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Hematology					
Week 1	9	10	10	9	9
Week 6	8	8	10	10	9
Month 3	10	9	10	10	10
Hematocrit (%)					
Week 1	41.9 ± 0.5	43.0 ± 0.6	42.3 ± 0.6	42.6 ± 0.5	42.2 ± 1.0
Week 6	47.0 ± 0.4	43.5 ± 0.4**	44.1 ± 0.4**	44.4 ± 0.5**	44.5 ± 0.7*
Month 3	47.0 ± 0.5	45.8 ± 0.6	46.5 ± 0.5	46.9 ± 0.4	45.1 ± 0.4*
Hemoglobin (g/dL)					
Week 1	14.0 ± 0.1	14.3 ± 0.2	14.0 ± 0.1	14.2 ± 0.2	13.6 ± 0.3
Week 6	16.5 ± 0.1	15.1 ± 0.1**	15.2 ± 0.2**	15.3 ± 0.2**	15.1 ± 0.1**
Month 3	16.1 ± 0.2	15.8 ± 0.2	15.8 ± 0.2	15.9 ± 0.1	15.3 ± 0.1**
Erythrocytes (10^6/μL)					
Week 1	6.86 ± 0.09	7.04 ± 0.10	6.98 ± 0.07	7.05 ± 0.08	6.76 ± 0.15
Week 6	8.66 ± 0.05	8.02 ± 0.09**	7.94 ± 0.08**	7.72 ± 0.11**	6.80 ± 0.17**
Month 3	8.91 ± 0.08	8.48 ± 0.13**	8.34 ± 0.10**	8.16 ± 0.09**	7.52 ± 0.10**
Reticulocytes (10^5/μL)					
Week 1	3.36 ± 0.18	3.48 ± 0.24	3.86 ± 0.18	3.60 ± 0.30	4.86 ± 0.43**
Week 6	1.64 ± 0.12	2.86 ± 0.22**	3.52 ± 0.14**	4.56 ± 0.29**	8.83 ± 0.86**
Month 3	1.94 ± 0.15	2.92 ± 0.20**	3.38 ± 0.26**	4.22 ± 0.23**	4.93 ± 0.28**
Nucleated erythrocytes/100 leukocytes					
Week 1	0.44 ± 0.18	0.00 ± 0.00	0.60 ± 0.27	0.33 ± 0.17	2.22 ± 0.55*
Week 6	0.00 ± 0.00	0.50 ± 0.27	0.60 ± 0.22*	2.00 ± 0.56**	4.67 ± 1.01**
Month 3	0.20 ± 0.13	0.78 ± 0.32	1.20 ± 0.49	3.00 ± 0.88**	5.30 ± 1.63**
Mean cell volume (fL)					
Week 1	61.1 ± 0.4	61.0 ± 0.3	60.8 ± 0.4	60.6 ± 0.4	62.6 ± 0.3
Week 6	54.3 ± 0.2	54.1 ± 0.4	55.6 ± 0.3**	57.5 ± 0.4**	65.4 ± 1.1**
Month 3	52.9 ± 0.2	54.0 ± 0.2**	55.9 ± 0.3**	57.6 ± 0.2**	59.9 ± 0.5**
Mean cell hemoglobin (pg)					
Week 1	20.5 ± 0.2	20.3 ± 0.2	20.1 ± 0.1	20.2 ± 0.2	20.2 ± 0.2
Week 6	19.1 ± 0.1	18.9 ± 0.2	19.2 ± 0.2	19.8 ± 0.2**	22.4 ± 0.6**
Month 3	18.1 ± 0.1	18.6 ± 0.1**	19.0 ± 0.1**	19.5 ± 0.1**	20.3 ± 0.2**
Mean cell hemoglobin concentration (g/dL)					
Week 1	33.5 ± 0.2	33.3 ± 0.2	33.2 ± 0.3	33.4 ± 0.2	32.3 ± 0.2**
Week 6	35.2 ± 0.1	34.8 ± 0.2*	34.5 ± 0.2**	34.6 ± 0.2**	34.1 ± 0.4**
Month 3	34.3 ± 0.2	34.4 ± 0.2	34.1 ± 0.1	33.9 ± 0.2	33.9 ± 0.1
Platelets (10^3/μL)					
Week 1	949.2 ± 26.1	882.0 ± 82.5	985.1 ± 36.9	991.0 ± 22.8	1,046.0 ± 24.0
Week 6	728.9 ± 13.8	780.4 ± 12.9*	813.4 ± 9.8**	802.0 ± 48.7**	815.9 ± 17.1**
Month 3	671.6 ± 19.1	753.6 ± 12.1	787.5 ± 17.5**	785.1 ± 11.3**	707.3 ± 13.8
Leukocytes (10^3/μL)					
Week 1	8.12 ± 0.45	8.38 ± 0.41	8.21 ± 0.50	8.41 ± 0.61	13.22 ± 0.81**
Week 6	10.45 ± 0.69	10.58 ± 0.83	10.34 ± 0.52	9.87 ± 0.48	11.46 ± 0.96
Month 3	12.69 ± 0.52	12.10 ± 0.38	12.11 ± 0.75	12.20 ± 0.53	12.35 ± 0.48
Segmented neutrophils (10^3/μL)					
Week 1	1.01 ± 0.09	1.00 ± 0.09	1.15 ± 0.16	1.29 ± 0.18	1.92 ± 0.24**
Week 6	1.50 ± 0.11	1.74 ± 0.21	1.56 ± 0.13	1.48 ± 0.10	1.64 ± 0.18
Month 3	2.45 ± 0.17	2.29 ± 0.14	2.31 ± 0.17	2.27 ± 0.20	2.16 ± 0.16
Lymphocytes (10^3/μL)					
Week 1	7.00 ± 0.38	7.29 ± 0.34	6.94 ± 0.42	7.03 ± 0.48	11.15 ± 0.67**
Week 6	8.85 ± 0.78	8.72 ± 0.64	8.69 ± 0.55	8.29 ± 0.47	9.71 ± 0.81
Month 3	10.06 ± 0.38	9.59 ± 0.33	9.62 ± 0.80	9.68 ± 0.58	10.05 ± 0.47

TABLE F2
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male (continued)					
Hematology (continued)					
Week 1	9	10	10	9	9
Week 6	8	8	10	10	9
Month 3	10	9	10	10	10
Monocytes (10^3/μL)					
Week 1	0.09 ± 0.02	0.07 ± 0.01	0.07 ± 0.02	0.07 ± 0.01	0.09 ± 0.04
Week 6	0.04 ± 0.02	0.07 ± 0.02	0.02 ± 0.02	0.04 ± 0.02	0.05 ± 0.04
Month 3	0.09 ± 0.04	0.17 ± 0.05	0.07 ± 0.02	0.12 ± 0.05	0.08 ± 0.03
Eosinophils (10^3/μL)					
Week 1	0.03 ± 0.01	0.02 ± 0.01	0.06 ± 0.02	0.01 ± 0.01	0.07 ± 0.03
Week 6	0.07 ± 0.02	0.06 ± 0.02	0.07 ± 0.02	0.07 ± 0.03	0.05 ± 0.04
Month 3	0.09 ± 0.03	0.05 ± 0.03	0.12 ± 0.04	0.10 ± 0.04	0.06 ± 0.03
Methemoglobin (g/dL)					
Week 1	0.40 ± 0.03[b]	0.46 ± 0.02	0.64 ± 0.03**	0.71 ± 0.05**[b]	0.88 ± 0.04**[b]
Week 6	0.45 ± 0.03	0.51 ± 0.03	0.63 ± 0.04**	0.74 ± 0.04**	0.80 ± 0.04**
Month 3	0.45 ± 0.02	0.57 ± 0.02**	0.66 ± 0.03**	0.80 ± 0.02**	0.84 ± 0.05**
Heinz bodies (%)					
Week 1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 1.4
Week 6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2**	15.2 ± 2.3**
Month 3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.2**	12.7 ± 1.3**
Clinical Chemistry					
Week 1	10	10	10	10	10
Week 6	9	8	10	10	10
Month 3	10	9	10	10	10
Urea nitrogen (mg/dL)					
Week 1	19.5 ± 0.5	19.6 ± 0.5	20.1 ± 0.4	19.5 ± 0.5	19.2 ± 0.6
Week 6	21.4 ± 0.3	21.0 ± 0.4	22.1 ± 0.3	21.3 ± 0.4	21.5 ± 0.4
Month 3	21.5 ± 0.5	20.8 ± 0.5	20.9 ± 0.4	21.1 ± 0.5	20.1 ± 0.4
Creatinine (mg/dL)					
Week 1	0.59 ± 0.01	0.58 ± 0.01	0.61 ± 0.01	0.63 ± 0.02	0.60 ± 0.00
Week 6	0.71 ± 0.02	0.69 ± 0.01	0.68 ± 0.01	0.68 ± 0.01	0.70 ± 0.02
Month 3	0.71 ± 0.02	0.71 ± 0.02	0.68 ± 0.01	0.67 ± 0.02	0.69 ± 0.01
Total protein (g/dL)					
Week 1	6.0 ± 0.1	6.1 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.4 ± 0.1**
Week 6	7.0 ± 0.1	6.7 ± 0.1	6.7 ± 0.0*	7.0 ± 0.1	7.1 ± 0.1
Month 3	7.3 ± 0.1	7.1 ± 0.1	7.3 ± 0.1	7.3 ± 0.0	7.1 ± 0.1
Albumin (g/dL)					
Week 1	4.4 ± 0.0	4.5 ± 0.1	4.6 ± 0.1	4.6 ± 0.1*	4.7 ± 0.0**
Week 6	4.9 ± 0.1	4.8 ± 0.0	4.8 ± 0.0	5.0 ± 0.1	5.1 ± 0.1
Month 3	4.8 ± 0.1	4.9 ± 0.1	5.0 ± 0.0	5.0 ± 0.0**	5.0 ± 0.0**
Alanine aminotransferase (IU/L)					
Week 1	40 ± 1	43 ± 1	40 ± 1	44 ± 1	44 ± 2
Week 6	60 ± 4	53 ± 3	60 ± 4	43 ± 1**[c]	49 ± 4*
Month 3	87 ± 10	98 ± 13	72 ± 7	59 ± 4**	46 ± 2**

TABLE F2
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male (continued)					
Clinical Chemistry (continued)					
Week 1	10	10	10	10	10
Week 6	9	8	10	10	10
Month 3	10	9	10	10	10
Alkaline phosphatase (IU/L)					
Week 1	1,469 ± 12	1,487 ± 22	1,438 ± 26	1,457 ± 20	1,389 ± 36
Week 6	842 ± 27	793 ± 22	834 ± 27	776 ± 20	733 ± 16**
Month 3	630 ± 9	563 ± 19**	550 ± 16**	534 ± 25**	523 ± 15**
Creatine kinase (IU/L)					
Week 1	527 ± 61	562 ± 109	588 ± 138	549 ± 70	505 ± 114
Week 6	380 ± 17[d]	527 ± 109	402 ± 59	459 ± 23	538 ± 73
Month 3	268 ± 49	301 ± 42	284 ± 33	265 ± 43	307 ± 46
Sorbitol dehydrogenase (IU/L)					
Week 1	23 ± 1	22 ± 1	24 ± 1	28 ± 2*	25 ± 1*
Week 6	25 ± 1	26 ± 1	29 ± 3	24 ± 1[c]	30 ± 2
Month 3	28 ± 4	40 ± 5	27 ± 2	21 ± 2	20 ± 1
Bile acids (μmol/L)					
Week 1	25.4 ± 3.7[c]	34.0 ± 4.5	30.3 ± 5.5	37.7 ± 7.3	43.6 ± 5.3
Week 6	14.1 ± 1.5	17.6 ± 1.9	26.6 ± 5.0*	32.6 ± 3.5**	46.7 ± 2.7**
Month 3	16.9 ± 1.2[c]	21.1 ± 1.3	18.2 ± 0.8	21.2 ± 1.5	27.1 ± 4.2*
Female					
Week 1	10	10	10	10	10
Week 6	9	10	10	10	9
Month 3	10	10	10	9	10
Hematocrit (%)					
Week 1	43.4 ± 0.5	43.6 ± 0.6	43.9 ± 0.3	43.9 ± 0.7	42.2 ± 0.7
Week 6	44.8 ± 0.6	42.8 ± 0.4	43.3 ± 0.4	42.1 ± 0.6*	43.8 ± 0.6
Month 3	46.4 ± 0.6	45.9 ± 0.5	44.6 ± 0.2**	44.2 ± 0.4**	44.4 ± 0.4**
Hemoglobin (g/dL)					
Week 1	14.7 ± 0.1	14.6 ± 0.2	14.7 ± 0.1	14.5 ± 0.2	13.8 ± 0.2
Week 6	15.6 ± 0.2	14.9 ± 0.2*	14.8 ± 0.2**	14.4 ± 0.2**	14.9 ± 0.1**
Month 3	15.7 ± 0.1	15.3 ± 0.2*	15.0 ± 0.1**	14.9 ± 0.1**	14.9 ± 0.2**
Erythrocytes (10^6/μL)					
Week 1	7.06 ± 0.09	7.13 ± 0.11	7.14 ± 0.04	7.22 ± 0.11	6.81 ± 0.14
Week 6	7.69 ± 0.13	7.15 ± 0.07**	7.04 ± 0.07**	6.71 ± 0.10**	6.61 ± 0.09**
Month 3	7.88 ± 0.10	7.51 ± 0.08*	7.26 ± 0.06**	7.07 ± 0.06**	6.93 ± 0.10**
Reticulocytes (10^5/μL)					
Week 1	2.23 ± 0.14	1.97 ± 0.14	2.39 ± 0.09	2.50 ± 0.20	3.81 ± 0.48**
Week 6	1.34 ± 0.10	2.48 ± 0.23**	2.40 ± 0.12**	3.86 ± 0.28**	4.48 ± 0.45**
Month 3	1.42 ± 0.09	2.26 ± 0.19**	2.65 ± 0.25**	3.30 ± 0.14**	4.21 ± 0.26**
Nucleated erythrocytes/100 leukocytes					
Week 1	0.80 ± 0.33	0.20 ± 0.20	0.40 ± 0.22	1.60 ± 0.52	2.90 ± 1.14
Week 6	0.22 ± 0.15	1.00 ± 0.39	1.80 ± 0.55**	3.80 ± 1.27**	2.44 ± 0.97**
Month 3	0.30 ± 0.21	1.30 ± 0.50	1.00 ± 0.33	3.44 ± 1.23**	7.40 ± 2.27**

TABLE F2
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female (continued)					
Hematology (continued)					
Week 1	10	10	10	10	10
Week 6	9	10	10	10	9
Month 3	10	10	10	9	10
Mean cell volume (fL)					
Week 1	61.6 ± 0.5	61.3 ± 0.5	61.4 ± 0.3	60.8 ± 0.3	62.2 ± 0.4
Week 6	58.7 ± 0.4	59.9 ± 0.2*	61.7 ± 0.3**	62.9 ± 0.3**	66.3 ± 0.5**
Month 3	59.0 ± 0.2	61.2 ± 0.7**	61.5 ± 0.3**	62.7 ± 0.2**	64.3 ± 0.6**
Mean cell hemoglobin (pg)					
Week 1	20.8 ± 0.2	20.4 ± 0.1	20.6 ± 0.2	20.0 ± 0.2**	20.3 ± 0.2**
Week 6	20.3 ± 0.1	20.8 ± 0.1*	21.1 ± 0.1**	21.5 ± 0.1**	22.5 ± 0.1**
Month 3	19.9 ± 0.2	20.4 ± 0.1*	20.7 ± 0.1**	21.1 ± 0.1**	21.6 ± 0.3**
Mean cell hemoglobin concentration (g/dL)					
Week 1	33.8 ± 0.2	33.4 ± 0.2	33.5 ± 0.3	32.9 ± 0.3*	32.6 ± 0.2**
Week 6	34.7 ± 0.2	34.7 ± 0.1	34.2 ± 0.2*	34.2 ± 0.2*	33.9 ± 0.3*
Month 3	33.8 ± 0.3	33.4 ± 0.4	33.6 ± 0.2	33.7 ± 0.2	33.6 ± 0.3
Platelets (10^3/μL)					
Week 1	884.7 ± 11.6	853.6 ± 38.6	892.5 ± 40.1	908.1 ± 31.9	1,006.7 ± 33.9**
Week 6	709.3 ± 21.1	757.6 ± 14.9	783.0 ± 24.5*	815.8 ± 26.7**	825.6 ± 13.3**
Month 3	664.7 ± 14.3	723.3 ± 10.2**	746.8 ± 11.3**	769.2 ± 15.9**	785.9 ± 15.3**
Leukocytes (10^3/μL)					
Week 1	7.97 ± 0.54	6.73 ± 0.33	8.57 ± 0.55	8.10 ± 0.54	11.86 ± 0.91**
Week 6	8.17 ± 0.39	8.52 ± 0.31	8.31 ± 0.40[c]	8.55 ± 0.34	9.18 ± 0.35
Month 3	8.00 ± 0.39	7.99 ± 0.41	10.08 ± 0.62	8.10 ± 0.51	8.20 ± 0.51
Segmented neutrophils (10^3/μL)					
Week 1	0.92 ± 0.14	0.81 ± 0.08	1.00 ± 0.12	1.06 ± 0.14	1.61 ± 0.16**
Week 6	0.91 ± 0.08	0.99 ± 0.12	1.10 ± 0.19[c]	1.11 ± 0.14	1.14 ± 0.12
Month 3	1.51 ± 0.16	1.43 ± 0.20	1.88 ± 0.19	1.41 ± 0.23	1.38 ± 0.17
Lymphocytes (10^3/μL)					
Week 1	6.92 ± 0.52	5.87 ± 0.31	7.49 ± 0.46	6.94 ± 0.49	10.13 ± 0.92**
Week 6	7.25 ± 0.35	7.45 ± 0.29	7.11 ± 0.34[c]	7.38 ± 0.28	8.00 ± 0.37
Month 3	6.38 ± 0.40	6.49 ± 0.33	8.06 ± 0.50	6.66 ± 0.53	6.80 ± 0.45
Monocytes (10^3/μL)					
Week 1	0.08 ± 0.03	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.07 ± 0.02
Week 6	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01[c]	0.01 ± 0.01	0.03 ± 0.02
Month 3	0.03 ± 0.01	0.01 ± 0.01	0.05 ± 0.03	0.01 ± 0.01	0.01 ± 0.01
Eosinophils (10^3/μL)					
Week 1	0.06 ± 0.03	0.03 ± 0.02	0.04 ± 0.01	0.07 ± 0.02	0.05 ± 0.02
Week 6	0.01 ± 0.01	0.09 ± 0.03	0.10 ± 0.03*[c]	0.05 ± 0.01	0.01 ± 0.01
Month 3	0.08 ± 0.02	0.06 ± 0.03	0.09 ± 0.03	0.03 ± 0.01	0.02 ± 0.01
Methemoglobin (g/dL)					
Week 1	0.36 ± 0.03	0.45 ± 0.03*	0.51 ± 0.02**	0.68 ± 0.04**	0.78 ± 0.03**
Week 6	0.33 ± 0.02	0.46 ± 0.02**	0.57 ± 0.03**	0.63 ± 0.03**	0.83 ± 0.07**
Month 3	0.36 ± 0.02	0.64 ± 0.03**	0.73 ± 0.05**	0.86 ± 0.04**	1.10 ± 0.07**
Heinz bodies (%)					
Week 1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
Week 6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.6 ± 0.5**	5.0 ± 1.1**
Month 3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.2**	14.0 ± 2.3**

