NTP
National Toxicology Program
U.S. Department of Health and Human Services

NTP Technical Report on the Toxicity Studies of Malachite Green Chloride and Leucomalachite Green (CAS Nos. 569-64-2 and 129-73-7) Administered in Feed to F344/N Rats and B6C3F$_1$ Mice

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National Toxicology Program
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June 2004
NIH Publication No. 04-4416

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

FOREWORD

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

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

The studies described in this Toxicity Study Report were performed under the direction of the NCTR and were conducted in compliance with NTP laboratory health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals.

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

Details about ongoing and completed NTP studies are available at the NTP's World Wide Web site: http://ntp-server.niehs.nih.gov. Abstracts of all NTP Toxicity Study Reports and full versions of the most recent reports and other publications are available from the NIEHS's Environmental Health Perspectives (EHP) http://ehp.niehs.nih.gov (866-541-3841or 919-653-2590). In addition, printed copies of these reports are available from EHP as supplies last. A listing of all the NTP Toxicity Study Reports printed since 1991 appears on the inside back cover.

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Administered in Feed
to F344/N Rats and B6C3F$_1$ Mice

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June 2004

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

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

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PEER REVIEW

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

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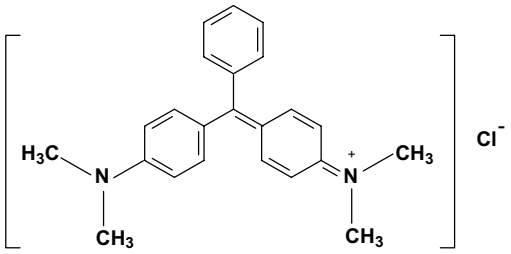
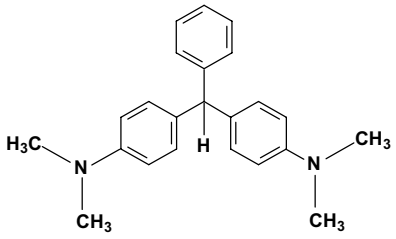
ABSTRACT



MALACHITE GREEN CHLORIDE

CAS No. 569-64-2

Chemical Formula: $C_{23}H_{25}ClN_2$ Molecular Weight: 364.92



LEUCOMALACHITE GREEN

CAS No. 129-73-7

Chemical Formula: $C_{23}H_{26}N_2$ Molecular Weight: 330.47

Malachite Green Chloride

Synonyms: *bis*[*p*-(Dimethylamino)phenyl]phenylmethylium chloride; *N*-[4-[[4-(dimethylamino)-phenyl]phenylmethylene]-2,5-cyclohexadien-1-ylidene]-*N*-methylmethanaminium chloride

Trade names: Aniline Green; Benzal Green; Benzaldehyde Green; China Green; C.I. Basic Green 4; C.I. 42000; Diamond Green B; Diamond Green Bx; Diamond Green P Extra; Fast Green; Light Green N; New Victoria Green Extra I; New Victoria Green Extra II; New Victoria Green Extra O; Solid Green O; Victoria Green B; Victoria Green WB

Leucomalachite Green

Synonym: *p,p′*-Benzylidenebis-*N,N*-dimethylaniline

Malachite green chloride is a triphenylmethane dye used in the fish and dye industries. Leucomalachite green is prepared by the reduction of malachite green chloride. Malachite green chloride was nominated for toxicity and carcinogenicity testing by the Food and Drug Administration and selected by the National Institutes of Environmental Health Sciences for carcinogenicity testing by the National Toxicology Program (NTP) due to the potential for significant worker and consumer exposure and lack of carcinogenicity data. The current 28-day studies were conducted as part of an overall effort by the NTP to determine the toxicity and carcinogenicity of malachite green chloride.

Male and female F344/N Nctr BR rats and B6C3F$_1$/Nctr BR (C57BL/6N × C3H/HeN MTV$^-$) mice were exposed to malachite green chloride (95% pure) or leucomalachite green (99% pure) (male rats and female mice only) in feed for 28 days. Animals were evaluated for clinical pathology and histopathology. Genetic toxicity studies for malachite green chloride were conducted *in vitro* in *Salmonella typhimurium* and *in vivo* in rat bone marrow

erythrocytes and in mouse peripheral blood erythrocytes. Genetic toxicity studies for leucomalachite green were conducted *in vivo* in mouse peripheral blood erythrocytes.

Groups of eight male and eight female rats and mice were fed diets containing 0, 25, 100, 300, 600, or 1,200 ppm malachite green chloride for 28 days. Additional groups of eight male and eight female rats designated for thyroid hormone assays were fed diets containing 0 or 1,200 ppm malachite green chloride. Groups of eight male rats and eight female mice were fed diets containing 0, 290, 580, or 1,160 ppm leucomalachite green for 28 days. Additional groups of eight male rats designated for thyroid hormone assays were fed diets containing 0 or 1,160 ppm leucomalachite green.

All rats and mice survived to the end of the studies. In the malachite green chloride study, the body weight gain of males rats in the 1,200 ppm group was significantly less than that of the controls. The final mean body weight of female rats and mice in the 1,200 ppm groups and the body weight gains of female rats and mice in the 600 (rats only) and 1,200 ppm groups were significantly less than those of the controls. In the leucomalachite green study, the final mean body weight of male rats and female mice in the 1,160 ppm groups and the mean body weight gains of male rats and female mice in the 580 and 1,160 ppm groups were significantly less than those of the control groups.

In the malachite green chloride study, feed consumption by all exposed groups of male and female rats and mice was generally similar to that by the control groups. Exposure concentrations of 25, 100, 300, 600, and 1,200 ppm resulted in average daily doses of 3 to 190 mg malachite green chloride/kg body weight to male and female rats and 5 to 250 mg/kg to male and female mice. In the leucomalachite green study, feed consumption by all groups of exposed male rats was similar to that by the controls. Dietary concentrations of 290, 580, and 1,160 ppm resulted in average daily doses of approximately 30, 60, and 115 mg leucomalachite green/kg body weight to male rats and approximately 62, 110, and 220 mg/kg to female mice.

In female rats exposed to malachite green chloride, there was a significant increases in γ-glutamyltransferase activities with an activity in 1,200 ppm females seven times greater than that in the controls. Likewise, γ-glutamyltransferase activity in male rats exposed to 1,160 ppm leucomalachite green was twice that in the controls. On days 4 and 21, the concentration of thyroxine was significantly decreased in male rats exposed to 1,160 ppm leucomalachite green and the concentration of thyroid-stimulating hormone was significantly increased.

In the malachite green chloride study, the relative liver weights of 600 and 1,200 ppm male rats and the relative and absolute liver weights of 300 ppm or greater female rats were generally significantly greater than those of the controls. In the leucomalachite green study, the relative liver weights of 290 ppm or greater male rats were significantly greater than those of the control group.

No gross lesions were observed in rats or mice and no microscopic lesions were observed in female mice that were attributed to malachite green chloride exposure. Microscopically, the incidences of hepatocyte cytoplasmic vacuolization were significantly increased in 1,200 ppm male and female rats exposed to malachite green chloride. No gross lesions were observed in rats or mice that could be attributed to leucomalachite green exposure. Microscopically, the incidences of hepatocyte cytoplasmic vacuolization were significantly increased in 580 and 1,160 ppm male rats. The incidence of multifocal apoptosis in the transitory epithelium of the urinary bladder was significantly increased in 1,160 ppm female mice exposed to leucomalachite green.

Malachite green chloride, tested at concentrations of 0.1 to 10 μg/plate, was not mutagenic in any of several strains of *Salmonella typhimurium*, with or without S9 metabolic activation. Negative results were also obtained in two *in vivo* micronucleus tests, one that assessed induction of micronuclei in rat bone marrow erythrocytes after three intraperitoneal injections of malachite green chloride, and a second study that determined the level of micronuclei in circulating erythrocytes of male and female mice following 28 days of exposure to malachite green chloride via dosed feed. The frequency of micronucleated normochromatic erythrocytes in peripheral blood was significantly increased in female mice exposed to leucomalachite green in feed for 28 days; no significant increases in micronucleus frequencies were observed in the polychromatic erythrocyte population.

INTRODUCTION

CHEMICAL AND PHYSICAL PROPERTIES

Malachite green chloride is a green crystal with a metallic luster; it is soluble in ethanol, methanol, and amyl alcohol and is very soluble in water. Neutral water solutions are blue-green, with an absorption maximum of 616.9 nm; aqueous solutions are yellow below pH 2 (*Merck Index*, 1996). The compound has a molecular weight of 364.92.

Leucomalachite green is a faint green solid with an absorption maximum of 266 nm in tetrahydrofuran and an extinction coefficient of 3.34×10^4 (ChemSyn Science Laboratories, unpublished data). The compound has a molecular weight of 330.47.

PRODUCTION, USE, AND HUMAN EXPOSURE

Malachite green chloride, a triphenylmethane dye, is prepared as a double salt with zinc chloride for dyeing purposes. It is prepared in a stepwise reaction that involves the condensation of benzaldehyde with *N*,*N*-dimethylalanine and oxidation of the resulting bis (*p*-dimethylaminophenyl) phenylmethane, followed by reaction of the product with hydrochloric acid (Nelson, 1974). Leucomalachite green is prepared by the reduction of malachite green.

The production and uses of malachite green chloride have been reviewed by Culp and Beland (1996). Malachite green is widely used in the dye industry and as an antifungal agent in fish hatcheries. Malachite green is not approved by the Food and Drug Administration or the Environmental Protection Agency for use on any aquatic species. However, it is relatively inexpensive, readily available, and highly efficacious; therefore, its continued use in some United States fisheries is likely. The chemical has been used routinely in aquaculture since the early 1930s and is considered by many in the fish industry as the most effective antifungal agent (Schnick, 1988). In a study of over 180 compounds tested for antifungal activity, none equaled malachite green for efficacy and low toxicity (Meyer and Schnick, 1989). A broad range of malachite green concentrations has been used to treat fungal and parasitic infections, with doses of 100 ppm for a few seconds of dip (Nelson, 1974) to a prolonged treatment of 0.1 ppm in ponds (Stoskopf, 1993). Because of its use in commercial fish hatcheries, workers in the dye and aquaculture industries may potentially be exposed to the chemical. The National Occupational Exposure Survey

conducted by the National Institute for Occupational Safety and Health between 1981 and 1983 estimated that more than 180,000 workers are potentially exposed to malachite green annually (NIOSH, 1990). The general public may become exposed to malachite green through the consumption of treated fish. Additional consumer exposure can occur via fish imported from Europe (Alabaster, 1982; Solbé, 1982; Schlotfeldt, 1992) and Canada (Thorburn and Moccia, 1993), where the use of malachite green has been documented. While fish sold in the United States have not been routinely tested for malachite green, random sampling from markets in the United Kingdom indicates the continued use of malachite green in aquaculture (Veterinary Medicines Directorate, 1996). Concern about the use of malachite green has also been raised in the United Kingdom. A report prepared by the Water Research Centre for the Department of the Environment, Transport, and the Regions of the United Kingdom recommended an annual average environmental quality standard of 500 ng/L malachite green for the protection of freshwater aquatic life, although no standards were recommended for drinking water due to a lack of data (Burchmore and Wilkinson, 1993).

Human exposure to leucomalachite green has been documented (Doerge *et al*., 1998; Veterinary Medicines Directorate, 1999). Doerge *et al*. (1998) analyzed edible flesh from trout purchased from retail outlets in the United Kingdom during 1994 and 1995 as part of nonregulatory food surveillance program. Eight of the 12 samples were positive for malachite green and leucomalachite green. Most noteworthy, the concentration of leucomalachite green in the samples was 12 to 37 times higher than that of malachite green.

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

Little is known about the metabolism of malachite green in fish or other species. Some studies have shown that malachite green is taken in and retained by the tissues of fish. For example, Alderman and Clifton-Hadley (1993) exposed trout to a 1.6 ppm malachite green bath treatment for 40 minutes to examine the uptake, distribution, and elimination of the dye. Maximum concentrations of malachite green in serum, liver, and kidney were detected immediately after exposure, with levels ranging from 7.8 to 34.0 ppm; a peak concentration of 10.8 ppm was reached in muscle 90 to 120 minutes after exposure.

Other data suggest that malachite green is reduced to leucomalachite green after entering the body, and that the dye persists in the body in this form. Werth and Boiteux (1968) reported the detection of leucomalachite green in liver, kidney, heart, lung, and muscle of rats 2 hours following an intravenous injection of malachite green and in Ehrlich's ascitic tumor cells 3 hours after intraperitoneal injection of malachite green. Bauer *et al*. (1988)

observed that trout excreted intact malachite green rapidly, but leucomalachite green was stored in muscle tissue for a relatively long time with a half-life of about 40 days. In another study, Law (1994) demonstrated a rapid absorption of malachite green in fingerling trout exposed to 2 ppm malachite green for 1 hour. The amount of chromatic malachite green (the ionic form) measured in whole tissue homogenates decreased with time, ranging from approximately 1 ppm 2 hours after treatment to 0.2 ppm 169 hours after treatment. However, the levels of leucomalachite green increased to 3.5 ppm 24 hours after treatment and remained steady for the remainder of the 7-day study. Analysis of methylene chloride extracts of liver, muscle, and skin 73 hours or more after treatment showed the presence of leucomalachite green but little or no chromatic malachite green.

Humans

No studies on the absorption, distribution, metabolism, or excretion of malachite green chloride or leucomalachite green in humans were found in a review of the literature.