TABLE F2
Hematology and Clinical Chemistry Data for Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female (continued)					
Clinical Chemistry					
Week 1	10	10	10	10	10
Week 6	9	10	10	10	9
Month 3	10	10	10	9	10
Urea nitrogen (mg/dL)					
Week 1	21.9 ± 0.8	22.3 ± 0.7	22.0 ± 0.6	20.0 ± 0.4	19.8 ± 0.4*
Week 6	23.9 ± 0.7	24.8 ± 0.4	23.4 ± 0.5	23.7 ± 0.8	24.1 ± 0.7
Month 3	19.4 ± 0.8	18.8 ± 0.7	19.4 ± 0.5	20.2 ± 0.6	21.0 ± 0.9
Creatinine (mg/dL)					
Week 1	0.64 ± 0.02	0.65 ± 0.02	0.66 ± 0.02	0.62 ± 0.01	0.61 ± 0.01
Week 6	0.72 ± 0.02	0.71 ± 0.01	0.70 ± 0.02	0.70 ± 0.00	0.69 ± 0.02
Month 3	0.70 ± 0.00	0.70 ± 0.02	0.69 ± 0.01	0.66 ± 0.02*	0.69 ± 0.01
Total protein (g/dL)					
Week 1	6.3 ± 0.1	6.4 ± 0.1	6.4 ± 0.1	6.5 ± 0.1	6.5 ± 0.1
Week 6	6.6 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.8 ± 0.1
Month 3	7.1 ± 0.1	7.1 ± 0.1	6.8 ± 0.1	6.9 ± 0.1	6.9 ± 0.1
Albumin (g/dL)					
Week 1	4.7 ± 0.0	4.9 ± 0.1	4.8 ± 0.1	4.9 ± 0.1	5.0 ± 0.0**
Week 6	4.9 ± 0.1	4.9 ± 0.0	4.9 ± 0.1	4.9 ± 0.0	5.1 ± 0.0
Month 3	5.1 ± 0.1	5.1 ± 0.1	4.9 ± 0.1	5.0 ± 0.0	5.0 ± 0.0
Alanine aminotransferase (IU/L)					
Week 1	38 ± 1	38 ± 1	43 ± 1	41 ± 2	44 ± 1*
Week 6	41 ± 2	40 ± 2	40 ± 2	40 ± 1	39 ± 1
Month 3	58 ± 4	52 ± 2	52 ± 2	55 ± 3	50 ± 2
Alkaline phosphatase (IU/L)					
Week 1	1,123 ± 20	1,199 ± 32	1,123 ± 39	1,079 ± 36	1,080 ± 37
Week 6	702 ± 22	626 ± 13	623 ± 21	582 ± 16**	646 ± 15
Month 3	498 ± 15	475 ± 19	447 ± 14	497 ± 23	508 ± 17
Creatine kinase (IU/L)					
Week 1	680 ± 134	607 ± 66	1,299 ± 283	1,074 ± 304	2,013 ± 567*
Week 6	473 ± 35	589 ± 86	409 ± 50[c]	482 ± 56	508 ± 74
Month 3	315 ± 29	282 ± 34	339 ± 25	348 ± 32	278 ± 26
Sorbitol dehydrogenase (IU/L)					
Week 1	17 ± 1	19 ± 1	21 ± 2	20 ± 1	21 ± 1**
Week 6	26 ± 1	23 ± 1	28 ± 2	26 ± 2	28 ± 2
Month 3	25 ± 2	21 ± 1	22 ± 1	25 ± 1	22 ± 1
Bile acids (µmol/L)					
Week 1	26.0 ± 4.6	26.8 ± 3.0	26.8 ± 4.3	29.8 ± 3.2	35.5 ± 2.2
Week 6	25.0 ± 3.8	16.2 ± 1.1[c]	23.0 ± 2.8	31.1 ± 3.3	34.1 ± 5.5
Month 3	20.9 ± 2.4	30.2 ± 2.4*	41.7 ± 4.7**	50.9 ± 5.3**	60.2 ± 4.2**

* 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=10
[c] n=9
[d] n=7

TABLE F3
Hematology and Urinalysis Data for Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Male				
Hematology				
Week 2	10	10	10	10
Month 3	10	10	10	10
Month 12	10	10	8	10
Month 18	9	10	8	9
Hematocrit (spun) (%)				
Week 2	42.9 ± 0.6	43.9 ± 0.4	42.9 ± 0.6	42.2 ± 0.5
Month 3	45.6 ± 0.4	46.2 ± 0.5	44.9 ± 0.4	43.8 ± 0.6*
Month 12	45.4 ± 0.3	45.4 ± 0.4	43.5 ± 0.4**	43.5 ± 0.4**
Month 18	45.6 ± 0.6	47.5 ± 1.0	46.3 ± 0.8	44.6 ± 0.3
Hematocrit (auto) (%)				
Week 2	43.1 ± 0.6	44.1 ± 0.5	42.8 ± 0.5	43.0 ± 0.5
Month 3	46.0 ± 0.3	46.3 ± 0.5	45.2 ± 0.4	43.9 ± 0.5**
Month 12	44.9 ± 0.3	45.0 ± 0.4	42.8 ± 0.4**	42.6 ± 0.3**
Month 18	46.0 ± 0.6	48.3 ± 1.1	47.2 ± 0.9	45.1 ± 0.5
Hemoglobin (g/dL)				
Week 2	14.5 ± 0.2	14.7 ± 0.1	14.2 ± 0.2	14.3 ± 0.2
Month 3	15.5 ± 0.1	15.5 ± 0.1	15.0 ± 0.1*	14.4 ± 0.2**
Month 12	15.1 ± 0.1	15.2 ± 0.1	14.4 ± 0.1**	14.2 ± 0.1**
Month 18	15.5 ± 0.2	16.2 ± 0.4	15.5 ± 0.3	14.7 ± 0.2*
Erythrocytes (10^6/µL)				
Week 2	7.49 ± 0.12	7.63 ± 0.08	7.32 ± 0.10	7.40 ± 0.10
Month 3	8.79 ± 0.06	8.82 ± 0.10	8.50 ± 0.08*	7.87 ± 0.07**
Month 12	8.82 ± 0.05	8.87 ± 0.09	8.31 ± 0.08**	8.02 ± 0.03**
Month 18	8.22 ± 0.11	8.66 ± 0.19	8.30 ± 0.13	7.68 ± 0.11*
Reticulocytes (10^5/µL)				
Week 2	4.64 ± 0.13	4.94 ± 0.25	4.93 ± 0.27	4.77 ± 0.27
Month 3	3.44 ± 0.11	3.29 ± 0.13	3.76 ± 0.07	4.86 ± 0.14**
Month 12	2.44 ± 0.08	2.42 ± 0.06	3.05 ± 0.11**	3.95 ± 0.09**
Month 18	2.84 ± 0.15	2.87 ± 0.18	3.77 ± 0.13**	4.87 ± 0.08**
Nucleated erythrocytes (10^3/µL)				
Week 2	0.30 ± 0.15	0.20 ± 0.13	0.40 ± 0.22	0.30 ± 0.21
Month 3	0.40 ± 0.16	0.00 ± 0.00	0.40 ± 0.16	0.90 ± 0.28
Month 12	0.10 ± 0.10	0.30 ± 0.15	0.88 ± 0.30*	1.70 ± 0.42**
Month 18	0.00 ± 0.00	0.20 ± 0.13	1.00 ± 0.38*	0.56 ± 0.34
Mean cell volume (fL)				
Week 2	57.6 ± 0.3	57.8 ± 0.2	58.5 ± 0.3	58.1 ± 0.3
Month 3	52.4 ± 0.1	52.4 ± 0.3	53.2 ± 0.2**	55.8 ± 0.2**
Month 12	50.9 ± 0.1	50.7 ± 0.2	51.4 ± 0.2	53.2 ± 0.3**
Month 18	56.0 ± 0.5	55.7 ± 0.4	56.9 ± 0.3	58.7 ± 0.4**
Mean cell hemoglobin (pg)				
Week 2	19.3 ± 0.1	19.2 ± 0.1	19.4 ± 0.1	19.3 ± 0.1
Month 3	17.6 ± 0.1	17.6 ± 0.1	17.7 ± 0.1	18.4 ± 0.2**
Month 12	17.1 ± 0.1	17.2 ± 0.1	17.3 ± 0.1	17.7 ± 0.1**
Month 18	18.9 ± 0.2	18.6 ± 0.2	18.7 ± 0.1	19.2 ± 0.2
Mean cell hemoglobin concentration (g/dL)				
Week 2	33.5 ± 0.1	33.2 ± 0.2	33.1 ± 0.1	33.2 ± 0.2
Month 3	33.6 ± 0.1	33.6 ± 0.1	33.2 ± 0.1*	32.9 ± 0.2**
Month 12	33.7 ± 0.1	33.8 ± 0.2	33.6 ± 0.1	33.3 ± 0.1
Month 18	33.7 ± 0.1	33.4 ± 0.2	32.9 ± 0.2**	32.7 ± 0.1**

TABLE F3
Hematology and Urinalysis Data for Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Male (continued)				
Hematology (continued)				
Week 2	10	10	10	10
Month 3	10	10	10	10
Month 12	10	10	8	10
Month 18	9	10	8	9
Platelets (10^3/μL)				
Week 2	837.2 ± 14.1	800.9 ± 17.5	874.2 ± 25.2	829.1 ± 21.6
Month 3	579.4 ± 20.1	565.3 ± 13.9	590.8 ± 31.5	627.1 ± 22.6
Month 12	635.2 ± 13.0	627.4 ± 15.0	665.3 ± 9.6	700.6 ± 18.1*
Month 18	547.2 ± 24.7	565.4 ± 18.2	591.1 ± 29.0	645.0 ± 38.4
Leukocytes (10^3/μL)				
Week 2	9.64 ± 0.35	9.26 ± 0.32	8.99 ± 0.45	9.57 ± 0.38
Month 3	8.15 ± 0.44	8.26 ± 0.27	8.35 ± 0.59	8.78 ± 0.49
Month 12	8.33 ± 0.19	8.59 ± 0.44	8.90 ± 0.58	8.24 ± 0.35
Month 18	8.47 ± 0.20	7.65 ± 0.58[b]	7.78 ± 0.53	7.70 ± 0.69
Segmented neutrophils (10^3/μL)				
Week 2	0.77 ± 0.04	0.76 ± 0.04	0.80 ± 0.05	0.80 ± 0.05
Month 3	1.14 ± 0.05	1.24 ± 0.04	1.20 ± 0.08	1.27 ± 0.08
Month 12	1.59 ± 0.06	1.79 ± 0.13	1.94 ± 0.22	1.43 ± 0.06
Month 18	1.90 ± 0.08	2.17 ± 0.28[b]	1.99 ± 0.12	1.82 ± 0.17
Lymphocytes (10^3/μL)				
Week 2	8.29 ± 0.29	7.96 ± 0.29	7.63 ± 0.41	8.25 ± 0.33
Month 3	6.39 ± 0.36	6.45 ± 0.26	6.55 ± 0.50	6.96 ± 0.42
Month 12	6.13 ± 0.14	6.16 ± 0.30	6.34 ± 0.40	6.26 ± 0.28
Month 18	5.94 ± 0.17	4.98 ± 0.34[b]	5.29 ± 0.43	5.46 ± 0.51
Activated lymphocytes (10^3/μL)				
Week 2	0.30 ± 0.02	0.26 ± 0.02	0.30 ± 0.03	0.27 ± 0.02
Month 3	0.31 ± 0.03	0.27 ± 0.02	0.27 ± 0.03	0.30 ± 0.04
Month 12	0.13 ± 0.01	0.16 ± 0.02	0.16 ± 0.02	0.16 ± 0.02
Month 18	0.14 ± 0.01	0.12 ± 0.03	0.11 ± 0.02	0.09 ± 0.01*
Monocytes (10^3/μL)				
Week 2	0.16 ± 0.01	0.17 ± 0.02	0.16 ± 0.01	0.15 ± 0.01
Month 3	0.17 ± 0.02	0.17 ± 0.01	0.15 ± 0.02	0.14 ± 0.02
Month 12	0.27 ± 0.01	0.27 ± 0.02	0.25 ± 0.02	0.19 ± 0.01**
Month 18	0.33 ± 0.01	0.27 ± 0.03[b]	0.27 ± 0.01*	0.21 ± 0.02**
Basophils (10^3/μL)				
Week 2	0.080 ± 0.011	0.072 ± 0.011	0.066 ± 0.007	0.060 ± 0.009
Month 3	0.062 ± 0.010	0.045 ± 0.004	0.055 ± 0.010	0.054 ± 0.009
Month 12	0.091 ± 0.008	0.103 ± 0.010	0.108 ± 0.010	0.116 ± 0.008
Month 18	0.078 ± 0.005	0.061 ± 0.009[b]	0.069 ± 0.015	0.066 ± 0.014
Eosinophils (10^3/μL)				
Week 2	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00
Month 3	0.09 ± 0.01	0.09 ± 0.01	0.13 ± 0.05	0.07 ± 0.01
Month 12	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.08 ± 0.01**
Month 18	0.08 ± 0.01	0.07 ± 0.01[b]	0.06 ± 0.01*	0.06 ± 0.01*
Methemoglobin (g/dL)				
Week 2	0.15 ± 0.02	0.15 ± 0.02	0.21 ± 0.05	0.19 ± 0.01
Month 3	0.21 ± 0.01	0.19 ± 0.01	0.23 ± 0.02	0.29 ± 0.02**
Month 12	0.16 ± 0.04	0.14 ± 0.02	0.25 ± 0.02**	0.32 ± 0.02**
Month 18	0.12 ± 0.02	0.16 ± 0.02	0.24 ± 0.03**	0.33 ± 0.02**

TABLE F3
Hematology and Urinalysis Data for Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Male (continued)				
Hematology (continued)				
Week 2	10	10	10	10
Month 3	10	10	10	10
Month 12	10	10	8	10
Month 18	9	10	8	9
Heinz bodies (%)				
Week 2	0.1 ± 0.0	0.1 ± 0.0[b]	0.1 ± 0.0[b]	0.2 ± 0.1
Month 3	0.4 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
Month 12	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1
Month 18	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
Urinalysis				
n	5	5	5	5
Creatinine (mg/dL)				
Month 3	161.54 ± 8.20	161.04 ± 14.30	177.68 ± 15.85	173.16 ± 28.13
Month 12	149.98 ± 15.59	170.00 ± 30.46	118.30 ± 16.34	106.96 ± 13.26
Month 18	132.44 ± 6.24	152.68 ± 16.56	167.50 ± 39.19	116.20 ± 11.12
Volume (mL)				
Month 3	4.1 ± 0.6	3.3 ± 0.3	3.2 ± 0.8	2.4 ± 0.2
Month 12	7.7 ± 0.7	4.6 ± 0.9	3.6 ± 0.7**	5.0 ± 0.4
Month 18	6.7 ± 0.7	6.0 ± 0.4	5.9 ± 1.8	6.7 ± 1.0
Female				
Hematology				
Week 2	10	10	10	10
Month 3	10	10	10	10
Month 12	10	10	9	9
Month 18	9	9	8	9
Hematocrit (spun) (%)				
Week 2	46.1 ± 0.6	45.2 ± 0.6	45.4 ± 0.4	45.3 ± 0.5
Month 3	44.8 ± 0.4	45.0 ± 0.4	44.5 ± 0.3	42.8 ± 0.4**
Month 12	44.9 ± 0.3	44.5 ± 0.4	43.4 ± 0.4*	41.4 ± 0.5**
Month 18	45.1 ± 0.3	44.9 ± 0.4	43.9 ± 0.4	43.1 ± 0.3*
Hematocrit (auto) (%)				
Week 2	46.9 ± 0.8	46.0 ± 0.6	46.0 ± 0.5	46.3 ± 0.6
Month 3	44.8 ± 0.4	45.0 ± 0.4	44.4 ± 0.4	43.1 ± 0.3*
Month 12	46.0 ± 0.3	45.6 ± 0.3	44.1 ± 0.2**	41.5 ± 0.4**
Month 18	43.7 ± 0.4	43.4 ± 0.5	42.3 ± 0.4*	41.2 ± 0.3**
Hemoglobin (g/dL)				
Week 2	14.8 ± 0.2	14.5 ± 0.2	14.6 ± 0.2	14.6 ± 0.2
Month 3	15.2 ± 0.1	15.3 ± 0.1	14.9 ± 0.1	14.2 ± 0.1**
Month 12	15.5 ± 0.1	15.4 ± 0.1	14.8 ± 0.1**	13.8 ± 0.1**
Month 18	14.9 ± 0.1	14.7 ± 0.2	14.2 ± 0.1**	13.6 ± 0.1**

Female (continued)

TABLE F3
Hematology and Urinalysis Data for Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Hematology (continued)				
Week 2	10	10	10	10
Month 3	10	10	10	10
Month 12	10	10	9	9
Month 18	9	9	8	9
Erythrocytes (10^6/μL)				
Week 2	8.22 ± 0.14	8.03 ± 0.10	8.11 ± 0.09	8.10 ± 0.10
Month 3	8.21 ± 0.06	8.27 ± 0.05	7.99 ± 0.06*	7.44 ± 0.06**
Month 12	8.27 ± 0.04	8.23 ± 0.06	7.67 ± 0.04**	7.04 ± 0.06**
Month 18	7.92 ± 0.07	7.91 ± 0.09	7.46 ± 0.04**	7.01 ± 0.06**
Reticulocytes (10^5/μL)				
Week 2	3.29 ± 0.24	3.33 ± 0.17	3.46 ± 0.15	3.63 ± 0.23
Month 3	3.10 ± 0.07	2.98 ± 0.12	3.48 ± 0.11*	4.55 ± 0.15**
Month 12	2.21 ± 0.09	2.21 ± 0.09	3.13 ± 0.15**	4.86 ± 0.12**
Month 18	2.64 ± 0.10	2.46 ± 0.06	3.52 ± 0.11**	4.87 ± 0.15**
Nucleated erythrocytes (10^3/μL)				
Week 2	0.10 ± 0.10	0.20 ± 0.13	0.20 ± 0.13	0.10 ± 0.10
Month 3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Month 12	1.10 ± 0.43	0.80 ± 0.25	3.22 ± 1.00	3.56 ± 0.88*
Month 18	1.33 ± 0.37	0.89 ± 0.39	1.25 ± 0.56	1.11 ± 0.26
Mean cell volume (fL)				
Week 2	57.1 ± 0.4	57.3 ± 0.3	56.7 ± 0.3	57.2 ± 0.3
Month 3	54.5 ± 0.1	54.5 ± 0.2	55.6 ± 0.1**	57.9 ± 0.2**
Month 12	55.6 ± 0.2	55.5 ± 0.1	57.6 ± 0.1**	58.9 ± 0.3**
Month 18	55.2 ± 0.2	55.0 ± 0.1	56.8 ± 0.2**	58.7 ± 0.2**
Mean cell hemoglobin (pg)				
Week 2	18.0 ± 0.1	18.1 ± 0.1	18.0 ± 0.1	18.0 ± 0.1
Month 3	18.5 ± 0.1	18.6 ± 0.1	18.6 ± 0.1	19.1 ± 0.1**
Month 12	18.8 ± 0.1	18.7 ± 0.1	19.3 ± 0.1**	19.6 ± 0.1**
Month 18	18.8 ± 0.1	18.6 ± 0.1	19.1 ± 0.1	19.5 ± 0.1**
Mean cell hemoglobin concentration (g/dL)				
Week 2	31.6 ± 0.3	31.6 ± 0.2	31.8 ± 0.1	31.5 ± 0.1
Month 3	34.0 ± 0.1	34.0 ± 0.1	33.5 ± 0.1*	33.1 ± 0.2**
Month 12	33.7 ± 0.2	33.7 ± 0.1	33.6 ± 0.2	33.3 ± 0.1
Month 18	34.1 ± 0.2	33.8 ± 0.1	33.6 ± 0.1*	33.1 ± 0.1**
Platelets (10^3/μL)				
Week 2	749.5 ± 33.8	789.3 ± 22.3	802.1 ± 26.7	775.9 ± 25.8
Month 3	623.1 ± 9.8	624.4 ± 12.3	621.8 ± 22.2	674.8 ± 22.6**
Month 12	602.2 ± 13.6	587.4 ± 15.6	692.0 ± 8.2**	760.1 ± 12.4**
Month 18	522.8 ± 14.6	481.8 ± 18.1	612.0 ± 17.8*	680.7 ± 22.6**
Leukocytes (10^3/μL)				
Week 2	10.64 ± 0.53	10.34 ± 0.49	10.40 ± 0.55	9.87 ± 0.53
Month 3	6.43 ± 0.37	7.86 ± 0.40*	8.17 ± 0.56*	7.37 ± 0.50
Month 12	5.09 ± 0.15	5.95 ± 0.24**	6.41 ± 0.36**	6.08 ± 0.33**
Month 18	4.74 ± 0.23	5.12 ± 0.35	6.39 ± 0.77**	6.49 ± 0.42**
Segmented neutrophils (10^3/μL)				
Week 2	0.83 ± 0.04	0.85 ± 0.06	0.95 ± 0.05	0.97 ± 0.12
Month 3	0.93 ± 0.07	1.51 ± 0.11**	1.43 ± 0.06**	1.10 ± 0.04
Month 12	1.06 ± 0.12	1.00 ± 0.05	1.09 ± 0.05	1.09 ± 0.08
Month 18	1.36 ± 0.13	1.35 ± 0.07	1.64 ± 0.11	1.58 ± 0.13

Female (continued)

TABLE F3
Hematology and Urinalysis Data for Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Hematology (continued)				
Week 2	10	10	10	10
Month 3	10	10	10	10
Month 12	10	10	9	9
Month 18	9	9	8	9
Lymphocytes ($10^3/\mu L$)				
Week 2	9.27 ± 0.45	8.99 ± 0.44	8.98 ± 0.47	8.42 ± 0.51
Month 3	4.93 ± 0.30	5.58 ± 0.28	6.10 ± 0.51	5.76 ± 0.46
Month 12	3.54 ± 0.10	4.36 ± 0.19**	4.77 ± 0.30**	4.49 ± 0.27**
Month 18	3.01 ± 0.09	3.32 ± 0.26	4.31 ± 0.67**	4.55 ± 0.28**
Activated lymphocytes ($10^3/\mu L$)				
Week 2	0.25 ± 0.03	0.23 ± 0.03	0.22 ± 0.04	0.21 ± 0.03
Month 3	0.30 ± 0.06	0.41 ± 0.05	0.33 ± 0.03	0.28 ± 0.03
Month 12	0.13 ± 0.02	0.19 ± 0.03	0.16 ± 0.03	0.24 ± 0.06
Month 18	0.07 ± 0.01	0.11 ± 0.03	0.11 ± 0.02	0.08 ± 0.01
Monocytes ($10^3/\mu L$)				
Week 2	0.18 ± 0.02	0.17 ± 0.01	0.16 ± 0.01	0.18 ± 0.02
Month 3	0.14 ± 0.02	0.22 ± 0.03*	0.19 ± 0.02	0.12 ± 0.01
Month 12	0.18 ± 0.01	0.21 ± 0.01	0.18 ± 0.02	0.14 ± 0.01
Month 18	0.20 ± 0.01	0.23 ± 0.03	0.20 ± 0.02	0.18 ± 0.02
Basophils ($10^3/\mu L$)				
Week 2	0.056 ± 0.010	0.052 ± 0.006	0.048 ± 0.009	0.041 ± 0.006
Month 3	0.066 ± 0.015	0.093 ± 0.014	0.052 ± 0.006	0.044 ± 0.007
Month 12	0.124 ± 0.013	0.132 ± 0.010	0.136 ± 0.012	0.096 ± 0.009
Month 18	0.050 ± 0.006	0.062 ± 0.011	0.085 ± 0.012	0.064 ± 0.010
Eosinophils ($10^3/\mu L$)				
Week 2	0.06 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.05 ± 0.01
Month 3	0.06 ± 0.01	0.06 ± 0.00	0.08 ± 0.01	0.06 ± 0.01
Month 12	0.06 ± 0.00	0.07 ± 0.00	0.08 ± 0.01	0.04 ± 0.00*
Month 18	0.06 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.01
Methemoglobin (g/dL)				
Week 2	0.19 ± 0.02	0.20 ± 0.02	0.23 ± 0.02	0.43 ± 0.22
Month 3	0.16 ± 0.02	0.16 ± 0.02	0.20 ± 0.03	0.27 ± 0.02**
Month 12	0.19 ± 0.01	0.19 ± 0.01	0.28 ± 0.02**	0.32 ± 0.02**
Month 18	0.14 ± 0.02	0.18 ± 0.02	0.29 ± 0.01**	0.32 ± 0.02**
Heinz bodies (%)				
Week 2	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.7 ± 0.1
Month 3	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.2
Month 12	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
Month 18	0.1 ± 0.0	0.0 ± 0.0	0.4 ± 0.1**	7.1 ± 1.7**

TABLE F3
Hematology and Urinalysis Data for Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
Female (continued)				
Urinalysis				
n	5	5	5	5
Creatinine (mg/dL)				
Month 3	129.92 ± 24.13	95.76 ± 17.17	127.04 ± 16.88	123.90 ± 18.32
Month 12	84.98 ± 10.38	101.74 ± 18.51	84.12 ± 11.13	112.54 ± 22.20
Month 18	145.58 ± 20.07	130.46 ± 38.34	97.70 ± 13.09	87.36 ± 9.45
Volume (mL)				
Month 3	0.9 ± 0.3	1.8 ± 0.5	1.8 ± 0.7	2.0 ± 0.7
Month 12	4.0 ± 0.7	4.6 ± 0.8	5.7 ± 1.1	4.2 ± 0.8
Month 18	3.5 ± 0.4	3.7 ± 1.5	5.3 ± 0.9	5.4 ± 1.0

* 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 F4
Hematology and Urinalysis Data for Rats in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	5 mg/kg	25 mg/kg	50 mg/kg
n	10		10	8
Male				
Hematocrit (%)	50.4 ± 0.7		35.1 ± 1.3**	24.5 ± 2.3**
Hemoglobin (g/dL)	17.0 ± 0.2		14.4 ± 0.3**	10.2 ± 1.2**
Erythrocytes (10^6/µL)	9.91 ± 0.13		6.00 ± 0.34**	3.62 ± 0.52**
Reticulocytes (10^6/µL)	0.20 ± 0.02		0.53 ± 0.05**	0.59 ± 0.12**
Nucleated erythrocytes (10^3/µL)	0.00 ± 0.00		0.00 ± 0.00	0.00 ± 0.00[b]
Mean cell volume (fL)	50.9 ± 0.1		59.1 ± 1.4**	72.0 ± 4.5**
Mean cell hemoglobin (pg)	17.1 ± 0.1		24.5 ± 1.1**	29.5 ± 1.7**
Mean cell hemoglobin concentration (g/dL)	33.7 ± 0.3		41.4 ± 1.2**	41.2 ± 1.4**
Platelets (10^3/µL)	1,053.3 ± 30.1		1,162.2 ± 18.1**	1,391.0 ± 45.3**
Leukocytes (10^3/µL)	4.05 ± 0.45		4.59 ± 0.50	9.46 ± 1.40**[b]
Segmented neutrophils (10^3/µL)	0.76 ± 0.11		0.81 ± 0.21	2.14 ± 0.77[b]
Lymphocytes (10^3/µL)	3.25 ± 0.39		3.73 ± 0.33	7.28 ± 0.94**[b]
Monocytes (10^3/µL)	0.01 ± 0.01		0.00 ± 0.00	0.01 ± 0.01[b]
Eosinophils (10^3/µL)	0.03 ± 0.01		0.05 ± 0.01	0.03 ± 0.01[b]
Methemoglobin (g/dL)	0.13 ± 0.04		0.34 ± 0.05**	0.31 ± 0.04**
Heinz bodies (%)	0.0 ± 0.0		32.4 ± 2.8**	25.9 ± 3.3**
Female				
Hematocrit (%)	47.7 ± 0.7		39.0 ± 0.8**	30.9 ± 1.6**
Hemoglobin (g/dL)	16.5 ± 0.2		15.5 ± 0.2**	12.3 ± 0.5**
Erythrocytes (10^6/µL)	9.36 ± 0.16		6.79 ± 0.18**	4.34 ± 0.30**
Reticulocytes (10^6/µL)	0.21 ± 0.01		0.74 ± 0.08**	0.99 ± 0.11**
Nucleated erythrocytes (10^3/µL)	0.00 ± 0.00		0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	51.1 ± 0.3		57.6 ± 0.6**	71.9 ± 2.4**
Mean cell hemoglobin (pg)	17.6 ± 0.2		22.9 ± 0.4**	28.7 ± 1.0**
Mean cell hemoglobin concentration (g/dL)	34.5 ± 0.3		39.8 ± 0.5**	39.9 ± 0.6**
Platelets (10^3/µL)	865.8 ± 10.2		991.2 ± 19.9**	1,124.0 ± 40.5**
Leukocytes (10^3/µL)	5.79 ± 0.32		8.13 ± 0.57**	13.36 ± 1.23**
Segmented neutrophils (10^3/µL)	0.99 ± 0.10		1.34 ± 0.14*	1.55 ± 0.22*
Lymphocytes (10^3/µL)	4.75 ± 0.24		6.74 ± 0.60*	11.78 ± 1.13**
Monocytes (10^3/µL)	0.00 ± 0.00		0.00 ± 0.00	0.00 ± 0.00
Eosinophils (10^3/µL)	0.06 ± 0.02		0.05 ± 0.02	0.03 ± 0.02
Methemoglobin (g/dL)	0.11 ± 0.02		0.28 ± 0.03**	0.28 ± 0.05**
Heinz bodies (%)	0.0 ± 0.0		26.4 ± 2.1**	22.0 ± 2.8**

* 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. No data presented for the 500, 1,000, and 2,000 mg/kg groups due to 100% mortality.
[b] n=7

TABLE F5
Hematology and Clinical Chemistry Data for Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
Hematology					
Week 1	10	10	10	10	10
Week 6	10	10	10	10	8
Month 3	9	10	9	10	9
Hematocrit (%)					
Week 1	50.8 ± 0.6	47.8 ± 0.5**	47.0 ± 0.9**	40.2 ± 0.6**	32.3 ± 0.7**
Week 6	46.6 ± 0.4	43.3 ± 0.9*	40.8 ± 0.4**	36.4 ± 1.0**	28.4 ± 1.7**
Month 3	48.1 ± 0.7	45.7 ± 0.9*	43.2 ± 0.7**	38.7 ± 0.5**	27.3 ± 0.9**
Hemoglobin (g/dL)					
Week 1	16.2 ± 0.2	15.4 ± 0.1**	15.3 ± 0.2**	15.3 ± 0.3**	14.8 ± 0.4**
Week 6	15.3 ± 0.1	14.7 ± 0.3	14.9 ± 0.2	14.8 ± 0.4	11.4 ± 0.6**
Month 3	15.9 ± 0.2	15.6 ± 0.2	16.5 ± 0.2	15.6 ± 0.2	11.4 ± 0.3**
Erythrocytes (10^6/μL)					
Week 1	10.06 ± 0.11	9.52 ± 0.11**	9.37 ± 0.19**	7.91 ± 0.11**	6.17 ± 0.15**
Week 6	9.47 ± 0.09	8.73 ± 0.21**	7.94 ± 0.07**	6.83 ± 0.17**	5.15 ± 0.31**
Month 3	9.83 ± 0.12	9.21 ± 0.17*	8.69 ± 0.14**	7.77 ± 0.11**	5.25 ± 0.21**
Reticulocytes (10^5/μL)					
Week 1	2.23 ± 0.19	2.29 ± 0.12	2.91 ± 0.18*	4.65 ± 0.61**	6.58 ± 0.49**
Week 6	2.19 ± 0.12	2.89 ± 0.23*	4.26 ± 0.34**	6.57 ± 0.50**	5.29 ± 0.82**
Month 3	2.37 ± 0.07	3.75 ± 0.33**	4.51 ± 0.29**	7.01 ± 0.42**	8.03 ± 1.43**
Nucleated erythrocytes/100 leukocytes					
Week 1	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.10
Week 6	0.00 ± 0.00	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Month 3	0.00 ± 0.00[b]	0.00 ± 0.00	0.11 ± 0.11	0.20 ± 0.13	3.00 ± 1.49**
Mean cell volume (fL)					
Week 1	50.4 ± 0.2	50.3 ± 0.2	50.3 ± 0.3	50.9 ± 0.2	52.5 ± 0.3**
Week 6	49.1 ± 0.2	49.6 ± 0.2	51.2 ± 0.1**	53.4 ± 0.3**	55.1 ± 0.6**
Month 3	48.8 ± 0.2	49.6 ± 0.2**	49.7 ± 0.2**	49.8 ± 0.3**	52.4 ± 1.1**
Mean cell hemoglobin (pg)					
Week 1	16.1 ± 0.1	16.2 ± 0.1	16.4 ± 0.1	19.4 ± 0.5**	24.1 ± 0.8**
Week 6	16.1 ± 0.2	16.9 ± 0.1**	18.8 ± 0.2**	21.7 ± 0.3**	22.2 ± 0.4**
Month 3	16.2 ± 0.0	17.0 ± 0.2**	19.0 ± 0.3**	20.2 ± 0.4**	22.0 ± 0.7**
Mean cell hemoglobin concentration (g/dL)					
Week 1	32.0 ± 0.2	32.2 ± 0.2	32.6 ± 0.2*	38.1 ± 1.0**	46.0 ± 1.3**
Week 6	32.8 ± 0.3	34.0 ± 0.2**	36.7 ± 0.4**	40.7 ± 0.6**	40.3 ± 0.6**
Month 3	33.0 ± 0.1	34.2 ± 0.4*	38.2 ± 0.5**	40.5 ± 0.8**	41.9 ± 0.6**
Platelets (10^3/μL)					
Week 1	902.7 ± 41.9	828.5 ± 73.9	846.4 ± 45.8	864.2 ± 55.3	1,071.8 ± 32.2
Week 6	822.1 ± 25.2	814.9 ± 32.2	820.5 ± 41.2	918.4 ± 30.0	923.0 ± 35.1
Month 3	922.3 ± 28.9	982.8 ± 28.9	943.7 ± 54.9	1,055.1 ± 40.6**	1,219.7 ± 55.0**
Leukocytes (10^3/μL)					
Week 1	4.63 ± 0.41	5.49 ± 0.34	4.54 ± 0.20	4.95 ± 0.73	6.13 ± 1.15
Week 6	5.47 ± 0.96	5.60 ± 0.64	3.82 ± 0.45	2.51 ± 0.43**	2.60 ± 0.56*
Month 3	4.01 ± 0.20	4.31 ± 0.31	3.16 ± 0.35	4.42 ± 0.90	6.36 ± 1.41
Segmented neutrophils (10^3/μL)					
Week 1	0.85 ± 0.12	1.01 ± 0.23	0.67 ± 0.10	0.45 ± 0.07*	0.81 ± 0.27
Week 6	0.66 ± 0.15	0.67 ± 0.14	0.53 ± 0.12	0.26 ± 0.06	0.36 ± 0.08
Month 3	0.63 ± 0.09	0.51 ± 0.08	0.39 ± 0.07	0.49 ± 0.20*	1.32 ± 0.79
Lymphocytes (10^3/μL)					
Week 1	3.73 ± 0.30	4.40 ± 0.16	3.82 ± 0.13	4.47 ± 0.66	5.26 ± 1.06
Week 6	4.71 ± 0.80	4.86 ± 0.57	3.21 ± 0.35	2.24 ± 0.39**	2.22 ± 0.48*
Month 3	3.35 ± 0.16	3.76 ± 0.26	2.74 ± 0.28	3.92 ± 0.77	5.04 ± 0.69