TOXICITY

Experimental Animals

The toxicity data on malachite green is not complete because most reports do not adequately identify the purity of the malachite green used or the counterion with which the dye is associated. Due to extensive use of malachite green in aquaculture, the majority of toxicity studies have been conducted with fish. Results from a number of these studies are summarized below. More extensive listings can be found in Nelson (1974), Bills *et al.* (1977), and Burchmore and Wilkinson (1993).

Bills *et al.* (1977) determined the acute toxicity of malachite green chloride to fingerling fish and nontarget aquatic organisms. After 96 hours of exposure, the LC_{50} values in fish ranged from 30.5 to 383 µg/L, with bluegills being the most sensitive and Coho salmon being the most resistant. Asiatic clams tolerated more than 100 mg/L, with a 96-hour LC_{50} value of 122 mg/L. The toxicity in all species tested increased with lengthening exposures. With a 3-hour or 6-hour treatment, the toxicity to some of the species was greater in warm water (17° or 22° C) than in cool water (7° or 12° C). Only channel catfish were more sensitive to increased water temperatures during 96 hours of exposure.

Rather than mortality, biological effects have been the focus of many studies. Gerundo *et al*. (1991) treated rainbow trout with 1.6 ppm malachite green (source unknown) for 40 minutes once every 7 days for 7 weeks. After the third exposure, a fairly consistent pattern of increasing pathological changes was observed in most livers.

These included sinusoidal congestion, focal coagulative necrosis, diffuse degenerative changes, and cytoplasmic vacuolation. Similar tissue changes have been reported in fish exposed to other toxins. At the ultrastructural level, mitochondrial damage was evident and was thought to be due to the dye's action as a respiratory enzyme poison. The gills generally demonstrated lesions and necrosis; the latter was more evident following longer periods of exposure.

Physiological changes in fish blood have been reported by a number of investigators. Grizzle (1977) continuously exposed fingerling channel catfish to 100 µg/L malachite green (source unknown) for up to 28 days and assayed blood samples at various times. Compared to the controls, a large increase in neutrophils was measured in exposed catfish 1 and 3 days after treatment and was thought to be indicative of an inflammatory response. Increases were also observed in erythrocyte counts and hemoglobin concentrations. The latter effect was attributed to impairment of gas exchange by the gills due to a thickening of the lamellar epithelium. In an earlier study, Glagoleva and Malikova (1968) found extensive leukopenia and slight erythropenia in Baltic salmon exposed to 1.33 mg/L malachite green for 20 minutes. After 6 days, the number of erythrocytes returned to normal levels, but the number of leukocytes remained low. Hlavek and Bulkley (1980) repeated the experiments and did not observe a difference in the number of leukocytes in exposed fish when compared to controls. Leukocyte counts in both groups declined during the 24-hour period following treatment and recovered within the next 4 days. These researchers concluded that the leukocyte changes were due to nonspecific vertebrate stress syndrome as opposed to toxicity from exposure to malachite green chloride. Other blood chemistry parameters, including potassium, glucose, sodium, calcium, magnesium, and chloride concentrations, were measured in Coho salmon 28 days after exposure to 100 µg/L malachite green chloride (Bills and Hunn, 1976). An increase was found in potassium concentrations after exposure, while the other constituents remained unchanged.

The therapeutic use of malachite green is not restricted to freshwater species and has been extended to control fungal and epibiotic growth on eggs and larvae of cultured American lobsters. Fisher *et al*. (1976) noted that American lobster larvae exhibited decreased survival when treated with concentrations of malachite green (source unknown) greater than 8 ppm for 16 minutes every other day during their larval rearing period. Brief exposures to 20 ppm resulted in a delay in molting and a decrease in survival, with the majority of the dead animals showing the absence of one or more appendages.

In mammalian studies, male and female Wistar rats were administered aqueous solutions of malachite green oxalate by gavage; the animals were observed over a 14-day period (Clemmensen *et al*., 1984). Acute effects included reduced motor activity on the first day and hyperemia and atonia of the intestinal walls where the dye had reached before the death of the animal. Survivors were free of symptoms after 2 days. The oral LD_{50} was

calculated to be 275 mg/kg body weight. These investigators also reported an LD_{50} of 50 mg/kg body weight for NMRI mice. The acute oral toxicity of malachite green has also been determined in female Sprague-Dawley rats. Meyer and Jorgenson (1983) administered 300, 450, 600, or 750 mg malachite green oxalate/kg body weight, presumably by gavage. The 24-hour LD_{50} value for malachite green was determined to be 520 mg/kg. Effects observed included depression, prostration, emaciation, coma, and death.

In a 28-day study, Wistar rats were exposed to 0, 10, 100, or 1,000 ppm malachite green oxalate in feed (Clemmensen *et al*., 1984). The animals exposed to 1,000 ppm showed significant decreases in feed consumption and weight gain and increased hyperactivity. In addition, females exposed to 1,000 ppm showed an increase in lymphocytes and decreases in neutrophils and packed cell volume. Males exposed to 1,000 ppm showed a significant increase in plasma urea.

Meyer and Jorgenson (1983) reported that nonpregnant New Zealand white rabbits were able to tolerate 13 consecutive daily gavage doses of 50 mg malachite green oxalate/kg body weight. Pregnant rabbits were also dosed with 5, 10, or 20 mg malachite green/kg body weight by gavage on days 6 through 18 of gestation and observed daily for external signs of toxicity. Feed consumption was reduced in treated animals and the average total body weight was consistently lower after 29 days, although there were no overt signs of toxicity. Females in the untreated group gained an average of 230 g. The animals given 5 mg/kg malachite green gained an average of 60 g, while those given 10 or 20 mg/kg lost 30 g and 60 g, respectively.

Lavender and Pullman (1964) infused malachite green (source unknown) into the renal arteries of dogs and found marked increases in the urinary excretion of water, sodium, potassium, chloride, calcium, and phosphate. The dye was localized primarily in the renal cortex, indicating proximal or distal tubular uptake. In addition, it appeared to cause a direct vasoconstriction of the renal arterioles.

The instillation of an aqueous solution of 8% malachite green oxalate into the eyes of rabbits resulted in marked edema, substantial discharge, and slight hyperemia of the conjunctiva (Clemmensen *et al*., 1984). Treatment with fine crystals of malachite green oxalate caused total opacification and bright red and edematous conjunctivae that lasted for 2 weeks. Clemmensen *et al*. (1984) also treated the skin of guinea pigs and rats with 400 µL of a 20% suspension of malachite green oxalate and found no visible erythema or edema. In a study with humans, six of 11 eczema patients were found to be sensitized to patch tests using a 2% aqueous solution of malachite green (Bielicky and Novak, 1969).

No data were found in the literature on the toxicity of leucomalachite green.

Humans

Malachite green has been reported to be injurious to the human eye (Grant, 1974). No toxicity studies or reports of health effects related to exposure to leucomalachite green in humans were found in the literature.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Experimental Animals

Meyer and Jorgenson (1983) observed significant teratologic effects in New Zealand white rabbits administered 0, 5, 10, or 20 mg malachite green oxalate/kg body weight by gavage on days 6 through 18 of gestation. At all three doses there were significant increases in preimplantation losses, primarily due to early resorption of fetuses and decreases in the number of living fetuses. The body weights of the progeny were less than those in the controls, with the differences being significant in the 5 and 20 mg/kg groups. Developmental anomalies were observed in all treated groups. Skeletal deviations were the most common abnormality and included incomplete ossification of vertebrae and phalanges and malformed skulls. Enlargement of the liver, heart, and abdominal cavity was also observed. The percent of progeny with abnormalities were 18.5%, 38.0%, 33.9%, and 47.0% in the 0, 5, 10, and 20 mg/kg treatment groups, respectively. Thalidomide (150 mg/kg body weight), which was used as a positive control, caused similar types of changes in 94% of the progeny.

Damage can also occur to rainbow trout eggs upon exposure to malachite green. Using treatment regimens similar to those used in fisheries, Meyer and Jorgenson (1983) reported a delay in hatching, reduction in the average size of fry, and a significant increase in the percentage of fry with deformities. The abnormalities included head and jaw deformities, curvatures of the spine, missing fins, and a bobtailed condition. On the other hand, an increase in the percentage of eggs that hatched was increased in the treated groups compared to the untreated groups.

No data were found in the literature on the reproductive and developmental toxicity of leucomalachite green.

Humans

No studies of reproductive or developmental effects of malachite green chloride or leucomalachite green in humans were found in a review of the literature.

CARCINOGENICITY

Experimental Animals

The data relating to the carcinogenicity of malachite green are extremely limited. However, malachite green enhanced the formation of hepatic tumors in rats initiated with diethylnitrosamine (Fernandes *et al*., 1991). There is also suggestive evidence of carcinogenicity based on structure-activity relationships (reviewed by Culp and Beland, 1996).

No data were found in the literature on the carcinogenicity of leucomalachite green.

Humans

No epidemiology studies of malachite green chloride or leucomalachite green were found in a review of the literature.

GENETIC TOXICITY

There are little published mutagenicity data for malachite green. Clemmensen *et al.* (1984) reported that malachite green oxalate was mutagenic in *Salmonella typhimurium* strain TA98 in the presence of S9 activation enzymes, but they observed no mutagenicity in TA100, TA1535, or TA1537, with or without S9. Another investigation of malachite green-induced mutagenicity in *Salmonella* found negative results in TA98, TA100, and TA1537, but these investigations were only conducted in the absence of S9 (Ferguson and Baguley, 1988). Wolfe (1977) reported that malachite green inhibited DNA replication processes in *Escherichia coli* that were catalyzed by polymerase I, and Panandiker *et al.* (1994) reported induction of DNA single-strand breaks in Syrian hamster embryo cells exposed *in vitro* to 1 µg/mL malachite green. However, Au and Hsu (1979) found no evidence of induced chromosomal aberrations in cultured Chinese hamster ovary cells incubated for 5 hours with 20 µM malachite green. Furthermore, no increase in micronucleated erythrocytes was observed in bone marrow of mice administered a single dose of 37.5 mg/kg malachite green oxalate by gavage; the frequency of micronucleated cells was assessed at 24, 42, and 66 hours posttreatment (Clemmensen *et al.*, 1984). The testing protocol used in this *in vivo* assay is not the currently accepted standard, but the results of this investigation were nonetheless clearly negative. Finally, in a brief abstract, negative results were reported in a mammalian spot test (*in vivo* mammalian mutation assay) conducted in mice treated with 10 to 40 mg/kg malachite green by gavage on days 8, 9, and 10 of pregnancy (Jensen, 1984); no increase in the number of recessive coat color spots was observed in the offspring of treated females.

STUDY RATIONALE AND DESIGN

In 1993, the FDA nominated malachite green as a priority chemical for carcinogenicity testing by the National Toxicology Program. In August 1994, the selection was reviewed by the Toxicology Study Selection and Review Committee, which oversees the Interagency Agreement between the NCTR and the National Institute for Environmental Health Sciences. The basis for the selection of malachite green was the potential for significant worker and consumer exposure (NIOSH, 1990), suggestive evidence of tumor promotion in rodent liver (Fernandes *et al.*, 1991), suspicion of carcinogenicity based on structure-activity relationships (IARC, 1978; Littlefield *et al.*, 1985), and inadequacy of existing data to evaluate its carcinogenicity (reviewed by Culp and Beland, 1996).

It has been reported that malachite green is reduced to and persists as leucomalachite green in the tissues of fish treated with malachite green. Doerge *et al.* (1998) showed that humans are exposed to greater amounts of leucomalachite green than malachite green via the consumption of treated fish. There are no data available on the adverse effects to any species from exposure to leucomalachite green. Therefore, 2-year feed studies were proposed as part of a research plan to determine the carcinogenic risk from exposure to malachite green and leucomalachite green. The present study was designed to evaluate the toxicity of malachite green chloride and leucomalachite green after 28 days of exposure, help assist in the dose selection for the 2-year bioassay, and provide fundamental information that could be used as the basis for additional mechanistic studies.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION

Malachite Green Chloride

Malachite green chloride was obtained from Chemsyn Science Laboratories (Lenexa, KS) in one lot (CSL-96-645-88-23). Identity and purity analyses were conducted by the manufacturer and the study laboratory. Reports on analyses performed in support of the malachite green chloride studies are on file at the National Center for Toxicological Research (NCTR).

Malachite green chloride, a green solid, was identified by the study laboratory using ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy and high-performance liquid chromatography (HPLC)/mass spectrometry (MS). The supplier also identified the chemical as malachite green chloride with ^{1}H-nuclear magnetic resonance and ultraviolet/visible spectroscopy.

The purity of malachite green chloride was determined with HPLC by the manufacturer, heavy metal and HPLC analyses by the study laboratory, and elemental and heavy metal analyses by Galbraith Laboratories, Inc. (Knoxville, TN). Heavy metal analyses by the study laboratory were conducted with inductively coupled plasma/atomic emission spectroscopy, normalized against standards provided by the National Institute of Standards and Technology (Gaithersburg, MD).

Elemental analyses for carbon, hydrogen, nitrogen, and chlorine (total halogens) were in agreement with the theoretical values for malachite green chloride. Results of heavy metal analyses by Galbraith Laboratories, Inc., indicated less than 20 ppm calculated as lead. Results of heavy metal analyses by the study laboratory indicated the following concentrations: tin, 25.0 ppm; zinc, 23.1 ppm; aluminum, 3.69 ppm; iron, 2.25 ppm; copper, 1.62 ppm; and magnesium, 1.17 ppm. HPLC analyses conducted by the supplier indicated one major peak and four impurities with a combined area of approximately 5.1% of the total peak area. Further HPLC analyses, conducted by the study laboratory, indicated one major peak and seven impurities with a combined area of approximately 4.7% of the total peak area; two of the impurities were identified as leucomalachite green and the desmethyl analogue of malachite green, present at concentrations of approximately 1% each based on peak areas, retention times, and spectral characteristics. The overall purity was determined to be at least 95%.