TABLE F5
Hematology and Clinical Chemistry Data for Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male (continued)					
Hematology (continued)					
Week 1	10	10	10	10	10
Week 6	10	10	10	10	8
Month 3	9	10	9	10	9
Monocytes ($10^3/\mu L$)					
Week 1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Month 3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Eosinophils ($10^3/\mu L$)					
Week 1	0.05 ± 0.03	0.08 ± 0.03	0.05 ± 0.02	0.04 ± 0.02	0.05 ± 0.02
Week 6	0.10 ± 0.03	0.08 ± 0.03	0.08 ± 0.02	0.01 ± 0.01*	0.03 ± 0.01
Month 3	0.03 ± 0.01	0.04 ± 0.02	0.02 ± 0.02	0.01 ± 0.01*	0.00 ± 0.00**
Methemoglobin (g/dL)					
Week 1	0.24 ± 0.02	0.42 ± 0.03**	0.72 ± 0.04**	0.78 ± 0.04**[c]	0.86 ± 0.05**
Week 6	0.21 ± 0.01	0.44 ± 0.02**	0.54 ± 0.05**	0.60 ± 0.05**	0.61 ± 0.07**
Month 3	0.19 ± 0.01[b]	0.51 ± 0.02**	0.57 ± 0.04**[b]	0.60 ± 0.04**	0.60 ± 0.04**
Heinz bodies (%)					
Week 1	0.0 ± 0.0	6.2 ± 2.0**	37.6 ± 2.9**	89.0 ± 1.1**	88.6 ± 0.9**
Week 6	0.0 ± 0.0	20.5 ± 2.7**	37.4 ± 1.4**	89.3 ± 0.6**	89.3 ± 1.6**
Month 3	0.0 ± 0.0[b]	24.8 ± 2.0**	51.5 ± 3.5**	90.2 ± 0.6**	84.6 ± 2.3**
Clinical Chemistry					
Week 1	1	3	1	3	7
Week 6	7	5	9	5	6
Month 3	6	10	8	10	9
Urea nitrogen (mg/dL)					
Week 1	37.0	29.0 ± 2.1	24.0	19.7 ± 2.2	23.5 ± 3.4[d]
Week 6	23.7 ± 1.2[d]	26.4 ± 2.4	26.3 ± 1.6	21.6 ± 0.5	22.2 ± 1.1
Month 3	24.8 ± 0.3	24.7 ± 1.0[e]	23.4 ± 0.9	23.0 ± 0.8	25.6 ± 0.8
Creatinine (mg/dL)					
Week 1	0.70	0.53 ± 0.03	0.60	0.53 ± 0.03	0.52 ± 0.06[d]
Week 6	0.40 ± 0.00[d]	0.40 ± 0.06[f]	0.45 ± 0.03[g]	0.40 ± 0.00	0.42 ± 0.02
Month 3	0.47 ± 0.02	0.49 ± 0.01[e]	0.50 ± 0.00[e]	0.48 ± 0.01	0.48 ± 0.02
Total protein (g/dL)					
Week 1	5.7 ± 0.3[h]	5.5 ± 0.0[i]	5.6 ± 0.1[f]	5.4 ± 0.1[i]	5.2 ± 0.1
Week 6	4.9 ± 0.1	5.0 ± 0.3	5.2 ± 0.1	4.3 ± 0.3	5.0 ± 0.1[e]
Month 3	6.3 ± 0.1	6.3 ± 0.1[c]	6.2 ± 0.1	6.3 ± 0.1	5.9 ± 0.1
Albumin (g/dL)					
Week 1	3.8	3.9 ± 0.1	3.9	3.9 ± 0.1	3.8 ± 0.1
Week 6	3.2 ± 0.1	3.4 ± 0.2	3.5 ± 0.1	3.1 ± 0.1	3.6 ± 0.1
Month 3	4.5 ± 0.1	4.5 ± 0.0[g]	4.4 ± 0.1	4.5 ± 0.0	4.3 ± 0.1
Alanine aminotransferase (IU/L)					
Week 1	33 ± 5[h]	46 ± 4[j]	33 ± 1[i]	32 ± 4[d]	28 ± 2
Week 6	30 ± 3	34 ± 6	32 ± 2	26 ± 1	31 ± 2[e]
Month 3	38 ± 2[c]	46 ± 7	38 ± 2	40 ± 4	46 ± 5
Alkaline phosphatase (IU/L)					
Week 1	393 ± 5[f]	401 ± 11[d]	347 ± 19[i]	373 ± 7[e]	302 ± 17**
Week 6	175 ± 8	180 ± 17[d]	183 ± 10	162 ± 9	153 ± 8[g]
Month 3	189 ± 6[c]	204 ± 6	192 ± 5	178 ± 6	150 ± 5**

TABLE F5
Hematology and Clinical Chemistry Data for Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male (continued)					
Clinical Chemistry (continued)					
Week 1	1	3	1	3	7
Week 6	7	5	9	5	6
Month 3	6	10	8	10	9
Creatine kinase (IU/L)					
Week 1	1,199	2,012 ± 305**	1,190	615 ± 161**	517 ± 99**
Week 6	469 ± 109[j]	195[k]	747 ± 134[e]	785 ± 191[i]	1,069 ± 230
Month 3	976 ± 238[i]	606 ± 137[e]	773 ± 135[e]	969 ± 301	1,011 ± 287
Sorbitol dehydrogenase (IU/L)					
Week 1	71 ± 3[i]	61 ± 3[e]	71 ± 2[d]	70 ± 3[e]	66 ± 2[g]
Week 6	65 ± 3[g]	66 ± 2[g]	68 ± 2[b]	59 ± 2[c]	58 ± 3[g]
Month 3	71 ± 2[c]	85 ± 10	72 ± 2	63 ± 2	65 ± 3
Bile acids (µmol/L)					
Week 1	15.2 ± 0.6[d]	14.9 ± 1.3[e]	13.6 ± 0.9[e]	15.7 ± 2.5[e]	13.8 ± 2.1[g]
Week 6	68.0 ± 4.2[b]	61.1 ± 3.2	56.0 ± 6.6[b]	74.8 ± 5.5[c]	93.1 ± 14.0[g]
Month 3	15.4 ± 0.6[b]	17.4 ± 2.6	13.9 ± 0.4	13.5 ± 0.9	19.9 ± 3.4
Female					
Hematology					
Week 1	9	10	10	10	8
Week 6	10	10	10	10	10
Month 3	10	8	10	10	10
Hematocrit (%)					
Week 1	48.1 ± 0.5	46.9 ± 0.8	44.3 ± 0.4**	39.9 ± 0.7**	31.5 ± 0.8**
Week 6	48.4 ± 0.8	43.8 ± 0.5**	42.4 ± 0.5**	40.8 ± 0.8**	34.0 ± 1.3**
Month 3	47.9 ± 0.6	44.7 ± 0.5**	41.5 ± 0.4**	38.4 ± 0.7**	30.7 ± 0.6**
Hemoglobin (g/dL)					
Week 1	15.7 ± 0.2	15.1 ± 0.2*	14.6 ± 0.1**	14.8 ± 0.3**	14.6 ± 0.3**
Week 6	16.2 ± 0.2	14.8 ± 0.2**	15.1 ± 0.1**	15.9 ± 0.3**	13.3 ± 0.4**
Month 3	15.9 ± 0.1	14.9 ± 0.2**	14.9 ± 0.2**	15.2 ± 0.2**	12.5 ± 0.3**
Erythrocytes (10^6/µL)					
Week 1	9.63 ± 0.10	9.42 ± 0.16	8.85 ± 0.09**	7.86 ± 0.09**	5.90 ± 0.17**
Week 6	9.72 ± 0.18	8.61 ± 0.10**	8.18 ± 0.11**	7.82 ± 0.16**	6.45 ± 0.27**
Month 3	9.73 ± 0.13	9.06 ± 0.09**	8.45 ± 0.08**	7.59 ± 0.15**	5.78 ± 0.12**
Reticulocytes (10^5/µL)					
Week 1	1.58 ± 0.07	1.92 ± 0.21	2.65 ± 0.17**	4.37 ± 0.79**	6.50 ± 0.44**[e]
Week 6	2.01 ± 0.25	2.99 ± 0.26**	4.01 ± 0.46**	5.73 ± 0.50**	9.86 ± 0.76**
Month 3	2.16 ± 0.17	2.70 ± 0.20	4.90 ± 0.17**	7.52 ± 0.77**	14.26 ± 1.31**
Nucleated erythrocytes/100 leukocytes					
Week 1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.16*
Week 6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Month 3	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.10	0.00 ± 0.00	0.30 ± 0.15
Mean cell volume (fL)					
Week 1	50.1 ± 0.2	49.8 ± 0.2	50.1 ± 0.1	50.8 ± 0.4	53.6 ± 0.5**
Week 6	49.8 ± 0.3	51.1 ± 0.3**	52.0 ± 0.2**	52.3 ± 0.3**	52.7 ± 0.5**
Month 3	49.3 ± 0.2	49.3 ± 0.3	49.2 ± 0.3	50.6 ± 0.3**	53.3 ± 0.7**

TABLE F5
Hematology and Clinical Chemistry Data for Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female (continued)					
Hematology (continued)					
Week 1	9	10	10	10	8
Week 6	10	10	10	10	10
Month 3	10	8	10	10	10
Mean cell hemoglobin (pg)					
Week 1	16.3 ± 0.1	16.1 ± 0.1	16.5 ± 0.1	18.9 ± 0.3**	24.8 ± 0.6**
Week 6	16.7 ± 0.1	17.2 ± 0.1*	18.5 ± 0.1**	20.4 ± 0.3**	20.7 ± 0.4**
Month 3	16.4 ± 0.1	16.4 ± 0.1	17.7 ± 0.3**	20.1 ± 0.3**	21.7 ± 0.5**
Mean cell hemoglobin concentration (g/dL)					
Week 1	32.8 ± 0.2	32.2 ± 0.2	33.0 ± 0.2	37.2 ± 0.4**	46.3 ± 0.8**
Week 6	33.6 ± 0.2	33.9 ± 0.1	35.6 ± 0.3**	39.0 ± 0.5**	39.2 ± 0.5**
Month 3	33.3 ± 0.3	33.3 ± 0.2	36.0 ± 0.5**	39.6 ± 0.4**	40.8 ± 0.8**
Platelets (10^3/µL)					
Week 1	846.7 ± 24.3	796.7 ± 32.8	848.8 ± 22.4	884.6 ± 33.0	876.9 ± 27.4
Week 6	746.0 ± 51.6	959.9 ± 22.4**	847.7 ± 40.3	963.8 ± 30.8**	883.2 ± 24.3
Month 3	832.2 ± 32.4	956.8 ± 30.1	879.7 ± 27.7	992.1 ± 44.9**	1,013.8 ± 36.3**
Leukocytes (10^3/µL)					
Week 1	4.83 ± 0.49	4.32 ± 0.31[c]	4.72 ± 0.32[c]	5.09 ± 0.74[g]	3.11 ± 0.61
Week 6	5.35 ± 0.41	6.18 ± 0.72	4.73 ± 0.68	4.53 ± 0.62	4.88 ± 0.59
Month 3	3.47 ± 0.13	3.41 ± 0.18	3.28 ± 0.45	3.64 ± 0.38	2.95 ± 0.33
Segmented neutrophils (10^3/µL)					
Week 1	0.71 ± 0.10	0.81 ± 0.18[c]	0.75 ± 0.09[c]	0.80 ± 0.17[g]	0.66 ± 0.28
Week 6	0.46 ± 0.06	0.46 ± 0.10	0.33 ± 0.06	0.40 ± 0.09	0.32 ± 0.05
Month 3	0.45 ± 0.06	0.49 ± 0.04	0.29 ± 0.05	0.42 ± 0.12	0.27 ± 0.05
Lymphocytes (10^3/µL)					
Week 1	4.10 ± 0.43	3.48 ± 0.30[c]	3.93 ± 0.26[c]	4.22 ± 0.60[g]	2.43 ± 0.37*
Week 6	4.84 ± 0.37	5.65 ± 0.65	4.37 ± 0.63	4.11 ± 0.55	4.51 ± 0.55
Month 3	2.97 ± 0.12	2.88 ± 0.17	2.97 ± 0.39	3.20 ± 0.34	2.67 ± 0.32
Monocytes (10^3/µL)					
Week 1	0.00 ± 0.00	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00[g]	0.00 ± 0.00
Week 6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Month 3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Eosinophils (10^3/µL)					
Week 1	0.02 ± 0.01	0.03 ± 0.01[c]	0.04 ± 0.02[c]	0.07 ± 0.04[g]	0.02 ± 0.02
Week 6	0.05 ± 0.02	0.07 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	0.05 ± 0.02
Month 3	0.05 ± 0.02	0.05 ± 0.02	0.03 ± 0.01	0.02 ± 0.02	0.01 ± 0.01*
Methemoglobin (g/dL)					
Week 1	0.29 ± 0.01[b]	0.35 ± 0.05	0.56 ± 0.06**[c]	0.84 ± 0.07**[c]	0.98 ± 0.08**
Week 6	0.27 ± 0.02	0.50 ± 0.02**	0.46 ± 0.03**	0.64 ± 0.05**	0.56 ± 0.07**
Month 3	0.26 ± 0.03	0.54 ± 0.03**	0.64 ± 0.03**	0.67 ± 0.06**	0.42 ± 0.04
Heinz bodies (%)					
Week 1	0.0 ± 0.0	1.1 ± 0.6*	26.1 ± 5.0**	77.9 ± 5.3**	87.5 ± 0.6**[e]
Week 6	0.0 ± 0.0	13.8 ± 1.4**	26.8 ± 3.2**	68.0 ± 7.2**	84.4 ± 1.2**
Month 3	0.0 ± 0.0	18.7 ± 4.7**	51.9 ± 3.1**	80.1 ± 3.7**	75.2 ± 2.1**

TABLE F5
Hematology and Clinical Chemistry Data for Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female (continued)					
Clinical Chemistry					
Week 1	5	6	6	6	5
Week 6	6	2	5	3	8
Month 3	10	8	10	8	10
Urea nitrogen (mg/dL)					
Week 6	22.5 ± 0.5[h]	30.5 ± 0.5	19.4 ± 1.4	18.7 ± 4.1	20.0 ± 1.1
Month 3	20.2 ± 1.5[j]	23.0 ± 0.8[d]	18.7 ± 0.8[d]	19.8 ± 0.5	16.9 ± 0.6*[g]
Creatinine (mg/dL)					
Week 6	0.50 ± 0.00[h]	—[l]	0.50 ± 0.03	0.53 ± 0.03	0.45 ± 0.04
Month 3	0.48 ± 0.03[i]	0.50 ± 0.00[j]	0.48 ± 0.02[j]	0.44 ± 0.02	0.41 ± 0.01[g]
Total protein (g/dL)					
Week 6	5.4 ± 0.1[f]	6.4 ± 0.5	5.6 ± 0.3	5.8 ± 0.2	5.7 ± 0.2
Month 3	6.4 ± 0.1[g]	6.2 ± 0.1[e]	6.0 ± 0.1**[g]	6.0 ± 0.1*	5.9 ± 0.1**[c]
Albumin (g/dL)					
Week 6	3.0 ± 1.0[f]	4.9 ± 0.4	4.2 ± 0.2	4.4 ± 0.2	4.3 ± 0.1
Month 3	4.8 ± 0.1[e]	4.7 ± 0.0[d]	4.5 ± 0.0[e]	4.6 ± 0.1	4.5 ± 0.1[g]
Alanine aminotransferase (IU/L)					
Week 6	24 ± 4	32 ± 3	26 ± 3	31 ± 5[d]	25 ± 3
Month 3	31 ± 2[c]	36 ± 4	31 ± 2	25 ± 1	32 ± 3
Alkaline phosphatase (IU/L)					
Week 6	348 ± 24	289 ± 45[f]	306 ± 18[d]	299 ± 15[j]	289 ± 17
Month 3	271 ± 13[c]	278 ± 10	255 ± 7	230 ± 15	218 ± 10**
Creatine kinase (IU/L)					
Week 6	628 ± 224[h]	—	342 ± 248[f]	273 ± 124	171 ± 24[f]
Month 3	749 ± 209[f]	1,184 ± 320[i]	727 ± 492[f]	512 ± 84	283 ± 36*[d]
Sorbitol dehydrogenase (IU/L)					
Week 6	54 ± 3	59 ± 2[f]	56 ± 3[d]	61 ± 4[d]	57 ± 2[c]
Month 3	54 ± 2	53 ± 3	55 ± 2	54 ± 2[c]	55 ± 2
Bile acids (µmol/L)					
Week 1	17.2 ± 1.0	13.8 ± 1.4	13.0 ± 0.8	11.8 ± 1.0*	16.2 ± 0.9
Week 6	32.4 ± 8.2[e]	20.8 ± 4.5[i]	35.1 ± 11.7[g]	25.1 ± 6.0[c]	41.3 ± 8.0[c]
Month 3	16.2 ± 0.6	16.8 ± 0.7	16.2 ± 0.7	19.4 ± 0.5**[c]	22.2 ± 1.6**

* 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=10
[c] n=9
[d] n=6
[e] n=7
[f] n=3
[g] n=8
[h] n=2
[i] n=4
[j] n=5
[k] n=1
[l] No measurement taken

TABLE F6
Hematology and Urinalysis Data for Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Male				
Hematology				
Week 2	10	10	10	10
Month 3	9	10	9	10
Month 12	9	9	8	9
Month 18	10	9	8	8
Hematocrit (spun) (%)				
Week 2	49.9 ± 0.8	50.3 ± 0.7	49.6 ± 1.0	49.4 ± 0.9
Month 3	49.6 ± 0.7	48.6 ± 0.8	48.5 ± 0.8	46.3 ± 0.5**
Month 12	46.7 ± 0.6[b]	46.9 ± 0.4	46.7 ± 0.8	45.0 ± 0.8
Month 18	41.6 ± 1.0	45.5 ± 1.8	42.8 ± 1.4[c]	42.2 ± 1.2
Hematocrit (auto) (%)				
Week 2	50.6 ± 0.7	50.6 ± 0.9	50.4 ± 1.0	49.9 ± 1.0
Month 3	50.4 ± 0.8	49.5 ± 0.8	49.2 ± 0.8	46.9 ± 0.7**
Month 12	46.3 ± 0.5	46.7 ± 0.4	46.5 ± 0.9	44.2 ± 0.7
Month 18	41.4 ± 1.0	44.6 ± 1.8	42.4 ± 1.2	41.3 ± 1.0
Hemoglobin (g/dL)				
Week 2	16.9 ± 0.3	16.9 ± 0.3	16.8 ± 0.4	16.6 ± 0.4
Month 3	17.0 ± 0.2	16.6 ± 0.3	16.4 ± 0.3	15.4 ± 0.2**
Month 12	15.5 ± 0.3	15.5 ± 0.2	15.3 ± 0.3	14.6 ± 0.2
Month 18	14.0 ± 0.3	15.0 ± 0.6	14.5 ± 0.4	13.7 ± 0.3
Erythrocytes (10^6/μL)				
Week 2	10.68 ± 0.17	10.65 ± 0.21	10.64 ± 0.24	10.64 ± 0.26
Month 3	10.98 ± 0.19	10.70 ± 0.17	10.63 ± 0.15	10.11 ± 0.13**
Month 12	10.02 ± 0.11	10.19 ± 0.16	10.13 ± 0.20	9.43 ± 0.13*
Month 18	9.20 ± 0.26	10.29 ± 0.58	9.91 ± 0.52	9.07 ± 0.34
Reticulocytes (10^5/μL)				
Week 2	4.13 ± 0.15	4.62 ± 0.10*	4.19 ± 0.11	3.93 ± 0.17
Month 3	4.37 ± 0.13	4.08 ± 0.12	4.06 ± 0.15	4.99 ± 0.15
Month 12	2.75 ± 0.10	3.07 ± 0.23	3.39 ± 0.11**	4.23 ± 0.12**
Month 18	2.72 ± 0.17	3.25 ± 0.41	2.79 ± 0.25	3.41 ± 0.35
Nucleated erythrocytes (10^3/μL)				
Week 2	0.30 ± 0.15	0.50 ± 0.22	0.40 ± 0.22	0.20 ± 0.13
Month 3	0.00 ± 0.00	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00
Month 12	0.00 ± 0.00[b]	0.00 ± 0.00	0.00 ± 0.00	0.22 ± 0.22
Month 18	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)				
Week 2	47.4 ± 0.1	47.6 ± 0.2	47.4 ± 0.3	47.0 ± 0.3
Month 3	45.9 ± 0.2	46.2 ± 0.1	46.2 ± 0.2	46.4 ± 0.2
Month 12	46.2 ± 0.3	45.9 ± 0.4	45.9 ± 0.2	46.8 ± 0.3
Month 18	45.1 ± 0.5	43.7 ± 0.7	43.3 ± 1.3	45.7 ± 0.5
Mean cell hemoglobin (pg)				
Week 2	15.8 ± 0.1	15.9 ± 0.1	15.8 ± 0.1	15.6 ± 0.1
Month 3	15.5 ± 0.1	15.5 ± 0.1	15.4 ± 0.1	15.3 ± 0.2
Month 12	15.4 ± 0.1	15.2 ± 0.2	15.1 ± 0.1	15.5 ± 0.1
Month 18	15.3 ± 0.2	14.7 ± 0.2	14.7 ± 0.3	15.1 ± 0.2
Mean cell hemoglobin concentration (g/dL)				
Week 2	33.4 ± 0.2	33.4 ± 0.2	33.4 ± 0.2	33.3 ± 0.2
Month 3	33.8 ± 0.2	33.5 ± 0.3	33.3 ± 0.3	32.9 ± 0.4**
Month 12	33.4 ± 0.4	33.1 ± 0.2	33.0 ± 0.1	33.0 ± 0.1
Month 18	33.8 ± 0.3	33.6 ± 0.3	34.1 ± 0.5	33.0 ± 0.2