Reports on liquid chromatography/MS, electrospray ionization (ESI)/MS, and HPLC/ESI/MS analyses performed in support of the malachite green chloride studies are on file at the NCTR.

The bulk chemical was stored in the original amber bottle in the dark at room temperature. Analyses performed after the completion of the 28-day studies indicated no degradation of the bulk chemical; the stability of malachite green chloride was monitored at 6-month intervals over a 2-year period using HPLC with post-column oxidation.

Leucomalachite Green

Leucomalachite green was obtained from Chemsyn Science Laboratories in one lot (CSL-95-583-08-09). Identity, purity, and stability analyses were conducted by the manufacturer and the study laboratory. Reports on analyses performed in support of the leucomalachite green studies are on file at the NCTR.

Leucomalachite green, a faint green solid, was identified by the supplier using ^{1}H-nuclear magnetic resonance, infrared, and ultraviolet/visible spectroscopy and by the study laboratory using ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy.

The purity of leucomalachite green was determined by elemental analyses (performed by Oneida Research Services, Inc., Whitesboro, NY), heavy metal analyses (performed by Galbraith Laboratories, Inc.), and HPLC.

Elemental analyses for carbon, hydrogen, and nitrogen were in agreement with the theoretical values for leucomalachite green. Results of heavy metal analyses indicated less than 0.30 ppm lead and less than 1.0 ppm heavy metals calculated as lead. HPLC indicated one major peak and two impurities with a combined area of 0.24% of the total peak area at a wavelength of 254 nm.. One impurity was identified as malachite green based on retention time and spectral characteristics. The overall purity of lot CSL-95-583-08-09 was determined to greater than 99%.

Reports on liquid chromatography-atmospheric pressure chemical ionization/MS, direct exposure probe/electron ionization/MS, and HPLC/electrospray ionization/MS analyses performed in support of the leucomalachite green studies are on file at the NCTR.

The bulk chemical was stored in the original amber bottle with a double wrapping of Parafilm around the cap; the bottle was placed inside a plastic bag inside another plastic bag filled with a silica gel desiccant and stored at −20° C, protected from light. Analyses performed after the completion of the 28-day studies indicated no

degradation of the bulk chemical; the stability of leucomalachite green was monitored at 6-month intervals over a 2-year period using HPLC.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations for malachite green chloride were prepared on 6 days by dissolving the chemical in water and then mixing it with feed (Table F2). The 25 and 600 ppm dose formulations were prepared three times and the 100, 300, and 1,200 ppm dose formulations were prepared twice. The dose formulations for leucomalachite green were prepared by mixing the chemical with feed (Table F2). A premix was prepared by hand and blended with additional feed. The 96 and 290 ppm dose formulations for leucomalachite green were prepared once and the 580 and 1,160 ppm dose formulations were prepared twice. Dose formulations for each chemical were mixed in a Patterson-Kelly twin-shell blender with the intensifier bar on for 20 minutes. Dose formulations were stored in stainless steel feed cans at 4° ± 2° C for up to 92 days (malachite green chloride) or 95 days (leucomalachite green).

Homogeneity and stability studies of the 25 ppm malachite green chloride dose formulations were performed by the study laboratory using HPLC. Homogeneity was confirmed. Stability of the malachite green chloride formulation was confirmed for 92 days for dose formulations stored protected from light at 4° C and for 10 days for dose formulations stored at room temperature, either protected from light or open to air and light. Stability of the leucomalachite green formulation was confirmed for 95 days for dose formulations stored protected from light at up to 8° C and for 32 days for dose formulations stored at room temperature either protected from light or open to air.

Periodic analyses of the dose formulations of malachite green chloride were conducted by the study laboratory using HPLC. Analyses of the dose formulations of malachite green chloride were conducted on one batch each of 25, 100, and 1,200 ppm dose formulations, on both batches of the 300 ppm dose formulations, and on all three of the 600 ppm dose formulations (Table F3). During the 28-day studies, seven of eight dose formulations analyzed for rats and mice were within 10% of the target concentration, with no value greater than 103% of the target concentration. The formulation that was not within 10% of the target concentration was diluted with feed and remixed to provide a lower (300 ppm) concentration; the remix was analyzed and found to be within 10% of the target concentration. Analyses of the dose formulations of leucomalachite green were conducted by the study laboratory using HPLC. During the 28-day studies, all dose formulations were analyzed. All 6 dose formulations for rats and mice were within 10% of the target concentration (Table F4).

28-DAY STUDIES

Male and female F344/N Nctr BR rats and B6C3F$_1$/Nctr BR (C57BL/6N × C3H/HeN MTV$^-$) mice were obtained from the study laboratory's breeding colony. The rats and mice were 4 to 5 weeks old at allocation and 6 to 7 weeks old on the first day of exposure.

Groups of eight male and eight female rats and mice were fed diets containing 0, 25, 100, 300, 600, or 1,200 ppm malachite green chloride for 28 days. Additional groups of eight male and eight female rats designated for thyroid hormone assays were fed diets containing 0 or 1,200 ppm malachite green chloride. Groups of eight male rats and eight female mice were fed diets containing 0, 290, 580, or 1,160 ppm leucomalachite green for 28 days. Additional groups of eight male rats designated for thyroid hormone assays were fed diets containing 0 or 1,160 ppm leucomalachite green. Feed and water were available *ad libitum*. Rats were housed two per cage and mice four per cage. Clinical findings and feed consumption were recorded weekly. 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.

Clinical pathology studies were conducted on all core study animals at the end of the exposure period. The animals were anesthetized with carbon dioxide, and blood was collected by cardiac puncture (rats) or from the retroorbital sinus (mice). For hematology analyses, blood was placed in tubes containing EDTA as anticoagulant. Assessment of blood cells was determined by light microscopic examination of blood smears fixed in absolute methanol. For clinical chemistry analyses, blood samples were placed in tubes, allowed to clot, and then centrifuged, and the serum was collected. Parameters were measured with a Roche Diagnostic Cobas Mira-Plus analyzer (Roche Diagnostic Systems, Inc., Montclair, NJ). Reagents were obtained from the equipment manufacturer. All parameters measured are listed in Table 1.

Blood was collected by cardiac puncture from special study rats on days 4 and 21 for determination of thyroid-stimulating hormone (TSH), triiodothyronine (T_3), and thyroxine (T_4) concentrations. TSH was measured with double-antibody radioimmunoassay. Freshly prepared ^{125}I-TSH was allowed to react overnight with the specific antibody in the presence or absence of unlabeled hormone in the sample. An excess of secondary antibody containing polyethylene glycol was added. The bound and unbound ^{125}I-labeled hormones were separated by centrifugation and the radioactivity was measured in the precipitates. The amount of TSH was calculated from a standard curve. Total T_3 and total T_4 were determined with a "Coat-A-Count" procedure obtained from DPC (Los Angeles, CA). The procedure is a solid-phase radioimmunoassay, wherein ^{125}I-labeled T_3 or T_4 competes with the T_3 or T_4 in the sample for antibody sites. The parameters measured are listed in Table 1.

Necropsies were performed on all core study animals. The kidneys and liver 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 5 μm, and stained with hematoxylin and eosin. A complete histopathologic examination was performed on core study control animals, 1,200 ppm animals exposed to malachite green chloride, and 1,160 ppm animals exposed to leucomalachite green. The liver, pituitary gland, and thyroid gland from all core study animals and the urinary bladder from all core study mice were examined histopathologically. Table 1 lists the tissues and organs routinely examined.

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Malachite Green Chloride and Leucomalachite Green

Malachite Green Chloride	Leucomalachite Green
Study Laboratory National Center for Toxicological Research (Jefferson, AR)	National Center for Toxicological Research (Jefferson, AR)
Strain and Species Rats: F344/N Nctr BR Mice: B6C3F$_1$/Nctr BR (C57BL/6N × C3H/HeN MTV$^-$)	Rats: F344/N Nctr BR Mice: B6C3F$_1$/Nctr BR (C57BL/6N × C3H/HeN MTV$^-$)
Animal Source National Center for Toxicological Research (Jefferson, AR)	National Center for Toxicological Research (Jefferson, AR)
Time Held Before Studies 2 weeks	2 weeks
Average Age when Studies Began 6 to 7 weeks	6 to 7 weeks
Date of First Exposure of First Group 16 December 1996 (Start dates for other groups were staggered over the following 11 weeks.)	12 August 1996 (Start dates for other groups were staggered over the following 3 weeks.)
Duration of Exposure 28 days	28 days
Date of Last Exposure of First Group 12 January 1997 (Stop dates for other groups were staggered over the following 11 weeks.)	8 September 1996 (Stop dates for other groups were staggered over the following 3 weeks.)
Necropsy Dates 13 and 27 January; 4, 11, 18, and 25 February; and 3, 4, 24, and 25 March 1997	9, 10, 16, 17, and 24 September 1996
Average Age at Necropsy 10 to 11 weeks	10 to 11 weeks
Size of Study Groups Rats: 8 males and 8 females (core study); 16 males and 16 females (thyroid hormone assay) Mice: 8 males and females	Rats: 8 males (core study); 16 males (thyroid hormone assay) Mice: 8 females
Method of Distribution Animals were distributed randomly into groups of approximately equal initial mean body weights	Same as malachite green chloride studies
Animals per Cage Rats: 2 Mice: 4	Rats: 2 Mice: 4
Method of Animal Identification Ear clip	Ear clip

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Malachite Green Chloride and Leucomalachite Green

Malachite Green Chloride	Leucomalachite Green
Diet NIH-31 open formula meal (pellets were autoclaved, then ground to powder) (Purina Mills, Richmond, IN), available *ad libitum*	Same as malachite green chloride studies
Water Millipore-filtered water (Jefferson municipal supply) via 16-oz water bottle, available *ad libitum*	Same as malachite green chloride studies
Cages Polycarbonate (Allentown Caging Equipment Co., Allentown, NJ), changed twice weekly (rats) or weekly (mice); cages rotated weekly	Same as malachite green chloride studies
Bedding Hardwood chips (Northeastern Products Inc., Warrensburg, NY), changed twice weekly (rats) or weekly (mice)	Same as malachite green chloride studies
Cage Bonnets Microisolator tops (Lab Products, Inc., Maywood, NJ)	Same as malachite green chloride studies
Racks Metal animal cage racks (Allentown Caging Equipment Co., Allentown, NJ), changed weekly	Same as malachite green chloride studies
Animal Room Environment Average temperature: rats: 72.0° F mice: 74.3° F Average relative humidity: rats: 47.5% mice: 50.6%	Average temperature: rats: 73.8° F mice: 71.4° F Average relative humidity: rats: 51.5% mice: 52.0%
Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Same as malachite green chloride studies
Exposure Concentrations 0, 25, 100, 300, 600 or 1,200 ppm in feed, available *ad libitum*	0, 290, 580, or 1,160 ppm in feed, available *ad libitum*
Type and Frequency of Observation Animals were observed twice daily; animals were weighed initially, weekly, and at the end of the studies. Feed consumption and clinical findings were recorded weekly.	Same as malachite green chloride studies
Method of Sacrifice Asphyxiation with carbon dioxide, following overnight fasting	Same as malachite green chloride studies
Necropsy Necropsies were performed on all core study animals. Organs weighed were kidneys and liver.	Same as malachite green chloride studies

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Malachite Green Chloride and Leucomalachite Green

Malachite Green Chloride	Leucomalachite Green
Clinical Pathology Blood was collected by cardiac puncture (rats) or from the retroorbital sinus (mice) at the end of the exposure period for hematology and clinical chemistry and by cardiac puncture from special study rats for thyroid hormone assays on days 4 and 21. ***Hematology*:** hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials ***Clinical chemistry*:** urea nitrogen, creatinine, total protein, alanine aminotransferase, alkaline phosphatase, and total bile acids for rats and mice; glucose, sodium, potassium, chloride, calcium, phosphorus, albumin, cholesterol, triglycerides, aspartate aminotransferase, creatine kinase, sorbitol dehydrogenase, γ-glutamyltransferase, thyroid-stimulating hormone (TSH), triiodothyronine (T_3), and thyroxine (T_4) for rats	Blood was collected by cardiac puncture or from the retroorbital sinus of core study male rats and female mice at the end of the exposure period for hematology and clinical chemistry and by cardiac puncture from special study male rats for thyroid hormone assays on days 4 and 21. ***Hematology*:** hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials ***Clinical chemistry:*** urea nitrogen, creatinine, total protein, alanine aminotransferase, and alkaline phosphatase for female mice; glucose, sodium, potassium, chloride, calcium, phosphorus, albumin, cholesterol, triglycerides, aspartate aminotransferase, creatine kinase, sorbitol dehydrogenase, γ-glutamyltransferase, total bile acids, for TSH, T_3, and T_4 for male rats
Histopathology Complete histopathology was performed on all core study rats and mice exposed to 0 or 1,200 ppm. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, coagulating gland, ear, esophagus, eye, gallbladder (mice), harderian gland, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, lacrimal gland, larynx, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, muscle, nose, ovary, pancreas, parathyroid gland, peripheral nerve, pituitary gland, preputial gland, prostate, salivary gland, skin, spinal cord, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, tongue, trachea, urinary bladder, uterus, vagina, and Zymbal's gland. In addition, the liver, pituitary gland, and thyroid gland of rats and mice and the urinary bladder of mice were examined in the lower exposure groups.	Complete histopathology was performed on all core study male rats and female mice exposed to 0 or 1,160 ppm. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, coagulating gland, ear, esophagus, eye, gallbladder (mice), harderian gland, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, lacrimal gland, larynx, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, muscle, nose, ovary, pancreas, parathyroid gland, penis, peripheral nerve, pituitary gland, preputial gland, prostate, salivary gland, skin, spinal cord, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, tongue, trachea, ureter, urinary bladder, uterus, vagina, and Zymbal's gland. In addition, the liver, pituitary gland, and thyroid gland of male rats and female mice and the urinary bladder of female mice were examined in the lower exposure groups.

STATISTICAL METHODS

Calculation and Analysis of Lesion Incidences

The incidences of lesions are presented in Appendices A and B as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. Fisher's exact test (Bradley, 1968) was used to compare the proportion of lesions in the control group to that in each of the exposed groups. The Cochran-Armitage test (Thomas *et al.*, 1977) for a dose-response trend on proportions was used when all four exposure groups were examined. An exact test for this procedure was used because there were fewer than 10 animals in each exposure group. All tests are one sided in that decreases in incidence with an increasing

exposure concentration were not considered. The P values within pairwise comparisons were adjusted by Holm's modification of the Bonferroni procedure as described by Wright (1992).

Analysis of Continuous Variables

Body weights were analyzed on a per cage basis. The mixed-models approach to repeated measures analysis was used to model the mean cage body weights. The weights were modeled using the fixed effects of exposure concentration, time, and exposure-by-time interaction. Dunnett's two-sided test was used to test for differences between the control group mean and the exposure group mean (Dunnett, 1955). Contrasts were used to test for exposure-related trends. Feed consumption was analyzed by a mixed analysis with repeated measures. Dunnett's test was used to test between the control group mean and the exposure group mean for each week.

A SAS GLM procedure was used to model the organ weights and the ratios of organ weights to body weights as functions of the exposure concentration, with the exposure concentration effect declared as a categorical variable. Dunnett's test was used to test for differences between the control group mean and each exposure group mean.

Differences in the amount of compound consumed for each of the exposure concentrations were compared at weekly intervals using a one-way analysis of variance (ANOVA). In instances where there was a non-normal distribution and/or an unequal variance, the analyses were conducted using a Kruskal-Wallis one-way ANOVA on ranks (Glantz, 1992). Differences between the control group mean or median and each treatment group mean or median were tested by Dunnett's method.

For most hematology and clinical chemistry data, the variables were analyzed using a one-way ANOVA with randomized blocks, a one-way ANOVA with randomized blocks and excluding data identified as a potential outlier, or a nonparametric analysis. Dunnett's test was used to test for differences between the control group mean and each treatment group mean. For leukocyte differentials and reticulocytes, the variables were analyzed using a one-way ANOVA, with differences between the control group mean and each exposure group mean being compared by Dunnett's method. In instances where there was a non-normal distribution and/or an unequal variance, the analyses were conducted using a Kruskal-Wallis one-way ANOVA on ranks, with differences between the control group median and each exposure group median being tested by Dunnett's method.

QUALITY ASSURANCE METHODS

The 28-day studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). The Quality Assurance Unit of the National Center for Toxicological Research performed audits and inspections of protocols, procedures, data, and reports throughout the course of the studies.

GENETIC TOXICOLOGY

Salmonella typhimurium Mutagenicity Test Protocol

Testing was performed as reported by Zeiger *et al*. (1992). Malachite green chloride was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains TA97, TA98, TA100, TA102, TA104, and TA1535 either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague Dawley rat or Syrian hamster liver) for 20 minutes at 37º 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 37º C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and five doses of malachite green chloride. The high dose was limited by toxicity.

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

Rat Bone Marrow Micronucleus Test Protocol

The standard three-exposure protocol is described in detail by Shelby *et al*. (1993). Male F344/N rats were injected intraperitoneally (three times at 24-hour intervals) with malachite green chloride dissolved in saline. Solvent control animals were injected with saline only. The positive control rats received injections of cyclophosphamide. The rats were killed 24 hours after the third injection, and blood smears were prepared from bone marrow cells obtained from the femurs. Air-dried smears were fixed and stained; 2,000 polychromatic erythrocytes (PCEs) were scored for frequency of micronucleated cells in each of five rats per dose group.

The results were tabulated as the mean of the pooled results from all animals within a treatment group plus or minus the standard error of the mean. The frequency of micronucleated cells among PCEs was analyzed by a statistical software package that tested for increasing trend over exposure groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons with each exposure 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 exposure group is less than or equal to 0.025 divided by the number of exposure 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, reproducibility of any effects observed, and the magnitudes of those effects.

Mouse Peripheral Blood Micronucleus Test

A detailed discussion of this assay is presented by MacGregor *et al*. (1990). At the end of the 28-day studies, peripheral blood smears were obtained from eight male and female mice exposed to malachite green chloride and eight female mice exposed to leucomalachite green. 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 micronuclei in 2,000 PCEs and 2,000 normochromatic erythrocytes (NCEs) in each of eight mice per exposure group. The results for PCEs and NCEs were analyzed as described for rat bone marrow PCEs.

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 results presented in the Abstract of this Toxicity Study Report represent a scientific judgement of the overall evidence for activity of the chemical in an assay.

RESULTS

RATS

All rats survived to the end of the studies (Tables 2 and 3). In the malachite green chloride study, the mean body weight gain of males in the 1,200 ppm group was significantly less than that of the controls. The final mean body weight of females in the 1,200 ppm group and the body weight gains of females in the 600 and 1,200 ppm groups were significantly less than those of the controls. In the leucomalachite green study, the final mean body weight of males in the 1,160 ppm group and the body weight gains of males in the 580 and 1,160 ppm groups were significantly less than those of the control group. There were no clinical findings related to exposure to malachite green chloride or leucomalachite green.

In the malachite green chloride study, feed consumption by all exposed groups of males and females was generally similar to that by the control groups. Exposure concentrations of 25, 100, 300, 600, and 1,200 ppm resulted in average daily doses of approximately 3, 12, 40, 70, and 175 mg malachite green chloride/kg body weight to males and 3, 12, 40, 75, and 190 mg/kg to females. In the leucomalachite green study, feed consumption by all groups of exposed rats was similar to that by the controls. Dietary concentrations of 290, 580, and 1,160 ppm resulted in average daily doses of approximately 30, 60, and 115 mg leucomalachite green/kg body weight.

TABLE 2
Survival, Body Weights, and Feed Consumption of Rats in the 28-Day Feed Study of Malachite Green Chloride

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 4
Male							
0	8/8	114 ± 7	229 ± 7	115 ± 4		19.3	20.6
25	8/8	116 ± 6	238 ± 7	123 ± 3	104	23.6	24.4
100	8/8	110 ± 9	222 ± 15	112 ± 7	97	18.5	20.0
300	8/8	115 ± 8	235 ± 10	121 ± 2	103	24.8	23.6
600	8/8	115 ± 6	232 ± 6	117 ± 5	101	17.6	20.8
1,200	8/8	115 ± 7	199 ± 8	84 ± 5***	87	21.9	23.1
Female							
0	8/8	99 ± 3	154 ± 3	56 ± 2		15.3	16.0
25	8/8	103 ± 4	155 ± 4	52 ± 2	100	17.0	16.3
100	8/8	101 ± 3	154 ± 4	54 ± 2	100	15.4	15.1
300	8/8	102 ± 3	158 ± 4	57 ± 2	103	17.7	15.6
600	8/8	101 ± 2	145 ± 3	44 ± 2***	94	18.2	13.4
1,200	8/8	102 ± 3	128 ± 3***	26 ± 2***	83	19.4	16.3

***Significantly different ($P \leq 0.001$) from the control group by Dunnett's test
[a] Number of animals surviving at 28 days/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Average feed consumption is expressed as grams per animal per day.

TABLE 3
Survival, Body Weights, and Feed Consumption of Male Rats in the 28-Day Feed Study of Leucomalachite Green

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 4
0	8/8	137 ± 5	253 ± 5	116 ± 2		20.1	22.8
290	8/8	137 ± 4	246 ± 6	109 ± 3	97	19.7	20.1
580	8/8	138 ± 4	239 ± 5	101 ± 3**	94	17.7	19.2
1,160	8/8	142 ± 4	231 ± 5*	89 ± 3***	91	16.9	21.0

* Significantly different (P≤0.05) from the control group by Dunnett's test
** P≤0.01
***P≤0.001
[a] Number of animals surviving at 28 days/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Average feed consumption is expressed as grams per animal per day.

Malachite Green Chloride: The hematology and clinical chemistry data are presented in Table C1. Hematology changes in male rats included a decrease in mean cell hemoglobin values in the 300 ppm or greater groups and a significant decrease in mean cell volumes in the 600 and 1,200 ppm groups compared to controls. In female rats, the erythrocyte count, hemoglobin and mean cell hemoglobin concentrations, and hematocrit and mean cell hemoglobin values in the 1,200 ppm group were significantly decreased. There were no significant changes in the other hematology parameters measured in female rats.

In male rats, there was a significant decrease in sorbitol dehydrogenase activity in the 1,200 ppm group compared to that in controls. In female rats, γ-glutamyltransferase activities were significantly increased in the 600 and 1,200 ppm groups and cholesterol concentrations were significantly increased in the 300 ppm or greater groups.

Additional groups of male and female rats were exposed to 0 or 1,200 ppm malachite green chloride for 4 or 21 days and blood was collected for thyroid-stimulating hormone, triiodothyronine, and thyroxine concentrations. The triiodothyronine concentration was significantly increased in 1,200 ppm female rats on day 21 (Table C2). Thyroxine concentrations were significantly decreased in 1,200 ppm females on days 4 and 21, while no significant differences were observed in thyroid-stimulating hormone concentrations. The thyroid-stimulating hormone concentration in 1,200 ppm males was significantly decreased on day 4 but not on day 21. There were no significant changes in triiodothyronine or thyroxine concentrations in exposed males compared to the controls.

The relative liver weights of 600 and 1,200 ppm males and the relative and absolute liver weights of 300 ppm or greater females were significantly greater than those of the controls (Table D1).

No gross lesions were observed that could be attributed to malachite green chloride exposure. Microscopically, the incidences of hepatocyte cytoplasmic vacuolization in 1,200 ppm males and females were significantly greater than those in the controls (males: 0 ppm, 0/8; 25 ppm, 0/8; 100 ppm, 0/8; 300 ppm, 0/8; 600 ppm, 1/8; 1,200 ppm, 4/8; females: 0/8, 0/8, 0/8, 0/8, 0/8, 7/8; Tables A1 and A2; statistical analyses not presented).

Leucomalachite Green: The hematology and clinical chemistry data are presented in Table C3. Significantly decreasing linear trends in the exposed groups were observed in erythrocyte counts, hemoglobin concentrations, and hematocrit values compared to controls (statistical analyses not presented). The hemoglobin concentration, hematocrit value, and erythrocyte count in the 1,160 ppm group were significantly lower than those in the control group. The alkaline phosphatase activity was significantly decreased in the 1,160 ppm group, while triglyceride, creatinine, albumin, and cholesterol concentrations and alanine aminotransferase activities were generally significantly decreased in all exposed groups. The phosphorus concentration and γ-glutamyltransferase activity were significantly increased in the 1,160 ppm group.

Additional groups of male rats were exposed to 0 or 1,160 ppm leucomalachite green for 4 or 21 days and blood was collected for thyroid-stimulating hormone, triiodothyronine, and thyroxine concentrations. There were significant decreases in thyroxine concentrations and significant increases in thyroid-stimulating hormone concentrations in the 1,160 ppm group at both time points (Table C4).

The absolute liver weights of 1,160 ppm males and the relative liver weights of all exposed groups were significantly greater than those of the control group (Table D2). No gross lesions were observed that could be attributed to leucomalachite green exposure. The incidences of hepatocyte cytoplasmic vacuolization were significantly increased in 580 and 1,160 ppm males (0 ppm, 0/8; 290 ppm, 2/8; 580 ppm, 5/8; 1,160 ppm, 7/8; Table A3; statistical analyses not presented). Two of eight rats exposed to 1,160 ppm and two of eight rats exposed to 580 ppm leucomalachite green had apoptotic follicular epithelial cells in the thyroid gland. Morphologic changes consisted of sloughed follicular cells with condensed nuclei located within follicles. No inflammatory reaction was present. There was evidence of follicular epithelium regeneration, since even most severely affected follicles were still lined by viable epithelium.

MICE

All mice survived to the end of the studies (Table 4 and 5). In the malachite green chloride study, the final mean body weight and body weight gain of females in the 1,200 ppm group were significantly less than those of the controls. In the leucomalachite green study, the final mean body weight of females in the 1,160 ppm group and body weight gains of females in the 580 and 1,160 ppm groups were significantly less than those of the controls. There were no clinical findings related to exposure to malachite green chloride or leucomalachite green.

In the malachite green chloride study, feed consumption by all exposed groups was similar to that by the controls. Exposure concentrations of 25, 100, 300, 600, and 1,200 ppm resulted in average daily doses of approximately 4, 18, 50, 100, and 220 mg malachite green chloride/kg body weight to males and 5, 20, 65, 120, and 250 mg/kg to females. In the leucomalachite green study, feed consumption by the 580 and 1,160 ppm mice was less than that by the controls. Dietary concentrations of 290, 580, and 1,160 ppm resulted in average daily doses of approximately 60, 110, and 220 mg leucomalachite green/kg body weight.