TABLE F6
Hematology and Urinalysis Data for Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Male (continued)				
Hematology (continued)				
Week 2	10	10	10	10
Month 3	9	10	9	10
Month 12	9	9	8	9
Month 18	10	9	8	8
Platelets (10^3/μL)				
Week 2	834.1 ± 37.0	807.3 ± 21.1	794.5 ± 41.5	922.3 ± 75.2
Month 3	863.1 ± 32.0	823.3 ± 34.0	810.3 ± 54.9	825.1 ± 23.3
Month 12	844.9 ± 59.9	1,028.9 ± 73.8	932.4 ± 45.0	938.3 ± 40.4
Month 18	1,272.7 ± 115.0	1,144.7 ± 76.0	1,280.9 ± 106.4	1,233.9 ± 54.8
Leukocytes (10^3/μL)				
Week 2	4.83 ± 0.37	5.36 ± 0.30	4.62 ± 0.23	5.03 ± 0.45
Month 3	4.82 ± 0.40	3.85 ± 0.37	4.75 ± 0.34	5.65 ± 0.32
Month 12	6.69 ± 0.38	6.82 ± 0.60	6.38 ± 0.46	7.32 ± 0.35
Month 18	7.58 ± 0.36	8.01 ± 0.56	7.73 ± 0.60	8.01 ± 0.56
Segmented neutrophils (10^3/μL)				
Week 2	0.56 ± 0.15	0.51 ± 0.07	0.57 ± 0.10	0.59 ± 0.06
Month 3	0.59 ± 0.04	0.52 ± 0.04	0.67 ± 0.08	0.66 ± 0.05
Month 12	1.09 ± 0.06	1.21 ± 0.16	1.19 ± 0.10	1.33 ± 0.07
Month 18	1.60 ± 0.17	1.60 ± 0.18	1.78 ± 0.38	1.67 ± 0.13
Lymphocytes (10^3/μL)				
Week 2	4.04 ± 0.27	4.65 ± 0.26	3.89 ± 0.20	4.26 ± 0.43
Month 3	4.00 ± 0.36	3.16 ± 0.33	3.84 ± 0.27	4.68 ± 0.27
Month 12	5.11 ± 0.34	5.13 ± 0.40	4.78 ± 0.37	5.57 ± 0.31
Month 18	5.58 ± 0.33	5.98 ± 0.36	5.57 ± 0.50	5.91 ± 0.46
Activated lymphocytes (10^3/μL)				
Week 2	0.04 ± 0.01[c]	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
Month 3	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.05 ± 0.01
Month 12	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
Month 18	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01
Monocytes (10^3/μL)				
Week 2	0.08 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Month 3	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.15 ± 0.02*
Month 12	0.13 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01
Month 18	0.17 ± 0.02	0.22 ± 0.03	0.21 ± 0.02	0.20 ± 0.02
Basophils (10^3/μL)				
Week 2	0.017 ± 0.004	0.021 ± 0.005	0.011 ± 0.002	0.011 ± 0.002
Month 3	0.010 ± 0.002	0.008 ± 0.002	0.014 ± 0.004	0.016 ± 0.002
Month 12	0.022 ± 0.008	0.026 ± 0.003	0.016 ± 0.002	0.017 ± 0.004
Month 18	0.033 ± 0.004	0.041 ± 0.007	0.029 ± 0.004	0.029 ± 0.004
Eosinophils (10^3/μL)				
Week 2	0.08 ± 0.02	0.08 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
Month 3	0.10 ± 0.01	0.06 ± 0.01	0.09 ± 0.03	0.09 ± 0.02
Month 12	0.26 ± 0.05	0.22 ± 0.07	0.21 ± 0.03	0.21 ± 0.03
Month 18	0.15 ± 0.03	0.13 ± 0.01	0.11 ± 0.01	0.17 ± 0.03

TABLE F6
Hematology and Urinalysis Data for Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Male (continued)				
Hematology (continued)				
Week 2	10	10	10	10
Month 3	9	10	9	10
Month 12	9	9	8	9
Month 18	10	9	8	8
Methemoglobin (g/dL)				
Week 2	0.62 ± 0.10	0.59 ± 0.05	0.45 ± 0.07	0.58 ± 0.11
Month 3	0.33 ± 0.04	0.38 ± 0.03	0.44 ± 0.07	0.52 ± 0.05*
Month 12	0.38 ± 0.04	0.38 ± 0.04	0.40 ± 0.07	0.51 ± 0.04
Month 18	0.22 ± 0.04[c]	0.18 ± 0.05	0.30 ± 0.04	0.44 ± 0.10
Heinz bodies (%)				
Week 2	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	1.2 ± 0.1**
Month 3	0.0 ± 0.0	0.0 ± 0.0	2.2 ± 0.4**	23.3 ± 2.9**
Month 12	0.0 ± 0.0[b]	0.0 ± 0.0	2.3 ± 0.6**	13.3 ± 1.6**
Month 18	0.1 ± 0.0	0.2 ± 0.1	5.0 ± 0.5**	19.8 ± 1.1**
Urinalysis				
n	5	5	5	5
Creatinine (mg/dL)				
Month 3	36.36 ± 3.22	31.76 ± 1.57	30.50 ± 1.26	33.12 ± 3.30
Month 12	23.36 ± 0.78	23.40 ± 1.76	31.50 ± 2.63	28.96 ± 2.61
Month 18	45.36 ± 13.43	31.96 ± 4.69	26.55 ± 3.74[d]	26.36 ± 2.38
Volume (mL)				
Month 3	0.7 ± 0.2	0.8 ± 0.1	1.2 ± 0.1	1.0 ± 0.2
Month 12	2.5 ± 0.2	1.9 ± 0.3	1.4 ± 0.1*	2.0 ± 0.2
Month 18	1.5 ± 0.3	1.7 ± 0.2	1.7 ± 0.6[d]	1.5 ± 0.3
Female				
Hematology				
Week 2	10	10	10	10
Month 3	10	10	10	9
Month 12	10	9	10	9
Month 18	9	9	10	10
Hematocrit (spun) (%)				
Week 2	48.6 ± 0.9	48.8 ± 0.9	49.1 ± 0.6	48.6 ± 0.8
Month 3	47.2 ± 0.5	48.3 ± 0.7	47.0 ± 0.8	45.6 ± 0.7
Month 12	47.0 ± 0.4	47.1 ± 0.3[b]	45.2 ± 1.0	45.7 ± 0.5*[b]
Month 18	42.2 ± 0.5	42.0 ± 1.3	42.1 ± 0.8	43.1 ± 0.5
Hematocrit (auto) (%)				
Week 2	48.5 ± 0.8	48.5 ± 0.9	48.9 ± 0.6	48.1 ± 0.7
Month 3	48.1 ± 0.8	49.0 ± 0.7	47.7 ± 0.7	46.0 ± 0.8
Month 12	47.6 ± 0.4	48.0 ± 0.3	45.8 ± 1.1	46.1 ± 0.6
Month 18	41.9 ± 0.5	42.0 ± 1.3	41.5 ± 0.7	42.5 ± 0.5

TABLE F6
Hematology and Urinalysis Data for Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Female (continued)				
Hematology (continued)				
Week 2	10	10	10	10
Month 3	10	10	10	9
Month 12	10	9	10	9
Month 18	9	9	10	10
Hemoglobin (g/dL)				
Week 2	15.8 ± 0.2	15.8 ± 0.3	15.9 ± 0.2	15.6 ± 0.2
Month 3	16.3 ± 0.3	16.5 ± 0.2	15.8 ± 0.3	15.8 ± 0.2
Month 12	15.8 ± 0.1	16.0 ± 0.1	15.1 ± 0.3	15.2 ± 0.2
Month 18	14.2 ± 0.2	14.1 ± 0.6	13.9 ± 0.2	14.1 ± 0.2
Erythrocytes (10^6/μL)				
Week 2	10.56 ± 0.14	10.67 ± 0.21	10.73 ± 0.13	10.47 ± 0.18
Month 3	10.35 ± 0.17	10.50 ± 0.17	10.34 ± 0.14	9.86 ± 0.16
Month 12	9.82 ± 0.10	9.96 ± 0.07	9.41 ± 0.23	9.52 ± 0.11
Month 18	9.04 ± 0.14	9.03 ± 0.33	8.74 ± 0.23	9.12 ± 0.14
Reticulocytes (10^5/μL)				
Week 2	4.26 ± 0.20	4.60 ± 0.24	4.39 ± 0.19	4.43 ± 0.24
Month 3	4.53 ± 0.35	4.86 ± 0.18*	4.89 ± 0.18*	5.39 ± 0.36**
Month 12	2.79 ± 0.13	3.02 ± 0.11	4.04 ± 0.23**	4.60 ± 0.41**
Month 18	2.93 ± 0.20	4.10 ± 0.91	3.03 ± 0.23	3.21 ± 0.20
Nucleated erythrocytes (10^3/μL)				
Week 2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Month 3	0.00 ± 0.00	0.00 ± 0.00[b]	0.00 ± 0.00	0.00 ± 0.00[b]
Month 12	0.00 ± 0.00	0.00 ± 0.00[b]	0.00 ± 0.00	0.00 ± 0.00[b]
Month 18	0.11 ± 0.11	0.11 ± 0.11	0.00 ± 0.00	0.10 ± 0.10
Mean cell volume (fL)				
Week 2	45.9 ± 0.2	45.5 ± 0.1	45.6 ± 0.2	46.0 ± 0.2
Month 3	46.5 ± 0.2	46.6 ± 0.2	46.1 ± 0.2	46.7 ± 0.3
Month 12	48.4 ± 0.2	48.2 ± 0.2	48.7 ± 0.2	48.4 ± 0.3
Month 18	46.4 ± 0.3	46.6 ± 0.4	47.6 ± 0.6	46.7 ± 0.5
Mean cell hemoglobin (pg)				
Week 2	14.9 ± 0.1	14.8 ± 0.1	14.8 ± 0.1	14.9 ± 0.1
Month 3	15.7 ± 0.1	15.7 ± 0.1	15.3 ± 0.1**	16.0 ± 0.2
Month 12	16.1 ± 0.1	16.1 ± 0.1	16.1 ± 0.1	16.0 ± 0.1
Month 18	15.7 ± 0.1	15.6 ± 0.2	16.0 ± 0.2	15.5 ± 0.1
Mean cell hemoglobin concentration (g/dL)				
Week 2	32.6 ± 0.2	32.6 ± 0.2	32.5 ± 0.2	32.3 ± 0.2
Month 3	33.8 ± 0.1	33.7 ± 0.1	33.1 ± 0.2**	34.3 ± 0.4
Month 12	33.2 ± 0.1	33.4 ± 0.1	33.0 ± 0.1	33.1 ± 0.2
Month 18	33.8 ± 0.1	33.5 ± 0.5	33.5 ± 0.2	33.1 ± 0.2*
Platelets (10^3/μL)				
Week 2	646.2 ± 33.0	674.4 ± 58.1	599.2 ± 33.0	685.5 ± 40.1
Month 3	777.8 ± 53.6	657.3 ± 51.9	770.8 ± 51.5	751.6 ± 59.3
Month 12	895.4 ± 39.0	915.8 ± 50.4	980.5 ± 53.1	956.4 ± 46.5
Month 18	998.4 ± 49.2	821.6 ± 76.2	980.8 ± 60.5	964.6 ± 43.3
Leukocytes (10^3/μL)				
Week 2	4.60 ± 0.39	5.55 ± 0.54	5.13 ± 0.55	5.36 ± 0.55
Month 3	4.08 ± 0.31	3.83 ± 0.31	3.87 ± 0.49	4.30 ± 0.55
Month 12	5.52 ± 0.41	4.25 ± 0.33	5.65 ± 0.60	4.33 ± 0.23
Month 18	5.14 ± 0.41	5.34 ± 0.49	5.41 ± 0.90	4.89 ± 0.32

TABLE F6
Hematology and Urinalysis Data for Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Hematology (continued)				
Week 2	10	10	10	10
Month 3	10	10	10	9
Month 12	10	9	10	9
Month 18	9	9	10	10
Segmented neutrophils ($10^3/\mu L$)				
Week 2	0.51 ± 0.06	0.59 ± 0.07	0.53 ± 0.07	0.67 ± 0.12
Month 3	0.58 ± 0.08	0.51 ± 0.05	0.52 ± 0.07	0.76 ± 0.26
Month 12	1.14 ± 0.10	0.91 ± 0.05	1.47 ± 0.45	0.94 ± 0.14
Month 18	1.34 ± 0.13	1.56 ± 0.30	1.64 ± 0.51	1.17 ± 0.11
Lymphocytes ($10^3/\mu L$)				
Week 2	3.90 ± 0.35	4.73 ± 0.46	4.41 ± 0.48	4.49 ± 0.44
Month 3	3.32 ± 0.25	3.15 ± 0.27	3.18 ± 0.41	3.35 ± 0.33
Month 12	3.95 ± 0.33	3.04 ± 0.31	3.81 ± 0.35	3.10 ± 0.14
Month 18	3.49 ± 0.31	3.43 ± 0.50	3.35 ± 0.39	3.46 ± 0.26
Activated lymphocytes ($10^3/\mu L$)				
Week 2	0.03 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.05 ± 0.01
Month 3	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
Month 12	0.08 ± 0.01	0.07 ± 0.02	0.06 ± 0.01	0.05 ± 0.01
Month 18	0.04 ± 0.01	0.07 ± 0.05	0.05 ± 0.01	0.04 ± 0.01
Monocytes ($10^3/\mu L$)				
Week 2	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
Month 3	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0.01
Month 12	0.13 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.06 ± 0.01**
Month 18	0.13 ± 0.01	0.15 ± 0.02	0.23 ± 0.09	0.11 ± 0.02
Basophils ($10^3/\mu L$)				
Week 2	0.017 ± 0.004	0.029 ± 0.003*	0.014 ± 0.002	0.020 ± 0.003
Month 3	0.010 ± 0.003	0.012 ± 0.002	0.009 ± 0.002	0.008 ± 0.003
Month 12	0.014 ± 0.003	0.017 ± 0.006	0.015 ± 0.003	0.009 ± 0.001
Month 18	0.021 ± 0.002	0.029 ± 0.004	0.027 ± 0.007	0.019 ± 0.002
Eosinophils ($10^3/\mu L$)				
Week 2	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.02	0.07 ± 0.01
Month 3	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
Month 12	0.21 ± 0.04	0.12 ± 0.03	0.19 ± 0.03	0.17 ± 0.03
Month 18	0.11 ± 0.02	0.10 ± 0.01	0.12 ± 0.02	0.10 ± 0.02
Methemoglobin (g/dL)				
Week 2	0.47 ± 0.09	0.58 ± 0.11	0.56 ± 0.10	0.61 ± 0.08
Month 3	0.41 ± 0.05	0.46 ± 0.08	0.50 ± 0.10	0.51 ± 0.10
Month 12	0.34 ± 0.04	0.33 ± 0.05	0.41 ± 0.04	0.63 ± 0.05**
Month 18	0.22 ± 0.04	0.31 ± 0.04	0.34 ± 0.03*	0.52 ± 0.03**
Heinz bodies (%)				
Week 2	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1*	5.1 ± 0.9**
Month 3	0.1 ± 0.1	0.1 ± 0.1[b]	1.5 ± 0.2**	18.6 ± 1.5**
Month 12	0.1 ± 0.0	0.0 ± 0.0	1.0 ± 0.3**	6.2 ± 1.7**
Month 18	0.2 ± 0.1	0.1 ± 0.0	4.9 ± 1.0**	22.0 ± 3.6**[c]

TABLE F6
Hematology and Urinalysis Data for Mice in the 2-Year Gavage Study of Methylene Blue Trihydrate

	Vehicle Control	2.5 mg/kg	12.5 mg/kg	25 mg/kg
Female (continued)				
Urinalysis				
n	5	5	5	5
Creatinine (mg/dL)				
Month 3	44.92 ± 6.09	47.28 ± 5.81	46.42 ± 2.10	36.86 ± 3.75
Month 12	32.80 ± 5.20	42.22 ± 14.90	35.24 ± 14.54	20.50 ± 4.23
Month 18	41.68 ± 8.51	27.74 ± 3.11	25.96 ± 3.37	27.92 ± 3.28
Volume (mL)				
Month 3	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Month 12	0.7 ± 0.2	0.5 ± 0.1	1.7 ± 1.0	0.9 ± 0.1
Month 18	0.9 ± 0.3	1.5 ± 0.1	0.9 ± 0.2	1.2 ± 0.2

* 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=10
[c] n=9
[d] n=4

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 1-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg
Male				
n	10	10	10	8
Necropsy body wt	245 ± 2	233 ± 6	217 ± 4**	214 ± 6*
Heart				
Absolute	0.822 ± 0.014	0.802 ± 0.024	0.774 ± 0.020	0.844 ± 0.028
Relative	3.349 ± 0.046	3.434 ± 0.049	3.557 ± 0.033**	3.940 ± 0.081**
R. Kidney				
Absolute	1.026 ± 0.011	1.004 ± 0.026	0.960 ± 0.023	0.978 ± 0.022
Relative	4.183 ± 0.038	4.300 ± 0.042	4.418 ± 0.058**	4.571 ± 0.073**
Liver				
Absolute	11.18 ± 0.27	11.79 ± 0.38	11.19 ± 0.35	10.73 ± 0.49
Relative	45.550 ± 0.885	50.439 ± 0.766**	51.504 ± 1.285**	49.967 ± 1.182*
Lung				
Absolute	1.338 ± 0.046	1.234 ± 0.050	1.104 ± 0.065**	1.245 ± 0.042
Relative	5.453 ± 0.192	5.291 ± 0.175	5.057 ± 0.227	5.828 ± 0.196
Spleen				
Absolute	0.577 ± 0.007	0.819 ± 0.022**	1.116 ± 0.042**	1.559 ± 0.055**
Relative	2.351 ± 0.029	3.513 ± 0.080**	5.129 ± 0.150**	7.336 ± 0.365**
R. Testis				
Absolute	1.393 ± 0.013	1.301 ± 0.076	1.360 ± 0.021	1.349 ± 0.016
Relative	5.680 ± 0.073	5.587 ± 0.338	6.264 ± 0.083*	6.318 ± 0.126*
Thymus				
Absolute	0.372 ± 0.011	0.362 ± 0.014	0.279 ± 0.018**	0.282 ± 0.017**
Relative	1.516 ± 0.044	1.552 ± 0.048	1.279 ± 0.071**	1.312 ± 0.053*
Female				
n	10	10	10	6
Necropsy body wt	153 ± 2	149 ± 2	145 ± 2*	146 ± 3
Heart				
Absolute	0.583 ± 0.009	0.588 ± 0.017	0.558 ± 0.011	0.607 ± 0.017
Relative	3.808 ± 0.046	3.927 ± 0.077	3.839 ± 0.045	4.147 ± 0.078**
R. Kidney				
Absolute	0.620 ± 0.012	0.635 ± 0.014	0.624 ± 0.014	0.656 ± 0.02
Relative	4.048 ± 0.051	4.245 ± 0.058*	4.295 ± 0.054**	4.476 ± 0.081**
Liver				
Absolute	6.122 ± 0.155	6.439 ± 0.108	6.464 ± 0.185	7.179 ± 0.198**
Relative	39.950 ± 0.656	43.105 ± 0.680**	44.445 ± 0.778**	49.083 ± 0.884**
Lung				
Absolute	0.977 ± 0.032	1.000 ± 0.046	0.894 ± 0.029	0.930 ± 0.042
Relative	6.386 ± 0.217	6.675 ± 0.255	6.145 ± 0.139	6.346 ± 0.178
Spleen				
Absolute	0.399 ± 0.007	0.565 ± 0.012**	0.802 ± 0.021**	1.300 ± 0.072*
Relative	2.605 ± 0.032	3.781 ± 0.069**	5.522 ± 0.133**	8.879 ± 0.430**
Thymus				
Absolute	0.338 ± 0.008	0.306 ± 0.007*	0.278 ± 0.009**	0.271 ± 0.017**
Relative	2.206 ± 0.042	2.048 ± 0.049*	1.911 ± 0.052**	1.852 ± 0.105

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test
** $P \leq 0.01$
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data. No data presented for the 1,000 and 2,000 mg/kg groups due to 100% mortality.