TABLE 4
Survival, Body Weights, and Feed Consumption of Mice in the 28-Day Feed Study of Malachite Green Chloride

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 4
Male							
0	8/8	20.8 ± 0.4	25.3 ± 0.5	4.5 ± 0.4		3.2	3.6
25	8/8	20.7 ± 0.4	25.4 ± 0.4	4.7 ± 0.4	100	3.8	3.9
100	8/8	21.3 ± 0.4	25.8 ± 0.4	4.5 ± 0.4	102	3.8	4.4
300	8/8	21.1 ± 0.3	25.7 ± 0.4	4.6 ± 0.3	102	3.7	4.1
600	8/8	21.1 ± 0.7	25.0 ± 0.4	3.9 ± 0.5	99	3.5	3.4
1,200	8/8	20.9 ± 0.8	23.8 ± 0.8	2.9 ± 0.4	94	3.8	4.0
Female							
0	8/8	17.6 ± 0.4	19.3 ± 0.4	1.7 ± 0.3		3.2	3.2
25	8/8	17.0 ± 0.3	19.2 ± 0.3	2.2 ± 0.2	99	3.8	3.8
100	8/8	17.4 ± 0.4	19.6 ± 0.4	2.2 ± 0.2	102	3.9	3.9
300	8/8	17.2 ± 0.4	19.0 ± 0.3	1.8 ± 0.2	98	4.0	4.0
600	8/8	16.9 ± 0.2	18.5 ± 0.2	1.6 ± 0.1	96	3.8	3.2
1,200	8/8	17.3 ± 0.3	17.7 ± 0.3*	0.4 ± 0.3*	92	3.7	3.5

* Significantly different ($P \leq 0.05$) from the control group by Dunnett's test
[a] Number of animals surviving at 28 days/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Average feed consumption is expressed as grams per animal per day.

TABLE 5
Survival, Body Weights, and Feed Consumption of Female Mice in the 28-Day Feed Study of Leucomalachite Green

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 4
0	8/8	16.7 ± 0.4	19.2 ± 0.4	2.5 ± 0.3		3.9	4.4
290	8/8	17.6 ± 0.3	19.1 ± 0.3	1.5 ± 0.3	99	3.2	3.3
580	8/8	17.3 ± 0.3	18.3 ± 0.4	1.0 ± 0.3**	95	3.5	3.6
1,160	8/8	17.4 ± 0.4	17.9 ± 0.4*	0.5 ± 0.4***	93	3.7	3.6

* Significantly different ($P \leq 0.05$) from the control group by Dunnett's test
** $P \leq 0.01$
***$P \leq 0.001$
[a] Number of animals surviving at 28 days/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Average feed consumption is expressed as grams per animal per day.

Malachite Green Chloride: The hematology and clinical chemistry data are presented in Table C5. In male and female mice, there were significant linear decreases in erythrocyte counts, hematocrit values, and hemoglobin concentrations (statistical analyses not presented). The erythrocyte counts, hematocrit values, and hemoglobin concentrations were significantly decreased in 300 ppm or greater males compared to the controls. The mean cell volume, mean cell hemoglobin value, and reticulocyte count were significantly increased in 1,200 ppm males. The erythrocyte counts were significantly decreased in 100 ppm or greater females compared to the controls. Hemoglobin concentrations were significantly decreased in 300 ppm or greater females and hematocrit values were significantly decreased in the 600 and 1,200 ppm females. The mean cell volumes and reticulocyte counts were significantly increased in 300 ppm or greater females.

In male mice, creatinine concentrations were significantly decreased in the 300 ppm or greater groups compared to the controls. Creatinine concentrations in 600 and 1,200 ppm females were significantly decreased, while bile acid concentrations were significantly decreased in 300 ppm or greater females.

Significant decreases in absolute kidney weight occurred in female mice exposed to 600 or 1,200 ppm malachite green chloride, which may reflect the overall decrease in mean body weight. No gross or microscopic lesions were observed that could be attributed to malachite green chloride exposure.

Leucomalachite Green: The hematology and clinical chemistry data are presented in Table C6. Total protein concentrations were significantly decreased in the 580 and 1,160 ppm groups compared to those in the controls. There were no significant changes in other parameters measured in mice.

No gross lesions were observed that could be attributed to leucomalachite green exposure. The incidence of multifocal apoptosis in the transitional epithelium of the urinary bladder was significantly increased in 1,160 ppm females (0 ppm, 0/8; 290 ppm, 0/8; 580 ppm, 0/7; 1,160 ppm, 8/8; Table B3; statistical analyses not presented).

GENETIC TOXICOLOGY

Malachite green chloride was tested for mutagenicity in bacteria and for chromosomal effects in mammalian cells *in vivo*; all results were negative. Malachite green chloride (0.1-10.0 μg/plate) was not mutagenic in *Salmonella typhimurium* strain TA97, TA98, TA100, TA102, TA104, or TA1535, with or without induced rat or hamster liver S9 activation enzymes (Table E1). Results of a micronucleus test with malachite green chloride in rat bone marrow cells following three intraperitoneal injections at doses ranging from 1.094 to 8.750 mg/kg were negative (Table E2). Although the frequency of micronucleated polychromatic erythrocytes (PCEs) at the intermediate dose of 4.375 mg/kg malachite green chloride was significantly greater than the control frequency, the increase was very small; also, the frequency of micronucleated PCEs was not significant at 8.750 mg/kg and no bone marrow toxicity was detected at this dose. Therefore, the bone marrow micronucleus test in rats was judged to be negative overall. A peripheral blood micronucleus test was performed in male and female mice after 28 days of exposure to malachite green chloride in feed (25-1,200 ppm), and results in males and females were negative for normochromatic erythrocytes (NCEs) (Table E3). A peripheral blood micronucleus test was also performed in female mice after 28 days of exposure to leucomalachite green in feed; significant increases in the frequency of micronucleated NCEs were observed in the 290 and 580 ppm groups (Table E4). The trend P value was not significant due to a downturn in micronucleated NCEs in the 1,160 ppm group; dropping the 1,160 ppm data and reanalyzing the remaining data yielded a trend P value of 0.001, which is significant. The 28-day exposure period that was used in these studies is just short of the 30- to 35-day time period required for the circulating NCE population to attain equilibrium, a factor more critical to the interpretation of negative data than positive data. In an effort to confirm the results seen in NCE frequencies, the PCE populations were scored for frequency of micronucleated cells. For malachite green chloride, there was no indication of an increase and the frequency of micronucleated PCEs was unchanged, lending greater confidence to the negative call. For leucomalachite green, a small increase in the frequency of micronucleated PCEs was observed in the 290 and 580 ppm groups, patterning

the response seen in NCEs; however, the results were not significant. Nonetheless, the effects seen in NCEs were sufficient to conclude that the result of the micronucleus test with leucomalachite green was positive.

DISCUSSION

Male and female F344/N Nctr BR rats and B6C3F$_1$/Nctr BR (C57BL/6N × C3H/HeN MTV$^-$) mice were fed diets containing 0, 25, 100, 300, 600, or 1,200 ppm malachite green chloride or 0, 290, 580, or 1,160 ppm leucomalachite green (male rats and female mice only) for 28 days to determine the toxicity of malachite green chloride and leucomalachite green, to determine the appropriate doses to be used in 2-year studies, and to compare the biological effects of the administration of malachite green chloride to those of leucomalachite green.

A significant reduction in mean body weight gain occurred in male and female rats exposed to 1,200 ppm malachite green chloride and in female rats exposed to 600 ppm malachite green chloride compared to that in the control group. Further data indicate that the decreases in mean body weight gain was due to a toxic response. Increased liver-weight-to-body weight ratios were present in 600 and 1,200 ppm male and 300 ppm or greater female rats, suggesting a liver abnormality. Liver toxicity was indicated by the significantly increased incidences of hepatocyte cytoplasmic vacuolization in 1,200 ppm male and female rats. The increase in γ-glutamyltransferase activities in female rats exposed to 600 or 1,200 ppm malachite green chloride probably reflect liver toxicity seen microscopically. Furthermore, in a 28-day ancillary study by Culp *et al.* (1999), demethylated derivatives of malachite and leucomalachite green were observed by mass spectrometry and high-performance liquid chromatography analyses of livers from rats exposed to malachite green and male rats exposed to leucomalachite green. In addition, ^{32}P-postlabeling of liver DNA from rats exposed to either malachite green or leucomalachite green indicated the formation of a single major DNA adduct, which increased with increasing dose.

In addition to the pathologic changes observed in the present study for rats exposed to malachite green chloride, a number of statistically significant clinical chemistry changes were observed in the dosed groups as compared to the control groups. In 1,200 ppm female rats, significant decreases in three related parameters (erythrocyte count, hemoglobin concentration, and hematocrit value) were observed. In addition, a significant decreasing trend was observed for these parameters (statistical analyses not presented). These data are indicative of anemia.

In female rats exposed to 1,200 ppm malachite green chloride, decreased thyroxine concentrations were observed, suggesting thyroid dysfunction. However, thyroid-stimulating hormone concentrations were unchanged and triiodothyronine concentrations increased, which is not consistent with primary thyroid abnormality. Male rats

exposed to leucomalachite green had decreased thyroxine concentrations, while thyroid-stimulating hormone concentrations were significantly increased, indicating a primary thyroid abnormality.

In male and female mice exposed to malachite green chloride, there were no changes in feed consumption, although sporadic increases were observed throughout the study. The intermittent changes may reflect the small number of cages (2 for each exposure group) and the tendency of young rodents to scatter their feed. A significant reduction in mean body weight gain was observed in female mice exposed to 1,200 ppm malachite green chloride at week 4 compared to that in the control group. No significant decrease was observed in the male mice exposed to malachite green chloride, although the mean body weight of 1,200 ppm males was generally lower than that of the control group. Significant decreases in absolute kidney weight occurred in the female mice exposed to 600 or 1,200 ppm malachite green chloride, which may reflect the overall decrease in mean body weight.

In mice exposed to malachite green chloride, a number of significant clinical chemistry changes were observed in exposed groups. As observed in female rats exposed to malachite green chloride, a significant decrease was observed for three related parameters (erythrocyte counts, hematocrit values, and hemoglobin concentrations) in male and female mice exposed to malachite green chloride. Decreasing dose trends in these parameters were also observed in male and female mice (statistical analyses not presented). These data indicate a mild anemia. A significant increase in reticulocyte counts suggests a regenerative anemia. Additional changes to clinical chemistry parameters did not appear to be biologically significant, as they did not fit a pattern of abnormalities that would indicate a specific clinical condition.

As summarized in Tables 6 and 7, female rats and mice appear to be more sensitive than males to the effects of malachite green chloride. A number of effects observed in female rats exposed to malachite green chloride were not seen in male rats. In mice exposed to malachite green chloride, similar effects were observed in both sexes. However, when no outliers were excluded from the analysis, effects were generally observed at a lower dose in female mice than in male mice (statistical analyses not presented). This indicates that, at a minimum, female rats and mice should be included in a 2-year study.

The data also indicate that an exposure concentration of 1,200 ppm is too high for a 2-year study in rats exposed to malachite green chloride. Mean body weights of male and female rats exposed to 1,200 ppm malachite green chloride were decreased. In addition, changes in the erythrocyte count; hematocrit and mean cell hemoglobin values; hemoglobin, mean cell hemoglobin, triidothyronine and thyroxine concentrations; and γ-glutamyltransferase activity as well as hepatocyte vacuolization occurred in female rats exposed to 1,200 ppm malachite green chloride. Therefore, it is recommended that the highest exposure concentration selected for a

TABLE 6
Summary of Effects Observed in Male and Female Rats Exposed to Malachite Green Chloride or Leucomalachite Green for 28 Days

Effect	Lowest Dose Producing a Significant Effect[a]		
	Male Rats Exposed to Malachite Green Chloride	Female Rats Exposed to Malachite Green Chloride	Male Rats Exposed to Leucomalachite Green
Body weight	decrease, 1,200 ppm	decrease, 1,200 ppm	decrease, 580 ppm
Relative liver weight	increase, 600 ppm	increase, 300 ppm	increase, 290 ppm
Erythrocyte count	ns[b]	decrease, 1,200 ppm	decrease, 1,160 ppm
Hemoglobin concentration	decrease, 1,200 ppm	decrease, 1,200 ppm	decrease, 1,160 ppm
Hematocrit value	ns	decrease, 1,200 ppm	decrease, 1,160 ppm
Mean cell hemoglobin value	decrease, 300 ppm	decrease, 1,200 ppm	ns
Mean cell hemoglobin concentration	decrease, 1,200 ppm	decrease, 1,200 ppm	ns
Phosphorus concentration	ns	ns	increase, 1,160 ppm
γ-Glutamyltransferase activity	ns	increase, 600 ppm	increase, 1,160 ppm
Triiodothyronine concentration[c]	ns	increase, 1,200 ppm	ns
Thyroxine concentration[c]	ns	decrease, 1,200 ppm	decrease, 1,160 ppm
Thyroid-stimulating hormone concentration[c]	increase, 1,200 ppm	ns	increase, 1,160 ppm
Hepatocyte vacuolization	increase, 1,200 ppm[d]	increase, 1,200 ppm[d]	increase, 580 ppm[d]

[a] Dose at which the effect observed becomes significantly different from that of the control group
[b] No significant difference from the control group
[c] Measured for rats exposed to 0 or 1,200 ppm malachite green chloride and 0 or 1,160 ppm leucomalachite green
[d] Statistical analyses not presented

TABLE 7
Summary of Effects Observed in Male and Female Mice Exposed to Malachite Green Chloride or Leucomalachite Green for 28 Days

Effect	Lowest Dose Producing a Significant Effect[a]		
	Male Mice Exposed to Malachite Green Chloride	Female Mice Exposed to Malachite Green Chloride	Female Mice Exposed to Leucomalachite Green
Body weight	ns[b]	decrease, 1,200 ppm	decrease, 580 ppm
Relative liver weight	ns	ns	increase, 1,160 ppm
Erythrocyte count	decrease, 300 ppm	decrease, 100 ppm	ns
Hemoglobin concentration	decrease, 300 ppm	decrease, 300 ppm	ns
Hematocrit value	decrease, 300 ppm	decrease, 600 ppm	ns
Reticulocyte count	increase, 1,200 ppm	increase, 300 ppm	ns
Apoptosis of the bladder	not observed	not observed	increase, 1,160 ppm

[a] Dose at which the effect observed becomes significantly different from that of the control group
[b] No significant difference from the control group

2-year rat study not exceed 600 ppm malachite green chloride. In mice, 600 ppm is more problematic. In female mice exposed to 600 ppm malachite green chloride for 28 days, there were significant decreases in erythrocyte count, hemoglobin concentration, and hematocrit value. A significant increase in reticulocyte count was observed in female mice exposed to 300 ppm malachite green chloride. Based upon these observations, it is recommended that the highest exposure concentration selected for a 2-year mouse study not exceed 600 ppm malachite green chloride, with consideration given to the use of 450 ppm as the maximum dose.