TABLE G2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
n	10	9	10	10	10
Necropsy body wt	340 ± 4	348 ± 6	332 ± 7	326 ± 7	320 ± 6*
Heart					
Absolute	1.057 ± 0.025	1.027 ± 0.020	1.028 ± 0.015	1.083 ± 0.026	1.056 ± 0.031
Relative	3.109 ± 0.072	2.958 ± 0.045	3.105 ± 0.080	3.328 ± 0.040*	3.302 ± 0.062*
R. Kidney					
Absolute	1.231 ± 0.021	1.257 ± 0.030	1.217 ± 0.034	1.262 ± 0.035	1.232 ± 0.027
Relative	3.618 ± 0.038	3.617 ± 0.068	3.665 ± 0.088	3.874 ± 0.048**	3.857 ± 0.058**
Liver					
Absolute	12.89 ± 0.25	13.54 ± 0.41	13.75 ± 0.48	13.27 ± 0.37	14.10 ± 0.41
Relative	37.890 ± 0.558	38.954 ± 0.963	41.332 ± 0.958**	40.724 ± 0.520**	44.117 ± 0.872**
Lung					
Absolute	1.736 ± 0.099	1.552 ± 0.063	1.422 ± 0.049**	1.461 ± 0.051*	1.419 ± 0.048**
Relative	5.115 ± 0.308	4.468 ± 0.168	4.287 ± 0.138*	4.495 ± 0.141	4.438 ± 0.109*
Spleen					
Absolute	0.731 ± 0.010	0.834 ± 0.017	0.900 ± 0.018**	1.071 ± 0.032**	1.483 ± 0.071**
Relative	2.152 ± 0.037	2.400 ± 0.034	2.710 ± 0.037**	3.288 ± 0.054**	4.642 ± 0.204**
R. Testis					
Absolute	1.402 ± 0.024	1.423 ± 0.027	1.446 ± 0.018	1.426 ± 0.026	1.393 ± 0.034
Relative	4.121 ± 0.037	4.098 ± 0.063	4.363 ± 0.091**	4.387 ± 0.056**	4.359 ± 0.057**
Thymus					
Absolute	0.342 ± 0.015	0.312 ± 0.010	0.273 ± 0.014**	0.249 ± 0.008**	0.283 ± 0.015**
Relative	1.005 ± 0.044	0.898 ± 0.022	0.820 ± 0.030**	0.768 ± 0.029**	0.892 ± 0.053**
Female					
n	10	10	10	9	6
Necropsy body wt	201 ± 3	201 ± 4	198 ± 3	197 ± 3	201 ± 4
Heart					
Absolute	0.715 ± 0.012	0.719 ± 0.023	0.715 ± 0.025	0.710 ± 0.015	0.698 ± 0.013
Relative	3.570 ± 0.091	3.574 ± 0.093	3.596 ± 0.091	3.609 ± 0.079	3.473 ± 0.048
R. Kidney					
Absolute	0.738 ± 0.013	0.746 ± 0.014	0.747 ± 0.016	0.748 ± 0.013	0.787 ± 0.027
Relative	3.677 ± 0.060	3.713 ± 0.054	3.768 ± 0.064	3.802 ± 0.073	3.914 ± 0.099
Liver					
Absolute	7.119 ± 0.231	7.222 ± 0.116	7.415 ± 0.261	7.652 ± 0.157	8.107 ± 0.232**
Relative	35.390 ± 0.861	36.015 ± 0.842	37.350 ± 1.093	38.899 ± 0.857**	40.311 ± 0.656**
Lung					
Absolute	1.150 ± 0.038	1.099 ± 0.026	1.068 ± 0.024	1.162 ± 0.030	1.248 ± 0.040
Relative	5.730 ± 0.188	5.480 ± 0.164	5.389 ± 0.132	5.918 ± 0.205	6.232 ± 0.292
Spleen					
Absolute	0.487 ± 0.008[b]	0.538 ± 0.009	0.642 ± 0.012**[b]	0.769 ± 0.018**	1.080 ± 0.055**
Relative	2.432 ± 0.043[b]	2.684 ± 0.059	3.227 ± 0.049**[b]	3.912 ± 0.101**	5.371 ± 0.248**
Thymus					
Absolute	0.255 ± 0.009	0.261 ± 0.008	0.256 ± 0.012[b]	0.252 ± 0.014	0.258 ± 0.013
Relative	1.270 ± 0.040	1.301 ± 0.051	1.289 ± 0.060[b]	1.285 ± 0.081	1.283 ± 0.056

* Significantly different ($P \le 0.05$) from the vehicle control group by Williams' or Dunnett's test
** $P \le 0.01$
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=9

TABLE G3
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 1-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	125 mg/kg	250 mg/kg
n	10	10	8
Male			
Necropsy body wt	29.8 ± 0.4	29.5 ± 0.2	27.9 ± 0.8*
Heart			
Absolute	0.167 ± 0.005	0.166 ± 0.004	0.186 ± 0.008
Relative	5.593 ± 0.150	5.637 ± 0.136	6.670 ± 0.268**
R. Kidney			
Absolute	0.312 ± 0.008	0.313 ± 0.007	0.290 ± 0.012
Relative	10.476 ± 0.218	10.607 ± 0.220	10.378 ± 0.333
Liver			
Absolute	1.680 ± 0.051	1.614 ± 0.023	1.533 ± 0.033*
Relative	56.304 ± 1.172	54.748 ± 0.739	55.068 ± 1.089
Lung			
Absolute	0.246 ± 0.011	0.217 ± 0.005*	0.203 ± 0.005**
Relative	8.245 ± 0.358	7.368 ± 0.168	7.289 ± 0.254*
Spleen			
Absolute	0.075 ± 0.002	0.439 ± 0.050**	0.803 ± 0.068**
Relative	2.532 ± 0.065	14.927 ± 1.715**	28.880 ± 2.522**
R. Testis			
Absolute	0.108 ± 0.004	0.103 ± 0.004	0.098 ± 0.006
Relative	3.643 ± 0.129	3.491 ± 0.126	3.497 ± 0.155
Thymus			
Absolute	0.048 ± 0.002	0.041 ± 0.003	0.031 ± 0.005**
Relative	1.625 ± 0.082	1.380 ± 0.083	1.105 ± 0.141**
Female			
Necropsy body wt	24.7 ± 0.4	24.7 ± 0.3	23.5 ± 0.5
Heart			
Absolute	0.137 ± 0.003	0.151 ± 0.005*	0.160 ± 0.004**
Relative	5.552 ± 0.071	6.090 ± 0.178*	6.808 ± 0.207**
R. Kidney			
Absolute	0.200 ± 0.003	0.206 ± 0.004	0.218 ± 0.006**
Relative	8.101 ± 0.116	8.326 ± 0.091	9.312 ± 0.240**
Liver			
Absolute	1.348 ± 0.028	1.330 ± 0.033	1.292 ± 0.043
Relative	54.595 ± 0.684	53.743 ± 0.988	54.877 ± 0.876
Lung			
Absolute	0.228 ± 0.009	0.218 ± 0.005	0.196 ± 0.007*
Relative	9.242 ± 0.406	8.823 ± 0.215	8.348 ± 0.333
Spleen			
Absolute	0.093 ± 0.002	0.352 ± 0.021**	0.904 ± 0.066**
Relative	3.786 ± 0.072	14.273 ± 0.939**	38.361 ± 2.450**
Thymus			
Absolute	0.067 ± 0.001	0.058 ± 0.002	0.043 ± 0.006**
Relative	2.717 ± 0.067	2.340 ± 0.101*	1.817 ± 0.220**

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test
** $P \leq 0.01$
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data. No data presented for the 500, 1,000 and 2,000 mg/kg groups due to 100% mortality.

TABLE G4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male					
n	10	10	10	10	9
Necropsy body wt	36.8 ± 0.9	37.4 ± 1.1	37.5 ± 1.3	34.9 ± 0.7	33.1 ± 0.5*
Heart					
Absolute	0.149 ± 0.004	0.153 ± 0.004	0.152 ± 0.004	0.159 ± 00.005	0.175 ± 0.006**
Relative	4.078 ± 0.146	4.105 ± 0.137	4.085 ± 0.124	4.560 ± 0.090*	5.298 ± 0.208**
R. Kidney					
Absolute	0.272 ± 0.006	0.269 ± 0.008	0.269 ± 0.006	0.262 ± 0.008	0.266 ± 0.004
Relative	7.415 ± 0.212	7.195 ± 0.078	7.202 ± 0.173	7.515 ± 0.193	8.046 ± 0.119*
Liver					
Absolute	1.545 ± 0.045	1.523 ± 0.066	1.525 ± 0.056	1.426 ± 0.041	1.485 ± 0.036
Relative	42.043 ± 0.893	40.610 ± 0.996	40.711 ± 0.726	40.967 ± 1.054	44.918 ± 0.790
Lung					
Absolute	0.193 ± 0.007	0.183 ± 0.006	0.194 ± 0.007	0.188 ± 0.005	0.189 ± 0.004
Relative	5.281 ± 0.206	4.924 ± 0.219	5.243 ± 0.298	5.430 ± 0.224	5.719 ± 0.103
Spleen					
Absolute	0.064 ± 0.002	0.092 ± 0.004	0.139 ± 0.006*	0.274 ± 0.017**	0.595 ± 0.051**
Relative	1.741 ± 0.049	2.467 ± 0.077	3.711 ± 0.125	7.822 ± 0.409**	18.114 ± 1.769**
R. Testis					
Absolute	0.114 ± 0.003	0.117 ± 0.002	0.122 ± 0.003	0.117 ± 0.002	0.113 ± 0.002
Relative	3.116 ± 0.119	3.139 ± 0.068	3.265 ± 0.088	3.362 ± 0.084	3.424 ± 0.070
Thymus					
Absolute	0.046 ± 0.004	0.048 ± 0.003	0.053 ± 0.004	0.042 ± 0.003	0.039 ± 0.003
Relative	1.233 ± 0.083	1.289 ± 0.064	1.407 ± 0.082	1.217 ± 0.068	1.168 ± 0.079
Female					
n	10	9	10	10	10
Necropsy body wt	29.1 ± 0.9	29.9 ± 1.1	29.4 ± 0.8	28.1 ± 0.7	26.6 ± 0.5*
Heart					
Absolute	0.136 ± 0.004	0.133 ± 0.003	0.128 ± 0.004	0.136 ± 0.002	0.138 ± 0.003
Relative	4.691 ± 0.164	4.479 ± 0.148	4.352 ± 0.126	4.878 ± 0.137	5.193 ± 0.154
R. Kidney					
Absolute	0.175 ± 0.005	0.180 ± 0.006	0.177 ± 0.004	0.185 ± 0.003	0.169 ± 0.004
Relative	6.047 ± 0.159	6.054 ± 0.192	6.047 ± 0.153	6.610 ± 0.157*	6.362 ± 0.105
Liver					
Absolute	1.277 ± 0.052	1.317 ± 0.046	1.166 ± 0.037	1.253 ± 0.042	1.138 ± 0.027
Relative	43.912 ± 1.216	44.302 ± 1.543	39.673 ± 0.635*	44.754 ± 1.477	42.776 ± 0.733
Lung					
Absolute	0.201 ± 0.010	0.224 ± 0.014	0.212 ± 0.010	0.221 ± 0.009	0.194 ± 0.005
Relative	6.943 ± 0.312	7.591 ± 0.600	7.214 ± 0.263	7.932 ± 0.414	7.308 ± 0.213
Spleen					
Absolute	0.095 ± 0.004	0.113 ± 0.007	0.173 ± 0.009**	0.300 ± 0.019**	0.640 ± 0.030**
Relative	3.268 ± 0.097	3.783 ± 0.216	5.861 ± 0.193**	10.672 ± 0.542**	24.042 ± 0.958**
Thymus					
Absolute	0.063 ± 0.005	0.055 ± 0.004	0.053 ± 0.003*	0.046 ± 0.004**	0.041 ± 0.002**
Relative	2.168 ± 0.149	1.845 ± 0.124*	1.788 ± 0.068*	1.625 ± 0.106**	1.546 ± 0.053**

* Significantly different ($P \le 0.05$) from the vehicle control group by Williams' or Dunnett's test
** $P \le 0.01$
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.

APPENDIX H
REPRODUCTIVE TISSUE EVALUATIONS AND ESTROUS CYCLE CHARACTERIZATION

TABLE H1
Summary of Reproductive Tissue Evaluations for Male Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg
n	10	10	10	10
Weights (g)				
Necropsy body wt	340 ± 4	332 ± 7	326 ± 7	320 ± 6
L. Cauda epididymis	0.1633 ± 0.0045	0.1667 ± 0.0060	0.1537 ± 0.0054	0.1478 ± 0.0061
L. Epididymis	0.4612 ± 0.0093	0.4731 ± 0.0079	0.4343 ± 0.0215	0.4310 ± 0.0084
L. Testis	1.4859 ± 0.0262	1.5354 ± 0.0180	1.5051 ± 0.0280	1.4702 ± 0.0298
Spermatid measurements				
Spermatid heads (10^7/g testis)	9.47 ± 0.20	8.88 ± 0.20	9.30 ± 0.23	9.24 ± 0.38
Spermatid heads (10^7/testis)	14.07 ± 0.37	13.63 ± 0.35	14.21 ± 0.22	13.57 ± 0.57
Spermatid count (mean/10^{-4} mL suspension)	70.33 ± 1.83	68.15 ± 1.77	71.03 ± 1.10	67.85 ± 2.86
Epididymal spermatozoal measurements				
Motility (%)	68.72 ± 2.11	68.07 ± 0.88	68.01 ± 0.99	61.24 ± 3.60
Concentration (10^6/g cauda epididymal tissue)	317 ± 33	458 ± 21*	490 ± 119	296 ± 34

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunn's test
[a] Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (body and tissue weights) or Dunn's test (spermatid measurements and epididymal spermatozoal motility).

TABLE H2
Estrous Cycle Characterization for Female Rats in the 3-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg
n	10	10	9	10[b]
Necropsy body wt (g)	201 ± 3	198 ± 3	197 ± 3	200 ± 3
Estrous cycle length (days)	4.20 ± 0.11	4.35 ± 0.15	4.60 ± 0.26	4.40 ± 0.10
Estrous stages (% of cycle)				
Diestrus	27.5	31.7	34.2	35.8
Proestrus	23.3	20.0	20.8	20.0
Estrus	25.0	25.0	25.8	22.5
Metestrus	24.2	23.3	19.2	21.7

[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] Includes six core study and four clinical pathology study rats.

TABLE H3
Summary of Reproductive Tissue Evaluations for Male Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg
n	10	10	10	9
Weights (g)				
Necropsy body wt	36.8 ± 0.9	37.5 ± 1.3	34.9 ± 0.7	33.1 ± 0.5**
L. Cauda epididymis	0.0167 ± 0.0009	0.0180 ± 0.0006	0.0159 ± 0.0010	0.0158 ± 0.0005
L. Epididymis	0.0451 ± 0.0017	0.0490± 0.0014	0.0441 ± 0.0011	0.0425 ± 0.0009
L. Testis	0.1182 ± 0.0031	0.1285 ± 0.0032*	0.1221 ± 0.0021	0.1207 ± 0.0021
Spermatid measurements				
Spermatid heads (10^7/g testis)	15.99 ± 0.43	16.15 ± 0.46	16.19 ± 0.35	15.62 ± 0.59
Spermatid heads (10^7/testis)	1.88 ± 0.04	2.07 ± 0.07	1.98 ± 0.06	1.88 ± 0.07
Spermatid count (mean/10^{-4} mL suspension)	58.80 ± 1.11	64.68 ± 2.03	61.78 ± 1.73	58.81 ± 2.14
Epididymal spermatozoal measurements				
Motility (%)	68.70 ± 1.20	67.96 ± 1.15	65.94 ± 1.86	64.06 ± 1.60*
Concentration (10^6/g cauda epididymal tissue)	546 ± 78	588 ± 63	703 ± 63	834 ± 106*

* Significantly different ($P \le 0.05$) from the vehicle control group by Dunnett's test (left testis weight) or Shirley's test (epididymal spermatozoal measurements).
** Significantly different ($P \le 0.01$) from the vehicle control group by Williams' test.
[a] Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test (left caudal epididymal and epididymal weights) or Dunn's test (spermatid measurements).

TABLE H4
Estrous Cycle Characterization for Female Mice in the 3-Month Gavage Study of Methylene Blue Trihydrate[a]

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg
n	10	10	10	10
Necropsy body wt (g)	29.1 ± 0.9	29.4 ± 0.8	28.1 ± 0.7	26.6 ± 0.5*
Estrous cycle length (days)	4.20 ± 0.11	4.30 ± 0.13	4.61 ± 0.45[b]	4.63 ± 0.16[c]
Estrous stages (% of cycle)				
Diestrus	33.3	26.7	29.2	33.3
Proestrus	16.7	15.8	20.8	19.2
Estrus	27.5	35.8	30.0	29.2
Metestrus	22.5	21.7	20.0	18.3

* Significantly different ($P \le 0.05$) from the vehicle control group by Williams' test.
[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 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.
[c] Estrous cycle was longer than 12 days or unclear in 2 of 10 animals.

APPENDIX I
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION

Methylene Blue Trihydrate

Methylene blue trihydrate was obtained from Aldrich Chemical Company (Milwaukee, WI) in two lots (PY01917JX and 10306AF) and Sigma Chemical Co. (St. Louis, MO) in one lot (68H3728). Lot PY01917JX was used in the 1-month studies; lot 10306AF was used in the 3-month studies, and lot 68H3728 was used in the 2-year studies. Identity and purity analyses were conducted by the study laboratories, Battelle Columbus Operations (Columbus, OH; lots PY01917JX and 10306AF) and Southern Research Institute (Birmingham, AL; lot 68H3728), and by the analytical chemistry laboratory, Research Triangle Institute (Research Triangle Park, NC; lot 68H3728). Galbraith Laboratories, Inc. (Knoxville, TN), conducted melting point determination, elemental analyses, and Karl Fischer titration (lot 68H3728). Reports on analyses performed in support of the studies on methylene blue trihydrate are on file at the National Institute of Environmental Health Sciences.

Lots PY01917JX and 10306AF of methylene blue trihydrate, a green crystalline powder, were identified by the study laboratory using infrared (IR) spectroscopy. Lot 68H3728 was identified by the analytical chemistry laboratory and the study laboratory using IR and proton nuclear magnetic resonance spectroscopy (NMR). IR spectra were consistent with the literature spectra (*Aldrich*, 1981a,b) of methylene blue trihydrate, and IR and NMR spectra obtained from lot 68H3728 were consistent with spectra of a reference sample (different lot of methylene blue trihydrate) obtained from Aldrich Chemical Co. and a reference sample from the same lot. Representative IR and NMR spectra are presented in Figures I1 and I2.

The purities of lots PY01917JX and 10306AF were accepted as determined by the manufacturer using titration, melting point determination, elemental analyses, and ultraviolet/visible (UV/Vis) chromatography (200-700 nm). The purity of lot 10306AF was determined by the study laboratory using high-performance liquid chromatography (HPLC) by system A (Table I1). The purity of lot 68H3728 was determined by the analytical chemistry laboratory using elemental analysis, Karl Fischer titration, melting point determination, UV/Vis (200-750 nm) chromatography, and HPLC by systems B and C and by the study laboratory using HPLC by system D. The analytical chemistry laboratory performed additional analyses using HPLC/mass spectrometry (MS) by system E to identify the major impurity detected by HPLC.

For lot PY01917JX, elemental analysis showed good agreement between theoretical and observed percentages by weight for carbon (49.31%), hydrogen (4.71%), and nitrogen (10.73%); water content was 16.1%, 1.7% above theoretical; and the melting point was 192° C. The UV/Vis spectrum was consistent with the structure of methylene blue trihydrate.

For lot 10306AF, elemental analysis showed good agreement between theoretical and observed percentages by weight for carbon (49.31%), hydrogen (4.71%), and nitrogen (10.73%); water content was 16.1%, 1.7% above theoretical; and the melting point was 192° C. The UV/Vis spectrum was consistent with the structure of methylene blue trihydrate. HPLC by system A, at 290 nm, indicated one major peak and three impurities with relative peak areas of 0.16%, 0.12%, and 2.8%; at 665 nm, there was one major peak and one impurity with a relative area of 3.2%. The overall purity of lot 10306AF was determined to be greater than 95%.

For lot 68H3728, elemental analysis showed good agreement between theoretical and found percentages by weight for carbon (49.96%), hydrogen (6.73%), nitrogen (10.95%), sulfur (8.52%), and chlorine (8.97%); water content was 16.55%, 2.15% above theoretical; and the melting point was between 185° and 186° C, consistent for the chemical with water content of 16.55%. UV/Vis spectra were consistent with the structure of methylene blue

trihydrate. HPLC by system B indicated one major peak and three impurities with relative peak areas of 0.16%, 0.21%, and 6.55%. A second HPLC analysis by system C, designed to detect more impurities, indicated similar results. Additional analysis using HPLC/MS by system E was conducted by the analytical chemistry laboratory in an attempt to identify the 6.55% impurity. Interpretation of the fragmentation pattern indicated that this impurity was very similar to methylene blue trihydrate with the exception of one methyl group replaced by a proton. HPLC by system D indicated 102% relative purity compared to a reference standard from the same lot and greater than 94% purity using calculated peak areas. The overall purity of lot 68H3728 was determined to be greater than 91%. Stability studies conducted by the analytical chemistry laboratory demonstrated that the bulk chemical could be stored at room temperature (25° C).

For lots PY01917JX and 10306AF, the bulk chemical was reanalyzed at the end of each study by the study laboratory using HPLC by system F. For lot 68H3728, periodic reanalyses were conducted at least every 26 weeks and at the end of the study by the study laboratory using HPLC by system D. No degradation of the bulk chemical was observed.

Methylcellulose

For the 2-year studies, methylcellulose was obtained from Aldrich Chemical Company in two lots (11414HU and 128H0668). Identity was confirmed using IR; spectra were consistent with the structure of methylcellulose. The methoxyl content (29.6% and 32.7%, respectively) was determined by Galbraith Laboratories, Inc., according to specifications given in NTP (1992).

Preparation and Analysis of Dose Formulations

The vehicle was prepared by mixing methylcellulose with heated, deionized water with a magnetic stirrer to form a 0.5% solution, then cooled. For the 1- and 3-month studies, a slurry of the required amount of methylene blue trihydrate and 0.5% methylcellulose was prepared in a glass beaker, then filled to volume with 0.5% methylcellulose (with stirring), then mixed with an overhead stirrer for 30 minutes; for the 3-month studies, there was additional stirring with a magnetic stir bar (Table I2). For the 2-year studies, the required amount of methylene blue trihydrate was added to a volumetric flask, brought to volume with 0.5% methylcellulose, and mixed with a stir bar for at least 2 hours. The dose formulations were prepared once for the 1-month studies and every four weeks for the 3-month and 2-year studies. The dose formulations were stored in sealed amber glass bottles at room temperature (25° C) for up to 28 (1-month studies) or 35 (3-month and 2-year studies) days.

Prior to the 1-month studies, the study laboratory performed solubility, homogeneity, resuspendibility, and gavageability studies. Solubility studies demonstrated that dose formulations at concentrations up to 12.5 mg/mL were solutions (no homogeneity study necessary), and higher concentrations were classified as suspensions. Homogeneity studies of 25 and 200 mg/mL dose formulations and resuspendibility tests of a 200 mg/mL dose formulation were conducted using UV/Vis chromatography, measuring the absorbancy at 665 nm. Resuspendibility was tested after the formulation was stored in sealed glass bottles, protected from light, at room temperature for 28 days, sonicated for 15 minutes, mixed with a magnetic stir bar for 15 minutes, and analyzed. Gavageability of a 200 mg/mL dose formulation was tested using a 20 gauge needle. Stability studies of 12.5 mg/mL dose formulations were performed using HPLC by system F. Homogeneity was confirmed with the recommendation that dose formulations be stirred continuously while sampling and during administration; resuspendibility was confirmed; gavageability was confirmed; and stability was confirmed for up to 29 days for dose formulations stored in sealed glass bottles, protected from light, at 5° and 25° C and for 3 hours at simulated animal room conditions.

Prior to the 3-month studies, the study laboratory conducted homogeneity and gavageability studies of 40 mg/mL dose formulations and stability studies of 2.5 mg/mL dose formulations using HPLC by system G. Homogeneity and gavageability were confirmed, and stability was confirmed for up to 35 days for dose formulations stored in

amber glass bottles, protected from light at 5° and 25° C and for 3 hours at simulated animal room conditions.

Prior to the 2-year studies, the analytical chemistry laboratory tested the solubility, homogeneity, and stability of dose formulations. To check the solubility of methylene blue trihydrate in 0.5% methylcellulose, a 15.5 mg/mL dose formulation was visually examined after storage at 5° C for 24 hours. Homogeneity and stability studies of 0.10 mg/mL dose formulations were performed using HPLC by system H. The study laboratory conducted homogeneity studies of 0.25 and 10 mg/mL dose formulations using HPLC by system D. Solubility and homogeneity were confirmed, and stability was confirmed for up to 35 days for dose formulations stored in amber glass containers, sealed with Teflon®-lined lids and protected from light at –20°, 5°, and 22° C and for up to 3 hours at simulated animal room conditions.

Periodic analyses of the dose formulations of methylene blue trihydrate in 0.5% methylcellulose were conducted at the study laboratories. Dose formulations were analyzed once for the 1-month studies using HPLC by system D; animal room samples were also analyzed. Four of five dose formulations analyzed were within 10% of the target concentrations; all five animal room samples were within 10% of target concentrations (Table I3). Dose formulations were analyzed twice for the 3-month studies using HPLC by system G; animal room samples were also analyzed. All 10 dose formulations analyzed were within 10% of the target concentrations; 10 of 16 rat animal room samples and all eight mouse animal room samples were within 10% of the target concentrations (Table I4). Dose formulations were analyzed every 3 months for the 2-year studies using HPLC by system D; animal room samples were also analyzed. All 33 dose formulations analyzed were within 10% of the target concentrations; all 12 mouse animal room samples and all 12 rat animal room samples were within 10% of target concentrations (Table I5).

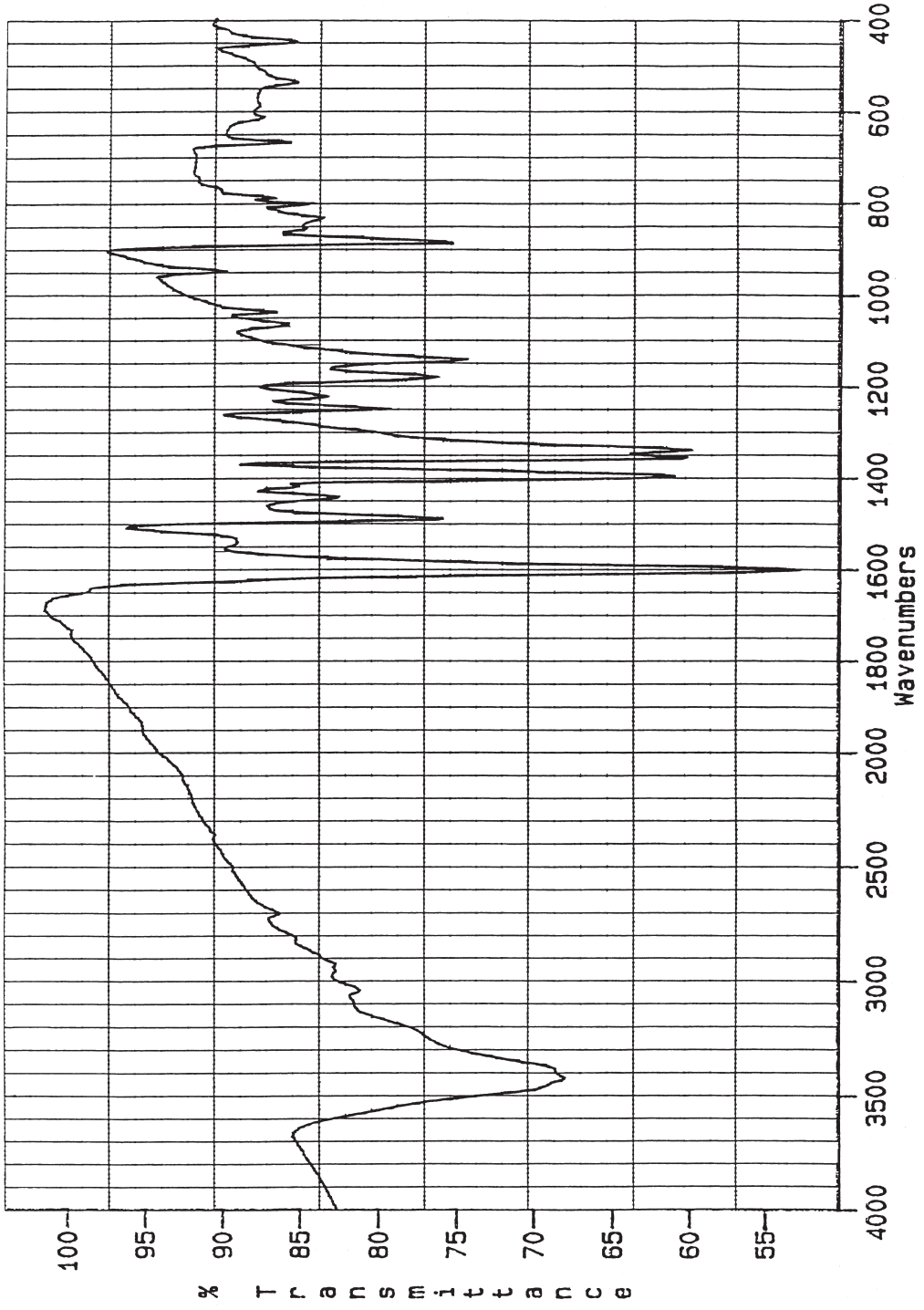

FIGURE I1
Infrared Spectrum of Methylene Blue Trihydrate

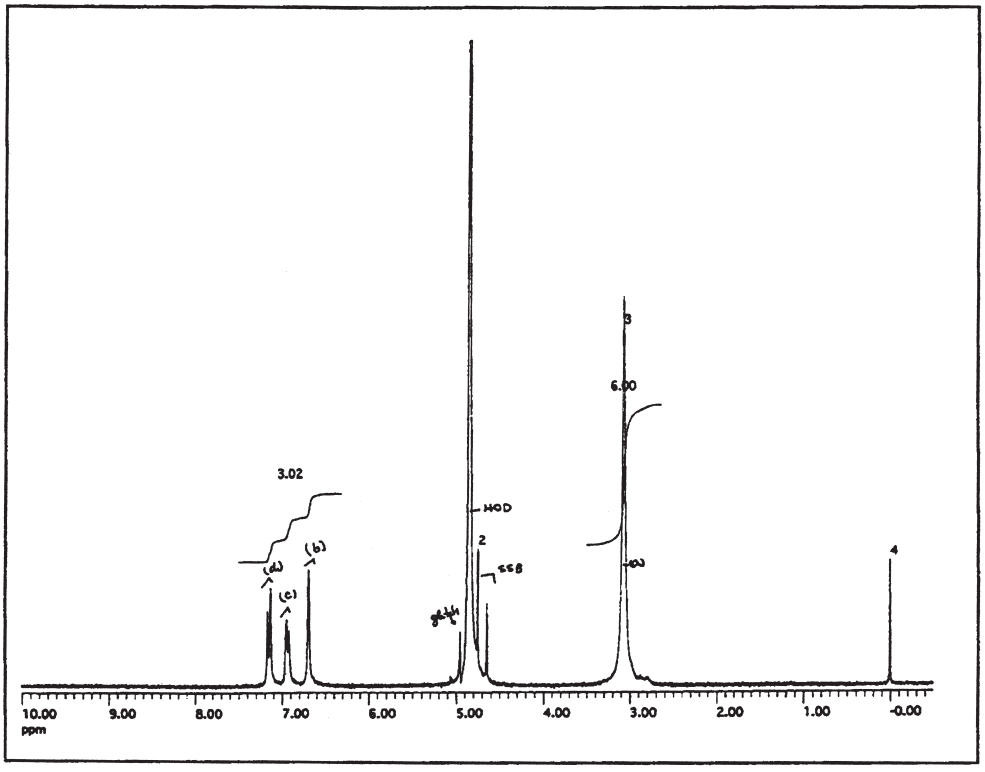

FIGURE I2
Proton Nuclear Magnetic Resonance Spectrum of Methylene Blue Trihydrate

TABLE I1
High-Performance Liquid Chromatography Systems Used in the Gavage Studies of Methylene Blue Trihydrate

Detection System	Column	Solvent System
System A		
Ultraviolet (290 nm) light Visible (665 nm) light	Meta-Chem ODS-2 (150 mm × 4.6 mm), 5-µm particle size	A) 75% 0.01 M ammonium acetate (pH 3): 25% acetonitrile and B) acetonitrile; isocratic 100% A for 6 minutes, then linear to 95% B in 25 minutes, then linear to 100% B, held 5 minutes; flow rate of 1.0 mL/minute
System B		
Visible (600 nm) light	Zorbax XDB-C8 (25 cm × 4.6 mm), 5-µm particle size (Agilent Technologies, Palo Alto, CA)	A) 0.1% trifluoroacetic acid (pH 4, adjusted with triethylamine) and B) acetonitrile; 70% A:30% B for 5 minutes, then linear to 10% A:90% B in 25 minutes, held 10 minutes; flow rate of 1.0 mL/minute
System C		
Visible (600 nm) light	Waters Nova-Pak CN HP, 4-µm particle size (Waters Corporation, Milford, MA)	A) 0.1% trifluoroacetic acid (pH 4, adjusted with triethylamine) and B) acetonitrile; 90% A:10% B for 5 minutes, then linear to 10% A:90% B in 25 minutes, held for 10 minutes; flow rate of 1.0 mL/minute
System D		
Visible (600 nm) light	Zorbax XDB-C8 (250 mm × 4.6 mm), 5-µm particle size (Agilent Technologies)	Isocratic 40% acetonitrile:60% 0.1% trifluoroacetic acid (pH 4, adjusted with triethylamine); flow rate of 0.8 mL/minute
System E		
Mass spectrometry	Zorbax XDB-C8 (25 cm × 4.6 mm), 5-µm particle size (Agilent Technologies)	A) 0.1% trifluoroacetic acid (pH 4, adjusted with triethylamine) and B) acetonitrile; 70% A:30% B for 5 minutes, then linear to 10% A:90% B in 25 minutes, held 10 minutes; flow rate of 1.0 mL/minute
System F		
Ultraviolet (292 nm) light	Hamilton PRP-1 (30.5 cm × 7 mm) (Hamilton Company)	A) 0.1 M tetrabutylammonium hydroxide (pH 2.5, adjusted with concentrated phosphoric acid) and B) 20% 0.5 M tetrabutylammonium hydroxide (pH 2.5, adjusted with concentrated phosphoric acid): 80% acetonitrile; 30% A:70% B; flow rate of 2 mL/minute
System G		
Ultraviolet (290 nm) light	Meta-Chem ODS-2 (150 mm × 4.6 mm)	A) 0.01 M or 0.5 M ammonium acetate (pH 3) and B) acetonitrile; isocratic (premixed); 75% A:25% B; flow rate of 1 mL/minute
System H		
Visible (600 nm) light	Zorbax XDB-C8 (250 mm × 4.6 mm), 5-µm particle size (Agilent Technologies)	45% 0.1% trifluoroacetic acid (pH 4, adjusted with triethylamine):55% acetonitrile; flow rate of 1.0 mL/minute

TABLE I2
Preparation and Storage of Dose Formulations in the Gavage Studies of Methylene Blue Trihydrate