In the leucomalachite green study, a significant reduction in mean body weight gain occurred in 580 and 1,160 ppm males compared to the controls. Other data indicate that the decrease in mean body weight gain was due to a toxic response. Specifically, increased liver-weight- to-body-weight ratios occurred in all exposed groups. Hepatocyte vacuolization was present in all groups of rats exposed to leucomalachite green, including seven of eight rats exposed to 1,160 ppm. Exposure concentration-related increases in γ-glutamyltransferase activities in the rats exposed to leucomalachite green may reflect liver changes seen microscopically.

Other histopathologic data revealed apoptotic follicular epithelial cells in the thyroid gland of two of eight rats in each of the groups exposed to 580 or 1,160 ppm leucomalachite green. Interestingly, thyroid gland tumors were observed in a 2-year study in male and female F344 rats exposed to gentian violet (Littlefield, 1988). Treatment-related increases in the incidences of follicular cell adenocarcinoma of the thyroid gland were observed in the males at incidences of 1%, 5%, 3%, and 6% and in females at incidences of 1%, 1%, 5%, and 8% after being exposed to 0, 100, 300, and 600 ppm gentian violet, respectively. In the present leucomalachite green study, thyroid-stimulating hormone, triiodothyronine, and thyroxine concentrations were analyzed on days 4 and 21 for male rats exposed to 0 or 1,160 ppm leucomalachite green. In the 1,160 ppm group, thyroxine concentrations were decreased while thyroid-stimulating hormone concentrations were significantly increased compared to controls. A decrease in thyroxine concentration is consistent with hypothyroidism, but does not preclude other causes of low circulating thyroxine, such as decreased pituitary function, alterations in protein binding of thyroxine, and alterations in peripheral metabolism of thyroxine. However, in combination with an increase in thyroid-stimulating hormone concentrations, these findings suggest that pituitary function was normal in rats with decreased thyroxine levels and that primary hypothyroidism was the most likely cause of reduced thyroxine concentrations. These data suggest leucomalachite green may have a potential harmful effect on the thyroid gland at high doses, as was the case with gentian violet.

In addition to the pathologic changes observed in male rats exposed to leucomalachite green, several statistically significant clinical chemistry changes were observed in exposed groups. There was a decreasing trend in three related parameters (erythrocyte count, hemoglobin concentration, and hematocrit value; Culp *et al.*, 1998). These

data indicate a mild normocytic-normochromic anemia. A significant increase in phosphorus concentration occurred, suggesting the anemia may have been due to intravascular hemolysis.

In female mice exposed to leucomalachite green, differences in feed consumption between the control and exposed groups were irregular throughout the 28-day study. This may reflect the small number of cages (two for each exposure group) and the tendency of young rodents to scatter their feed. A significant reduction in mean body weight gain was observed in female mice exposed to 580 or 1,160 ppm leucomalachite green as compared to the control group.

An increased liver-weight-to-body-weight ratio was observed in mice in the 1,160 ppm group, suggesting a toxic response to the liver at that exposure concentration. As noted earlier, demethylated derivatives of leucomalachite green and malachite green were observed by mass spectroscopy and high-performance liquid chromatography analyses of livers from mice fed leucomalachite green for 28 days (Culp *et al.*, 1999).

Other histopathologic data from the present leucomalachite green study revealed that all female mice exposed to 1,160 ppm leucomalachite green had apoptosis of the transitional epithelium of the urinary bladder. The apoptotic cells were phagocytized by neighboring transitional epithelial cells and appeared to undergo dissolution in phagocytic vacuoles (Culp *et al.*, 1999).

In addition to the pathologic changes observed in 1,160 ppm female mice, several statistically significant clinical chemistry changes were observed in exposed groups. The changes did not appear to be biologically significant, as they did not fit a pattern of abnormalities that would indicate a specific clinical condition.

Due to the decreased mean body weights and changes in clinical chemistry and pathology observed in the leucomalachite green rat and mouse studies, an exposure concentration of 1,160 ppm is considered too high for use in 2-year studies. Based on the body weight data, in which effects were observed at 580 ppm in rats, and exposure concentration-related changes in several clinical chemistry parameters, 580 ppm leucomalachite green is recommended as the maximum exposure concentration for a 2-year study.

In addition to determining recommended exposure concentrations for 2-year feed studies, the effects of malachite green chloride on rodents were compared to those of leucomalachite green. Male rats and female mice were exposed to similar molar concentrations of leucomalachite green as compared to malachite green. Specific changes were observed (Table 6). In general, leucomalachite green caused more changes in male rats and female mice than malachite green chloride. Moreover, all lesions observed in malachite green chloride-exposed animals were

observed in leucomalachite green-exposed animals (except anemia in mice exposed to malachite green chloride). Hepatocyte vacuolization was generally more extensive in leucomalachite green-exposed rats (observed in all exposed groups) compared to malachite green chloride-exposed rats (observed in male rats exposed to 600 and 1,200 ppm and female rats exposed to 1,200 ppm malachite green chloride), and additional lesions were observed in rodents exposed to leucomalachite green. Specifically, apoptosis of the transitional epithelium of the urinary bladder was observed in all female mice exposed to 1,160 ppm leucomalachite green, but not in female or male mice exposed to 1,200 ppm malachite green chloride. Changes in mean body weights and relative liver weights occurred at lower exposure concentrations of leucomalachite green than of malachite green chloride. Increased thyroid-stimulating hormone concentrations and decreased thyroxine concentrations indicated hypothyroidism in male rats exposed to 1,160 ppm leucomalachite green for 4 or 21 days. However, changes observed in thyroxine, thyroid-stimulating hormone, and triiodothyronine concentrations in rats exposed to malachite green chloride were not consistent with primary thyroid abnormality. These data substantiate a recommendation that a 2-year feed study be conducted with leucomalachite green and malachite green chloride and indicate that exposure to leucomalachite green may be more harmful to rodents.

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APPENDIX A
SUMMARY OF LESIONS IN RATS

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 28-Day Feed Study of Malachite Green Chloride[a]

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Disposition Summary						
Animals initially in study	8	8	8	8	8	8
Survivors						
Terminal sacrifice	8	8	8	8	8	8
Animals examined microscopically	8	8	8	8	8	8
Alimentary System						
Liver	(8)	(8)	(8)	(8)	(8)	(8)
Developmental malformation					3 (38%)	
Vacuolization cytoplasmic, hepatocyte					1 (13%)	4 (50%)
Salivary glands	(8)		(1)			(8)
Cytoplasmic alteration, parotid gland	5 (63%)		1 (100%)			7 (88%)
Inflammation, focal, parotid gland, interstitium						1 (13%)
Cardiovascular System						
Heart	(8)					(8)
Cardiomyopathy, focal	2 (25%)					1 (13%)
Cardiomyopathy, multifocal	3 (38%)					3 (38%)
Endocrine System						
Adrenal gland	(8)					(8)
Hyperplasia, focal, cortex	1 (13%)					
Vacuolization cytoplasmic, focal						1 (13%)
Pituitary gland	(8)	(8)	(8)	(8)	(8)	(6)
Cyst						1 (17%)
Degeneration, pars distalis				1 (13%)		
Thyroid gland	(8)	(7)	(8)	(8)	(8)	(8)
Degeneration, follicle	1 (13%)					1 (13%)
Ultimobranchial cyst		1 (14%)	1 (13%)			
General Body System						
None						
Genital System						
Coagulating gland	(8)					(6)
Inflammation, focal						1 (17%)
Preputial gland	(8)					(7)
Inflammation, multifocal	2 (25%)					
Prostate	(8)					(7)
Apoptosis						1 (14%)
Hematopoietic System						
None						
Integumentary System						
None						

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 28-Day Feed Study of Malachite Green Chloride

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Musculoskeletal System						
None						
Nervous System						
Brain, cerebrum	(8)					(8)
Hydrocephalus						1 (13%)
Respiratory System						
Lungs	(8)					(8)
Infiltration cellular, lymphocytic, focal, pleura						2 (25%)
Nose	(8)					(8)
Hyperplasia, multifocal, mucosa						1 (13%)
Trachea	(8)					(8)
Inflammation						1 (13%)
Special Senses System						
Eye	(8)					(8)
Degeneration, unilateral, retina						1 (13%)
Harderian gland	(8)					(8)
Inflammation, focal, interstitium	1 (13%)					3 (38%)
Inflammation, multifocal, interstitium						1 (13%)
Urinary System						
Kidney	(8)					(8)
Inflammation, focal	1 (13%)					1 (13%)

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

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 28-Day Feed Study of Malachite Green Chloride[a]

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Disposition Summary						
Animals initially in study	8	8	8	8	8	8
Survivors						
Terminal sacrifice	8	8	8	8	8	8
Animals examined microscopically	8	8	8	8	8	8
Alimentary System						
Liver	(8)	(8)	(8)	(8)	(8)	(8)
Developmental malformation				1 (13%)	1 (13%)	
Vacuolization cytoplasmic, hepatocyte						7 (88%)
Salivary glands	(8)					(8)
Cytoplasmic alteration, parotid gland	4 (50%)					8 (100%)
Cardiovascular System						
Heart	(8)					(7)
Cardiomyopathy, focal	2 (25%)					1 (14%)
Cardiomyopathy, multifocal	1 (13%)					
Endocrine System						
Pituitary gland	(8)	(8)	(8)	(8)	(8)	(8)
Degeneration, pars intermedia				1 (13%)		
Thyroid gland	(6)	(8)	(8)	(8)	(8)	(6)
Apoptosis, unilateral						1 (17%)
Ultimobranchial cyst		1 (13%)				1 (17%)
General Body System						
None						
Genital System						
Clitoral gland	(8)					(8)
Inflammation, multifocal, interstitium						2 (25%)
Ovary	(8)			(2)		(8)
Cyst				1 (50%)		
Hyperplasia, interstitial cell						2 (25%)
Uterus	(8)			(1)		(8)
Dilatation, bilateral				1 (100%)		
Hematopoietic System						
None						
Integumentary System						
None						
Musculoskeletal System						
None						

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 28-Day Feed Study of Malachite Green Chloride

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Nervous System						
None						
Respiratory System						
Lung, right	(8)					(8)
Infiltration cellular, histiocytic, focal						1 (13%)
Special Senses System						
Harderian gland	(8)					(8)
Inflammation, multifocal, interstitium	1 (13%)					4 (50%)
Lacrimal gland	(8)					(8)
Degeneration						1 (13%)
Inflammation, focal, interstitium	1 (13%)					
Inflammation, multifocal, interstitium	1 (13%)					
Urinary System						
Kidney	(8)					(8)
Inflammation, focal						1 (13%)
Mineralization	8 (100%)					6 (75%)
Urinary bladder	(8)					(8)
Inflammation, focal	1 (13%)					

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

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 28-Day Feed Study of Leucomalachite Green[a]

	0 ppm	290 ppm	580 ppm	1,160 ppm
Disposition Summary				
Animals initially in study	8	8	8	8
Survivors				
Terminal sacrifice	8	8	8	8
Animals examined microscopically	8	8	8	8
Alimentary System				
Liver	(8)	(8)	(8)	(8)
Vacuolization cytoplasmic, hepatocyte		2 (25%)	5 (63%)	7 (88%)
Cardiovascular System				
Heart	(8)			(8)
Cardiomyopathy, focal	1 (13%)			2 (25%)
Cardiomyopathy, multifocal	1 (13%)			1 (13%)
Endocrine System				
Pituitary gland	(8)	(6)	(8)	(7)
Cyst, pars distalis			1 (13%)	
Thyroid gland	(7)	(7)	(8)	(8)
Apoptosis, focal, follicular cell			1 (13%)	
Apoptosis, multifocal, follicular cell			1 (13%)	2 (25%)
Concretion, focal		1 (14%)		
General Body System				
None				
Genital System				
Prostate	(8)			(8)
Inflammation, multifocal, interstitium	2 (25%)			1 (13%)
Hematopoietic System				
None				
Integumentary System				
None				
Musculoskeletal System				
None				
Nervous System				
None				