1-Month Studies	3-Month Studies	2-Year Studies
Preparation		
The vehicle was prepared by mixing methylcellulose and heated, deionized water with a magnetic stirrer to form a 0.5% solution, then cooled. A slurry of the required amount of methylene blue trihydrate and 0.5% methylcellulose was prepared in a glass beaker, filled to volume with 0.5% methylcellulose (with stirring), then mixed with an overhead stirrer for 30 minutes. The dose formulations were prepared once.	The vehicle was prepared by mixing methylcellulose and heated, deionized water with a magnetic stirrer to form a 0.5% solution, then cooled. A slurry of the required amount of methylene blue trihydrate and 0.5% methylcellulose was prepared in a glass beaker, filled to volume with 0.5% methylcellulose (with stirring), then mixed with an overhead stirrer for 30 minutes, then stirred with a magnetic bar until foam subsided. The dose formulations were prepared every 4 weeks.	The vehicle was prepared by mixing methylcellulose and heated, deionized water with a magnetic stirrer to form a 0.5% solution, then cooled. The required amount of methylene blue trihydrate was added to a volumetric flask, brought to volume with 0.5% methylcellulose, and stirred with a stir bar for at least 2 hours. The dose formulations were prepared every 4 weeks or as needed.
Chemical Lot Number		
PY01917JX	10306AF	68H3728
Maximum Storage Time		
29 days	35 days	35 days
Storage Conditions		
Stored in sealed amber glass bottles, protected from light, at room temperature (25° C)	Stored in amber glass bottles with Teflon®-lined lids, protected from light, at room temperature (25° C)	Stored in amber glass bottles with Teflon®-lined lids, protected from light, at room temperature (25° C)
Study Laboratory		
Battelle Columbus Operations (Columbus, OH)	Battelle Columbus Operations (Columbus, OH)	Southern Research Institute (Birmingham, AL)

TABLE I3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 1-Month Gavage Studies of Methylene Blue Trihydrate

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
April 28, 1992	April 30, 1992	12.5	12.6	+1
		25	25.9	+4
		50	50.6	+1
		100	100.2	0
		200	226.9	+13[b]
	June 18, 1992[c]	12.5	12.8	+2
		25	25.8	+3
		50	53.5	+7
		100	105.0	+5
		200	218.0	+9

[a] Dosing volume=10 mL/kg; 12.5 mg/mL=125 mg/kg, 25 mg/mL=250 mg/kg, 50 mg/mL=500 mg/kg, 100 mg/mL=1,000 mg/kg, 200 mg/mL=2,000 mg/kg
[b] Formulation was outside the acceptable range of ± 10% of target concentration; NTP approved the use of the formulation.
[c] Animal room samples

TABLE I4
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 3-Month Gavage Studies of Methylene Blue Trihydrate

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
September 28, 1993	September 29-30, 1993	2.5	2.55	+2
		5	5.13	+3
		10	10.2	+2
		20	20.6	+3
		40	40.7	+2
	November 12-13, 1993[b]	5	7.11	+42
		10	11.5	+15
		20	20.0	0
		40	47.9	+20
	November 12-13, 1993[c]	2.5	2.40	–4
		5	5.11	+2
		10	9.63	–4
		20	21.3	+7
	November 23, 1993[d]	5	4.44	–11
		10	8.62	–14
		20	17.7	–12
		40	36.2	–10
	November 23, 1993[d]	5	4.97	–1
		10	9.99	0
		20	19.6	–2
		40	42.0	+5
December 20, 1993	December 21-22, 1993	2.5	2.64	+6
		5	5.03	+1
		10	10.5	+5
		20	20.0	0
		40	40.4	+1
	January 14, 1993[d]	5	4.82	–4
		10	10.1	+1
		20	19.1	–5
		40	40.3	+1
	January 14, 1993[c]	2.5	2.53	+1
		5	4.90	–2
		10	9.95	–1
		20	19.6	–2

[a] For rats, dosing volume=5 mL/kg; 5 mg/mL=25 mg/kg, 10 mg/mL=50 mg/kg, 20 mg/mL=100 mg/kg, 40 mg/mL=200 mg/kg; for mice, dosing volume =10 mL/kg; 2.5 mg/mL=25 mg/kg, 5 mg/mL=50 mg/kg, 10 mg/mL=100 mg/kg, 20 mg/mL=200 mg/kg
[b] Rat animal room samples
[c] Mouse animal room samples
[d] Rat animal room samples taken after more vigorous resuspension procedures were introduced after November 17, 1993

TABLE I5
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Gavage Studies of Methylene Blue Trihydrate

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
Rats				
June 19, 2000	June 20, 2000	1	0.985 ± 0.039	–2
		5	5.2 ± 0.21	+4
		10	10.0 ± 0.2	0
	July 24-25, 2000[b]	1	0.961 ± 0.0026	–4
		5	5.08 ± 0.0037	+2
		10	10.2 ± 0.37	+2
September 11, 2000	September 12, 2000	1	0.991 ± 0.030	–1
		5	5.04 ± 0.10	+1
		10	10.0 ± 0.024	0
December 4-5, 2000	December 6, 2000	1	0.992 ± 0.019	–1
		5	5.02 ± 0.13	0
		10	9.92 ± 0.22	–1
January 29, 2001	January 30, 2001	1	0.988 ± 0.0085	–1
		5	5.01 ± 0.043	0
		10	10.0 ± 0.67	0
	March 5-6, 2001[b]	1	0.989 ± 0.0048	–1
		5	5.11 ± 0.030	+2
		10	9.54 ± 0.17	–5
April 23, 2001	April 24, 2001	1	0.938 ± 0.067	–6
		5	5.04 ± 0.050	+1
		10	9.85 ± 0.16	–2
June 18, 2001	June 19, 2001	1	1.04 ± 0.017	+4
		5	5.07 ± 0.080	+1
		10	9.99 ± 0.35	0
September 10, 2001	September 11, 2001	1	0.981 ± 0.013	–2
		5	5.00 ± 0.016	0
		10	9.90 ± 0.011	–1
	October 15-16, 2001[b]	1	1.02 ± 0.0092	+2
		5	5.05 ± 0.00049	+1
		10	10.1 ± 0.14	+1
November 5, 2001	November 6-7, 2001	1	0.986 ± 0.017	–1
		5	4.87 ± 0.078	–3
		10	9.40 ± 0.86	–6
January 28, 2002	January 29, 2002	1	0.961 ± 0.023	–4
		5	4.92 ± 0.031	–2
		10	9.76 ± 0.24	–2

TABLE I5
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Gavage Studies of Methylene Blue Trihydrate

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
Rats (continued)				
March 25, 2002	March 26, 2002	1	0.950 ± 0.033	–5
		5	5.05 ± 0.069	+1
		10	9.93 ± 0.34	–1
March 25, 2002	April 29, 2002[b]	1	0.918 ± 0.021	–8
		5	5.31 ± 0.18	+6
		10	9.84 ± 0.16	–2
June 17, 2002	June 18-19, 2002	1	0.948 ± 0.037	–5
		5	4.83 ± 0.000035	–3
		10	9.82 ± 0.010	–2
Mice				
June 19, 2000	June 20, 2000	0.25	0.240 ± 0.010	–4
		1.25	1.24 ± 0.0067	–1
		2.5	2.45 ± 0.084	–2
	July 24-25, 2000[b]	0.25	0.232 ± 0.0023	–7
		1.25	1.23 ± 0.051	–2
		2.5	2.51 ± 0.065	0
September 11, 2000	September 12, 2000	0.25	0.25 ± 0.0022	0
		1.25	1.24 ± 0.014	–1
		2.5	2.50 ± 0.033	0
December 4-5, 2000	December 6, 2000	0.25	0.270 ± 0.00082	+8
		1.25	1.27 ± 0.016	+2
		2.5	2.63 ± 0.046	+5
January 29, 2001	January 30, 2001	0.25	0.261 ± 0.0023	+4
		1.25	1.26 ± 0.019	+1
		2.5	2.52 ± 0.012	+1
	March 5-6, 2001[b]	0.25	0.264 ± 0.0013	+6
		1.25	1.25 ± 0.030	0
		2.5	2.39 ± 0.023	–4
April 23, 2001	April 24, 2001	0.25	0.261 ± 0.003	+4
		1.25	1.23 ± 0.0099	–2
		2.5	2.52 ± 0.083	+1
June 18, 2001	June 19, 2001	0.25	0.271 ± 0.0029	+8
		1.25	1.27 ± 0.013	+2
		2.5	2.51 ± 0.027	0

TABLE I5
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Gavage Studies of Methylene Blue Trihydrate

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
Mice (continued)				
September 10, 2001	September 11, 2001	0.25	0.272 ± 0.005	+8
		1.25	1.29 ± 0.0033	+3
		2.5	2.53 ± 0.049	+1
	October 15-16, 2001[b]	0.25	0.267 ± 0.0014	+7
		1.25	1.30 ± 0.051	+4
		2.5	2.55 ± 0.028	+2
November 5, 2001	November 6-7, 2001	0.25	0.254 ± 0.00089	+2
		1.25	1.25 ± 0.0029	0
		2.50	2.46 ± 0.020	–2
January 28, 2002	January 29, 2002	0.25	0.264 ± 0.0013	+6
		1.25	1.22 ± 0.019	–2
		2.5	2.55 ± 0.037	+2
March 25, 2002	March 26, 2002	0.25	0.251 ± 0.0051	+0
		1.25	1.27 ± 0.0031	+2
		2.5	2.45 ± 0.073	–2
	April 29, 2002[b]	0.25	0.249 ± 0.00019	0
		1.25	1.28 ± 0.014	+2
		2.5	2.58 ± 0.091	+3
June 17, 2002	June 18-19, 2002	0.25	0.238 ± 0.0054	–5
		1.25	1.24 ± 0.022	–1
		2.5	2.56 ± 0.013	+2

[a] Results of duplicate analyses (mean ± standard deviation). For rats, dosing volume=5 mL/kg; 1 mg/mL=5 mg/kg, 5 mg/mL=25 mg/kg, 10 mg/mL=50 mg/kg; for mice, dosing volume =10 mL/kg; 0.25 mg/mL=2.5mg/kg, 1.25 mg/mL=12.5 mg/kg, 2.5 mg/mL=25 mg/kg
[b] Animal room samples

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

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
dl-α-Tocopheryl acetate	100 IU	
Niacin	23 mg	
Folic acid	1.1 mg	
d-Pantothenic acid	10 mg	*d*-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	*d*-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)	14.2 ± 0.64	15.7 – 13.3	24
Crude fat (% by weight)	8.1 ± 0.29	7.6 – 8.6	24
Crude fiber (% by weight)	9.1 ± 0.57	8.0 – 10.5	24
Ash (% by weight)	5.2 ± 0.25	4.8 – 5.8	24
Amino Acids (% of total diet)			
Arginine	0.748 ± 0.049	0.670 – 0.850	14
Cystine	0.224 ± 0.025	0.150 – 0.250	14
Glycine	0.702 ± 0.040	0.620 – 0.750	14
Histidine	0.368 ± 0.093	0.310 – 0.680	14
Isoleucine	0.534 ± 0.039	0.430 – 0.590	14
Leucine	1.079 ± 0.061	0.960 – 1.150	14
Lysine	0.704 ± 0.130	0.310 – 0.830	14
Methionine	0.400 ± 0.051	0.260 – 0.460	14
Phenylalanine	0.613 ± 0.036	0.540 – 0.660	14
Threonine	0.491 ± 0.041	0.430 – 0.590	14
Tryptophan	0.134 ± 0.018	0.110 – 0.160	14
Tyrosine	0.377 ± 0.050	0.280 – 0.460	14
Valine	0.658 ± 0.045	0.550 – 0.710	14
Essential Fatty Acids (% of total diet)			
Linoleic	3.89 ± 0.262	3.49 – 4.54	14
Linolenic	0.30 ± 0.036	0.21 – 0.35	14
Vitamins			
Vitamin A (IU/kg)	4,577 ± 701	3,060 – 5,870	24
Vitamin D (IU/kg)	1,000[a]		
ὰ-Tocopherol (ppm)	83.6 ± 17.07	52.0 – 110.0	14
Thiamine (ppm)[b]	7.1 ± 0.88	6.0 – 8.8	24
Riboflavin (ppm)	6.7 ± 2.17	4.20 – 11.20	14
Niacin (ppm)	79.3 ± 10.85	66.4 – 98.2	14
Pantothenic acid (ppm)	23.5 ± 3.49	17.4 – 29.1	14
Pyridoxine (ppm)[b]	9.24 ± 2.28	6.4 – 13.7	14
Folic acid (ppm)	1.76 ± 0.55	1.20 – 3.27	14
Biotin (ppm)	0.333 ± 0.12	0.225 – 0.704	14
Vitamin B_{12} (ppb)	62.8 ± 47.3	18.3 – 174.0	14
Choline (ppm)[b]	3,066 ± 280	2,700 – 3,790	14
Minerals			
Calcium (%)	1.041 ± 0.044	0.964 – 1.140	24
Phosphorus (%)	0.610 ± 0.035	0.552 – 0.701	24
Potassium (%)	0.667 ± 0.021	0.627 – 0.694	14
Chloride (%)	0.377 ± 0.042	0.300 – 0.474	14
Sodium (%)	0.192 ± 0.017	0.160 – 0.222	14
Magnesium (%)	0.202 ± 0.009	0.185 – 0.217	14
Sulfur (%)	0.170 ± 0.029	0.116 – 0.209	14
Iron (ppm)	176 ± 43.9	135 – 311	14
Manganese (ppm)	54.6 ± 8.02	42.1 – 73.1	14
Zinc (ppm)	54.3 ± 9.45	43.3 – 78.5	14
Copper (ppm)	6.37 ± 1.492	3.21 – 9.92	14
Iodine (ppm)	0.516 ± 0.229	0.233 – 0.972	14
Chromium (ppm)	0.544 ± 0.124	0.330 – 0.751	13
Cobalt (ppm)	0.25 ± 0.076	0.20 – 0.47	13

[a] From formulation
[b] As hydrochloride (thiamine and pyridoxine) or chloride (choline)

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

	Mean ± Standard Deviation[b]	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.21 ± 0.019	0.17 – 0.25	24
Cadmium (ppm)	0.04 ± 0.004	0.04 – 0.06	24
Lead (ppm)	0.09 ± 0.098	0.05 – 0.54	24
Mercury (ppm)	<0.02		24
Selenium (ppm)	0.23 ± 0.055	0.14 – 0.36	24
Aflatoxins (ppb)	<5.00		24
Nitrate nitrogen (ppm)[c]	12.0 ± 3.14	6.85 – 18.8	24
Nitrite nitrogen (ppm)[c]	<0.61		24
BHA (ppm)[d]	<1.0		24
BHT (ppm)[d]	<1.0		24
Aerobic plate count (CFU/g)	14.0 ± 13	10.0 – 70.0	24
Coliform (MPN/g)	3.2 ± 0.7	0.0 – 3.6	24
Escherichia coli (MPN/g)	<10		24
Salmonella (MPN/g)	Negative		24
Total nitrosoamines (ppb)[e]	4.8 ± 1.45	3.1 – 7.5	24
N-Nitrosodimethylamine (ppb)[e]	2.3 ± 0.56	1.2 – 3.2	24
N-Nitrosopyrrolidine (ppb)[e]	2.4 ± 1.17	1.0 – 5.1	24
Pesticides (ppm)			
α-BHC	<0.01		24
β-BHC	<0.02		24
γ-BHC	<0.01		24
δ-BHC	<0.01		24
Heptachlor	<0.01		24
Aldrin	<0.01		24
Heptachlor epoxide	<0.01		24
DDE	<0.01		24
DDD	<0.01		24
DDT	<0.01		24
HCB	<0.01		24
Mirex	<0.01		24
Methoxychlor	<0.05		24
Dieldrin	<0.01		24
Endrin	<0.01		24
Telodrin	<0.01		24
Chlordane	<0.05		24
Toxaphene	<0.10		24
Estimated PCBs	<0.20		24
Ronnel	<0.01		24
Ethion	<0.02		24
Trithion	<0.05		24
Diazinon	<0.10		24
Methyl chlorpyrifos	0.130 ± 0.076	0.020 – 0.288	24
Methyl parathion	<0.02		24
Ethyl parathion	<0.02		24
Malathion	0.191 ± 0.139	0.020 – 0.557	24
Endosulfan I	<0.01		24
Endosulfan II	<0.01		24
Endosulfan sulfate	<0.03		24

[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

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 five male and five female extra rats and mice at the start of the 1- and 3-month studies and from sentinel rats and mice at 4 weeks and at termination of the 3-month studies. During the 2-year studies, samples were collected from five male and five female sentinel rats and mice at 6, 12, and 18 months and from five male and five female 50 mg/kg rats and five male and five female 25 mg/kg mice at the end of the studies. Blood from each animal was collected and allowed to clot, and the serum was separated. The samples were processed appropriately and analyzed at the study laboratory or sent to MA Bioservices, Inc. (Rockville, MD; 1- and 3-month studies) or BioReliance (Rockville, MD; 2-year studies) 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	
1-Month Study	
ELISA	
PVM (pneumonia virus of mice)	Study start
RCV/SDA (rat coronavirus/sialodacryoadenitis virus)	Study start
Sendai	Study start
Hemagglutination Inhibition	
H-1 (Toolan's H-1 virus)	Study start
KRV (Kilham rat virus)	Study start
3-Month Study	
ELISA	
PVM	Study start, 1 month, study termination
RCV/SDA	Study start, 1 month, study termination
Sendai	Study start, 1 month, study termination
Hemagglutination Inhibition	
H-1	Study start, 1 month, study termination
KRV	Study start, 1 month, study termination

Method and Test	Time of Analysis
RATS (continued)	
2-Year Study	
ELISA	
Mycoplasma arthritidis	Study termination
Mycoplasma pulmonis	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	
M. arthritidis	Study termination
Parvovirus	6, 12, and 18 months, study termination
RCV/SDA	12 months
Sendai	12 months, study termination
MICE	
1-Month Study	
ELISA	
Ectromelia virus	Study start
GDVII (mouse encephalomyelitis virus)	Study start
LCM (lymphocytic choriomeningitis virus)	Study start
MHV (mouse hepatitis virus)	Study start
PVM	Study start
Reovirus 3	Study start
Sendai	Study start
Immunofluorescence Assay	
LCM	Study start
Hemagglutination Inhibition	
MVM (minute virus of mice)	Study start
Polyoma virus	Study start
3-Month Study	
ELISA	
Ectromelia virus	Study start, 1 month, study termination
EDIM (epizootic diarrhea of infant mice)	Study start, 1 month, study termination
GDVII	Study start, 1 month, study termination
LCM	Study start, 1 month, study termination
Mouse adenoma virus	Study start, 1 month, study termination
MHV	Study start, 1 month, study termination
PVM	Study start, 1 month, study termination
Reovirus 3	Study start, 1 month, study termination
Sendai	Study start, 1 month, study termination

Method and Test	Time of Analysis
MICE (continued)	
3-Month Study (continued)	
Immunofluorescence Assay	
EDIM	Study termination
Mouse adenoma virus	Study termination
Hemagglutination Inhibition	
K (papovavirus)	Study start, 1 month, study termination
MVM (minute virus of mice)	Study start, 1 month, study termination
Polyoma virus	Study start, 1 month, 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
M. arthritidis	Study termination
M. pulmonis	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	
MCVM (mouse cytomegalovirus)	Study termination
Parvovirus	6, 12, and 18 months, study termination
Sendai	Study termination

RESULTS

All test results were negative.