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 28-Day Feed Study of Leucomalachite Green

	0 ppm	290 ppm	580 ppm	1,160 ppm
Respiratory System				
Lungs	(8)			(8)
Infiltration cellular, histiocytic, focal, subpleura	1 (13%)			1 (13%)
Infiltration cellular, lymphocytic, focal, subpleura	2 (25%)			1 (13%)
Infiltration cellular, lymphocytic, multifocal, subpleura	1 (13%)			
Special Senses System				
Harderian gland	(8)			(8)
Inflammation, focal, interstitium				1 (13%)
Inflammation, multifocal, interstitium				2 (25%)
Urinary System				
None				

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

APPENDIX B
SUMMARY OF LESIONS IN MICE

TABLE B1
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 28-Day Feed Study of Malachite Green Chloride[a]

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Disposition Summary						
Animals initially in study	8	8	8	8	8	8
Survivors						
Terminal sacrifice	8	8	8	8	8	8
Animals examined microscopically	8	8	8	8	8	8
Alimentary System						
Gallbladder	(5)					(7)
Inflammation	1 (20%)					
Liver	(8)	(8)	(8)	(8)	(8)	(8)
Inflammation, focal						1 (13%)
Vacuolization cytoplasmic, focal	2 (25%)		1 (13%)	2 (25%)	2 (25%)	3 (38%)
Cardiovascular System						
None						
Endocrine System						
Adrenal gland	(8)					(8)
Cyst	1 (13%)					
Hyperplasia, focal, cortex						1 (13%)
Pituitary gland	(8)	(8)	(8)	(7)	(8)	(6)
Cyst	1 (13%)					
Thyroid gland	(4)	(8)	(6)	(8)	(8)	(3)
Cyst			1 (17%)			
General Body System						
None						
Genital System						
Epididymis	(8)					(8)
Aspermia	1 (13%)					
Preputial gland	(8)		(3)	(3)		(8)
Cyst	1 (13%)		3 (100%)	3 (100%)		
Prostate	(7)					(7)
Inflammation	1 (14%)					
Testes	(8)					(8)
Degeneration	3 (38%)					1 (13%)
Hematopoietic System						
None						
Integumentary System						
None						
Musculoskeletal System						
None						

TABLE B1
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 28-Day Feed Study of Malachite Green Chloride

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Nervous System						
None						
Respiratory System						
None						
Special Senses System						
None						
Urinary System						
Kidney	(8)					(8)
Inflammation	3 (38%)					4 (50%)
Urinary bladder	(8)	(8)	(8)	(8)	(8)	(6)
Inflammation	2 (25%)			1 (13%)	3 (38%)	

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

TABLE B2
Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Female Mice in the 28-Day Feed Study of Malachite Green Chloride[a]

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Disposition Summary						
Animals initially in study	8	8	8	8	8	8
Survivors						
Terminal sacrifice	8	8	8	8	8	8
Animals examined microscopically	8	8	8	8	8	8
Alimentary System						
Liver	(8)	(8)	(8)	(8)	(8)	(8)
Vacuolization cytoplasmic, focal	2 (25%)			2 (25%)		1 (13%)
Cardiovascular System						
None						
Endocrine System						
None						
General Body System						
None						
Genital System						
Uterus	(8)					(8)
Dilatation, bilateral	1 (13%)					
Vagina	(8)					(8)
Polyp	1 (13%)					
Hematopoietic System						
None						
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
Brain, cerebellum	(6)					(6)
Proliferation, focal, glial cell						1 (17%)

TABLE B2
Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Female Mice in the 28-Day Feed Study of Malachite Green Chloride

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Respiratory System						
Lung	(8)					(8)
Infiltration, cellular, lymphocytic, focal, subpleura	1 (13%)					
Nose	(8)					(8)
Exudate, nasolacrimal duct						1 (13%)
Special Senses System						
Harderian gland	(7)					(7)
Inflammation, multifocal, interstitium	1 (14%)					
Urinary System						
Kidney	(8)					(8)
Inflammation						1 (13%)
Urinary bladder	(8)	(8)	(8)	(8)	(8)	(8)
Inflammation	4 (50%)	4 (50%)	3 (38%)	1 (13%)		2 (25%)

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

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 28-Day Feed Study of Leucomalachite Green[a]

	0 ppm	290 ppm	580 ppm	1,160 ppm
Disposition Summary				
Animals initially in study	8	8	8	8
Survivors				
Terminal sacrifice	8	8	8	8
Animals examined microscopically	8	8	8	8
Alimentary System				
Liver	(8)	(8)	(8)	(8)
Left lateral lobe, necrosis, multifocal, hepatocyte	1 (13%)			
Median lobe, vacuolization cytoplasmic, focal, hepatocyte	1 (13%)	2 (25%)		2 (25%)
Salivary glands	(8)			(8)
Inflammation, focal, interstitium	1 (13%)			
Cardiovascular System				
Heart	(8)			(8)
Inflammation, focal, perivascular	1 (13%)			1 (13%)
Endocrine System				
Thyroid gland	(8)	(7)	(4)	(6)
Degeneration, focal, follicular cell				2 (33%)
General Body System				
None				
Genital System				
None				
Hematopoietic System				
None				
Integumentary System				
None				
Musculoskeletal System				
None				
Nervous System				
None				
Respiratory System				
Lung	(8)			(8)
Inflammation, multifocal, perivascular	1 (13%)			1 (13%)

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 28-Day Feed Study of Leucomalachite Green

	0 ppm	290 ppm	580 ppm	1,160 ppm
Special Senses System				
None				
Urinary System				
Kidney	(8)			(8)
Inflammation, focal, interstitium	2 (25%)			
Urinary bladder	(8)	(8)	(7)	(8)
Apoptosis, multifocal, transitional epithelium				8 (100%)

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

APPENDIX C
CLINICAL PATHOLOGY RESULTS

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 28-Day Feed Study of Malachite Green Chloride[a]

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Male						
Hematology						
n	8	8	8	8	7	7
Hematocrit (%)	46.5 ± 0.6	46.3 ± 0.3	46.2 ± 0.6	45.9 ± 0.6[b]	46.0 ± 0.6	44.9 ± 0.4
Hemoglobin (g/dL)	17.2 ± 0.3	17.0 ± 0.2	17.2 ± 0.2[b]	16.7 ± 0.3[b]	16.9 ± 0.3	16.3 ± 0.1*
Erythrocytes (10^6/µL)	8.81 ± 0.14	8.81 ± 0.08	8.87 ± 0.11[b]	8.75 ± 0.12[b]	8.84 ± 0.13	8.59 ± 0.09
Reticulocytes (%)[c]	2.0 ± 0.2	1.8 ± 0.2	2.3 ± 0.1	2.2 ± 0.2	2.0 ± 0.2	2.2 ± 0.3[d]
Mean cell volume (fL)	52.8 ± 0.2	52.6 ± 0.2	52.7 ± 0.3[e]	52.6 ± 0.2	52.0 ± 0.1*	52.1 ± 0.1*
Mean cell hemoglobin (pg)	19.5 ± 0.1	19.3 ± 0.1	19.5 ± 0.1[b]	19.0 ± 0.1***[b]	19.2 ± 0.1*[e]	19.0 ± 0.1***
Mean cell hemoglobin concentration (g/dL)	37.1 ± 0.2[b]	36.7 ± 0.2	37.0 ± 0.2[b]	36.3 ± 0.2***	36.7 ± 0.2	36.3 ± 0.2*
Platelets (10^3/µL)	677.6 ± 10.5	682.6 ± 12.7	716.5 ± 19.0[e]	704.6 ± 10.2	714.7 ± 12.3	695.3 ± 25.1[e]
Leukocytes (10^3/µL)	8.06 ± 0.42	6.53 ± 0.54	8.30 ± 0.50	5.43 ± 0.49**[b]	8.51 ± 0.40	6.45 ± 0.74[e]
Segmented neutrophils (%)[c]	13.9 ± 1.6	12.4 ± 1.0	12.3 ± 2.7	16.1 ± 1.6	11.4 ± 1.5	11.0 ± 0.5[d]
Lymphocytes (%)[c]	85.5 ± 1.8	86.6 ± 1.1	86.8 ± 2.9	83.4 ± 1.7	88.3 ± 1.5	88.6 ± 0.5[d]
Monocytes (%)[c]	0.30 ± 0.16	0.13 ± 0.13	0.80 ± 0.31	0.30 ± 0.16	0.00 ± 0.00	0.00 ± 0.00[d]
Eosinophils (%)[c]	0.4 ± 0.3	0.9 ± 0.3	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.2	0.4 ± 0.2[d]
Clinical Chemistry						
n	8	8	8	8	8	8
Urea nitrogen (mg/dL)	14.1 ± 0.4	14.8 ± 0.4	15.0 ± 0.4	13.9 ± 0.3[b]	15.5 ± 0.3[b]	15.8 ± 0.6[b]
Creatinine (mg/dL)	0.70 ± 0.03[b]	0.60 ± 0.02**	0.71 ± 0.03	0.67 ± 0.03[e]	0.66 ± 0.02	0.71 ± 0.03
Glucose (mg/dL)	104 ± 3	109 ± 4	116 ± 8[b]	97 ± 2	103 ± 5[b]	95 ± 6[b]
Sodium (mmol/L)	155 ± 1	155 ± 1[b]	155 ± 1	155 ± 1	154 ± 1[b]	154 ± 0[e]
Potassium (mmol/L)	6.5 ± 0.3	6.3 ± 0.2	6.5 ± 0.1	6.2 ± 0.1	6.6 ± 0.2[b]	6.3 ± 0.2
Chloride (mmol/L)	101 ± 1	100 ± 1	101 ± 1[b]	98 ± 2	100 ± 2	99 ± 1
Calcium (mg/dL)	11.13 ± 0.48[b]	12.10 ± 0.61	11.97 ± 0.27[b]	11.51 ± 0.47	11.61 ± 0.62	11.43 ± 0.40
Phosphorus (mg/dL)	9.1 ± 0.4	8.7 ± 0.7[e]	9.0 ± 0.3	9.2 ± 0.2	9.7 ± 0.6[e]	9.4 ± 0.3
Total protein (g/dL)	6.5 ± 0.1[e]	6.6 ± 0.1	6.5 ± 0.1	6.7 ± 0.1[b]	6.5 ± 0.1	6.4 ± 0.1[b]
Albumin (g/dL)	3.9 ± 0.1	3.9 ± 0.1	3.8 ± 0.1[b]	3.9 ± 0.1	3.8 ± 0.1	3.7 ± 0.1
Cholesterol (mg/dL)	49 ± 2	49 ± 1	55 ± 2*[e]	51 ± 1	44 ± 1	53 ± 1[b]
Triglycerides (mg/dL)	87 ± 8[d]	83 ± 3	100 ± 6	82 ± 7	84 ± 6[b]	91 ± 8
Alanine aminotransferase (µg/L)	45 ± 3	40 ± 4	44 ± 3	40 ± 2	38 ± 3[b]	40 ± 2
Alkaline phosphatase (µg/L)	292 ± 12	291 ± 10	266 ± 12	288 ± 10	283 ± 12	254 ± 12
Aspartate aminotransferase (µg/L)	89 ± 2	81 ± 4	90 ± 3	83 ± 4	82 ± 6[b]	88 ± 4[b]
Creatine kinase (µg/L)	241 ± 18	194 ± 26[b]	285 ± 32	209 ± 21[e]	208 ± 21[b]	295 ± 35
Sorbitol dehydrogenase (µg/L)	14 ± 1	15 ± 2	12 ± 1	15 ± 2[b]	14 ± 2	9 ± 3*
γ-Glutamyltransferase (µg/L)	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1[b]	0.5 ± 0.1[b]	0.5 ± 0.1	1.2 ± 0.3
Bile acids (µmol/L)	16.1 ± 2.6	22.0 ± 4.5	16.3 ± 3.0	14.2 ± 2.4*	15.7 ± 4.0**	15.3 ± 2.6

TABLE C1
Hematology and Clinical Chemistry Data for Rats in the 28-Day Feed Study of Malachite Green Chloride

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Female						
Hematology						
n	7	7	7	8	8	8
Hematocrit (%)	48.8 ± 0.4	48.8 ± 0.5	48.7 ± 0.3	47.5 ± 0.3[e]	47.3 ± 0.5	46.8 ± 0.4**[b]
Hemoglobin (g/dL)	17.8 ± 0.1	17.9 ± 0.1	17.8 ± 0.1	17.6 ± 0.2	17.4 ± 0.2[b]	16.4 ± 0.1***[b]
Erythrocytes (10^6/μL)	8.88 ± 0.07	8.96 ± 0.07	8.84 ± 0.05	8.66 ± 0.10[b]	8.66 ± 0.09	8.56 ± 0.07*[e]
Reticulocytes (%)[c]	2.0 ± 0.2	1.9 ± 0.1	1.8 ± 0.1	1.7 ± 0.2	1.8 ± 0.1	1.9 ± 0.2
Mean cell volume (fL)	54.9 ± 0.1	54.3 ± 0.2[d]	55.1 ± 0.2	55.5 ± 0.1[b]	54.8 ± 0.2[b]	54.3 ± 0.2
Mean cell hemoglobin (pg)	20.0 ± 0.1	20.0 ± 0.0	20.1 ± 0.1	20.1 ± 0.1[b]	20.0 ± 0.1[b]	19.3 ± 0.1***[b]
Mean cell hemoglobin concentration (g/dL)	36.5 ± 0.2	36.8 ± 0.1	36.5 ± 0.1	36.4 ± 0.2	36.5 ± 0.2	35.6 ± 0.2***[b]
Platelets (10^3/μL)	723.6 ± 16.5	694.3 ± 28.5	709.7 ± 15.2	714.3 ± 7.1	730.8 ± 11.6	658.5 ± 19.6[e]
Leukocytes (10^3/μL)	6.86 ± 0.52	6.74 ± 0.83	7.17 ± 0.43	7.90 ± 0.90[b]	6.26 ± 0.74	6.90 ± 0.95[b]
Segmented neutrophils (%)[c]	11.0 ± 1.5	10.7 ± 1.8	10.1 ± 1.5	13.6 ± 2.4	13.9 ± 1.4	9.4 ± 1.9
Lymphocytes (%)[c]	88.3 ± 1.6	88.1 ± 2.0	88.7 ± 1.6	85.1 ± 2.4	85.3 ± 1.5	89.6 ± 1.9
Monocytes (%)[c]	0.14 ± 0.14	0.60 ± 0.30	0.30 ± 0.18	0.30 ± 0.16	0.30 ± 0.16	0.30 ± 0.16
Eosinophils (%)[c]	0.6 ± 0.2	0.6 ± 0.3	0.9 ± 0.3	1.0 ± 0.5	0.6 ± 0.3	0.8 ± 0.3
Clinical Chemistry						
n	8	8	8	8	8	8
Urea nitrogen (mg/dL)	16.8 ± 0.6	17.0 ± 0.5	18.0 ± 0.7	17.0 ± 0.4	17.4 ± 0.8[d]	18.7 ± 0.4[b]
Creatinine (mg/dL)	0.61 ± 0.02	0.55 ± 0.02	0.69 ± 0.04*	0.70 ± 0.03***	0.61 ± 0.03[b]	0.64 ± 0.02
Glucose (mg/dL)	108 ± 5	110 ± 6	129 ± 9[e]	128 ± 5*	119 ± 6[e]	97 ± 3
Sodium (mmol/L)	156 ± 2	157 ± 2	156 ± 2[b]	156 ± 2	158 ± 2	156 ± 2
Potassium (mmol/L)	6.3 ± 0.1[b]	6.5 ± 0.2	6.5 ± 0.1[b]	6.6 ± 0.2[b]	6.4 ± 0.1[b]	6.6 ± 0.1
Chloride (mmol/L)	100 ± 1	100 ± 2	99 ± 2[b]	98 ± 1	99 ± 2	98 ± 2
Calcium (mg/dL)	11.36 ± 0.98[b]	10.39 ± 0.35[b]	11.04 ± 0.26	11.31 ± 0.14	10.70 ± 0.38[b]	11.29 ± 0.14[b]
Phosphorus (mg/dL)	9.8 ± 0.9[b]	12.3 ± 1.1	9.8 ± 0.6[b]	9.7 ± 0.5[b]	12.0 ± 1.2	9.6 ± 0.4
Total protein (g/dL)	6.4 ± 0.1	6.4 ± 0.1[b]	6.7 ± 0.1	6.8 ± 0.1**[b]	6.7 ± 0.1*	6.6 ± 0.1
Albumin (g/dL)	3.9 ± 0.1	4.1 ± 0.1[e]	4.2 ± 0.1[b]	4.0 ± 0.1	4.1 ± 0.1	4.0 ± 0.1
Cholesterol (mg/dL)	91 ± 4	103 ± 7[e]	95 ± 5	111 ± 2*[b]	110 ± 6*	128 ± 4***
Triglycerides (mg/dL)	100 ± 12	108 ± 20[b]	111 ± 14	141 ± 15	99 ± 14	118 ± 16
Alanine aminotransferase (μg/L)	43 ± 2	40 ± 2	48 ± 2	40 ± 2	37 ± 2	36 ± 1[b]
Alkaline phosphatase (μg/L)	208 ± 5	198 ± 8	220 ± 9[b]	200 ± 5[b]	199 ± 6[e]	206 ± 5
Aspartate aminotransferase (μg/L)	91 ± 6[b]	98 ± 7	98 ± 5	97 ± 7	95 ± 4[b]	91 ± 3[b]
Creatine kinase (μg/L)	227 ± 26	193 ± 11[b]	256 ± 21[b]	337 ± 38	279 ± 43[e]	293 ± 21
Sorbitol dehydrogenase (μg/L)	15 ± 1[b]	12 ± 1	15 ± 0[b]	14 ± 2[b]	12 ± 1[b]	13 ± 1
γ-Glutamyltransferase (μg/L)	0.6 ± 0.2[b]	1.1 ± 0.4	0.1 ± 0.0[b]	0.7 ± 0.2	1.7 ± 0.4*	4.2 ± 0.4***
Bile acids (μmol/L)	14.1 ± 0.8	14.5 ± 0.9	15.1 ± 1.0	13.3 ± 0.6	14.5 ± 1.2	13.1 ± 0.6[e]

* Significantly different ($P \leq 0.05$) from the control group by Dunnett's test
** $P \leq 0.01$
***$P \leq 0.001$
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=7
[c] Significant outliers were not excluded from statistical analyses.
[d] n=8
[e] n=6

TABLE C2
Thyroid Hormone Data for Rats in the 28-Day Feed Study of Malachite Green Chloride[a]

	0 ppm	1,200 ppm
n	8	8
Male		
Thyroid-stimulating hormone (ng/mL)		
Day 4	0.73 ± 0.06	1.14 ± 0.17*
Day 21	1.16 ± 0.15	1.18 ± 0.17
Triiodothyronine (ng/dL)		
Day 4	128.34 ± 6.48	134.70 ± 6.09
Day 21	105.97 ± 5.21	111.12 ± 7.60
Thyroxine (µg/dL)		
Day 4	3.76 ± 0.17	3.70 ± 0.05
Day 21	3.47 ± 0.05	3.14 ± 0.04
Female		
Thyroid-stimulating hormone (ng/mL)		
Day 4	0.86 ± 0.12	1.03 ± 0.12
Day 21	0.75 ± 0.09	0.97 ± 0.13
Triiodothyronine (ng/dL)		
Day 4	116.18 ± 5.41	118.69 ± 6.13
Day 21	105.42 ± 2.21	123.05 ± 4.12**
Thyroxine (µg/dL)		
Day 4	3.09 ± 0.14	2.55 ± 0.14*
Day 21	3.00 ± 0.14	2.52 ± 0.11*

* Significantly different ($P \leq 0.05$) from the control group by Dunnett's test
** $P \leq 0.01$
[a] Mean ± standard error. Statistical tests were performed on unrounded data.

TABLE C3
Hematology and Clinical Chemistry Data for Male Rats in the 28-Day Feed Study of Leucomalachite Green[a]

	0 ppm	290 ppm	580 ppm	1,160 ppm
Hematology				
n	6	8	6	8
Hematocrit (%)	49.1 ± 0.3	47.5 ± 0.5	48.0 ± 0.4	46.6 ± 0.5*
Hemoglobin (g/dL)	17.7 ± 0.2	17.1 ± 0.2	17.3 ± 0.1	16.8 ± 0.2*
Erythrocytes (10^6/μL)	9.21 ± 0.06	9.00 ± 0.09	9.13 ± 0.06	8.78 ± 0.09*
Reticulocytes (%)	2.97 ± 0.09	3.28 ± 0.16	2.93 ± 0.18	3.66 ± 0.26*
Mean cell volume (fL)	53.3 ± 0.2	52.8 ± 0.2*	52.5 ± 0.2*	53.3 ± 0.2
Mean cell hemoglobin (pg)	19.2 ± 0.1	18.9 ± 0.1	18.9 ± 0.1*	19.0 ± 0.1
Mean cell hemoglobin concentration (g/dL)	36.0 ± 0.2	36.0 ± 0.2	36.0 ± 0.1	35.9 ± 0.1
Platelets (10^3/μL)	600.2 ± 28.8	522.0 ± 72.0	684.0 ± 52.6	578.6 ± 85.0
Leukocytes (10^3/μL)	6.98 ± 1.12	6.84 ± 0.64	6.35 ± 1.50	7.81 ± 0.82
Segmented neutrophils (%)	19.50 ± 0.99	18.00 ± 1.30	19.33 ± 0.88	18.63 ± 1.27
Lymphocytes (%)	79.33 ± 0.80	81.13 ± 1.49	80.00 ± 0.82	80.38 ± 1.16
Monocytes (%)	0.50 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Basophils (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Eosinophils (%)	0.67 ± 0.33	0.88 ± 0.35	0.67 ± 0.33	1.00 ± 0.27
Clinical Chemistry				
n	8	8	7	8
Urea nitrogen (mg/dL)	16.5 ± 0.3	14.9 ± 0.5*	14.9 ± 0.8*	17.5 ± 0.8
Creatinine (mg/dL)	0.63 ± 0.02	0.55 ± 0.02*	0.54 ± 0.02*	0.51 ± 0.02*
Glucose (mg/dL)	148 ± 17	107 ± 5	120 ± 10	128 ± 12
Sodium (mmol/L)	153 ± 1	153 ± 1	155 ± 1	153 ± 1
Potassium (mmol/L)	5.9 ± 0.1	6.1 ± 0.1	6.2 ± 0.1	6.4 ± 0.2*
Chloride (mmol/L)	94 ± 1	90 ± 7	95 ± 2	90 ± 5
Calcium (mg/dL)	12.40 ± 0.33[b]	11.95 ± 0.14[b]	12.03 ± 0.17[b]	12.25 ± 0.19[b]
Phosphorus (mg/dL)	8.8 ± 0.2	9.0 ± 0.2	9.6 ± 0.3	9.7 ± 0.2*
Total protein (g/dL)	7.0 ± 0.1	6.8 ± 0.2	6.7 ± 0.1	6.9 ± 0.1
Albumin (g/dL)	4.2 ± 0.1	3.8 ± 0.0*	3.8 ± 0.1*	3.9 ± 0.1*
Cholesterol (mg/dL)	62 ± 2	47 ± 1*	46 ± 1*	55 ± 2*
Triglycerides (mg/dL)	142 ± 9	88 ± 7*	75 ± 4*	64 ± 7*
Alanine aminotransferase (μg/L)	56 ± 3	47 ± 1*	45 ± 2*	50 ± 2
Alkaline phosphatase (μg/L)	258 ± 8	256 ± 5	248 ± 7	216 ± 3*
Aspartate aminotransferase (μg/L)	114 ± 12	95 ± 6	96 ± 10	85 ± 7
Creatine kinase (μg/L)	480 ± 75	415 ± 29	500 ± 80	434 ± 64
Sorbitol dehydrogenase (μg/L)	11 ± 1	11 ± 1	11 ± 1	12 ± 1[c]
γ-Glutamyltransferase (μg/L)	0.6 ± 0.2	0.8 ± 0.3	0.7 ± 0.2	1.3 ± 0.2*
Bile acids (μmol/L)	24.5 ± 3.6	19.3 ± 1.7	19.4 ± 4.2	18.8 ± 1.1[c]

* Significantly different (P≤0.05) from the control group by Dunnett's test
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=4
[c] n=7

TABLE C4
Thyroid Hormone Data for Male Rats in the 28-Day Feed Study of Leucomalachite Green[a]

	0 ppm	1,160 ppm
n	8	8
Thyroid-stimulating hormone (ng/mL)		
Day 4	1.86 ± 0.16	3.04 ± 0.44*[b]
Day 21	3.64 ± 0.80	6.30 ± 1.58*
Triiodothyronine (ng/dL)		
Day 4	113.68 ± 5.25	107.96 ± 4.35
Day 21	97.48 ± 3.56	98.49 ± 4.06
Thyroxine (µg/dL)		
Day 4	4.95 ± 0.29	3.35 ± 0.18***
Day 21	2.96 ± 0.17	2.31 ± 0.13***

* Significantly different ($P \leq 0.05$) from the control group by Dunnett's test
***$P \leq 0.001$
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=7

TABLE C5
Hematology and Clinical Chemistry Data for Mice in the 28-Day Feed Study of Malachite Green Chloride[a]

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Male						
n	8	8	8	8	8	8
Hematology						
Hematocrit (%)	48.3 ± 0.5[b]	46.7 ± 0.5*	47.6 ± 0.6	46.7 ± 0.5*	46.5 ± 0.4*	45.7 ± 0.7**
Hemoglobin (g/dL)	17.0 ± 0.2[b]	16.3 ± 0.2*	16.8 ± 0.2	16.2 ± 0.2**	16.3 ± 0.2*	16.0 ± 0.2**
Erythrocytes (10^6/µL)	9.94 ± 0.12[b]	9.57 ± 0.13*	9.75 ± 0.11	9.52 ± 0.10*	9.50 ± 0.07**	9.27 ± 0.12***
Reticulocytes (%)[c]	2.5 ± 0.5	2.7 ± 0.2	3.4 ± 0.2	3.1 ± 0.2	3.8 ± 0.3	4.1 ± 0.4*
Mean cell volume (fL)	48.6 ± 0.2[b]	48.8 ± 0.2	48.8 ± 0.2	49.0 ± 0.2	48.9 ± 0.2	49.3 ± 0.2*
Mean cell hemoglobin (pg)	17.1 ± 0.1[b]	17.0 ± 0.1	17.2 ± 0.0	17.0 ± 0.1	17.1 ± 0.1	17.3 ± 0.1*
Mean cell hemoglobin concentration (g/dL)	35.1 ± 0.1[b]	34.9 ± 0.1	35.2 ± 0.1	34.7 ± 0.1**	35.1 ± 0.0	35.1 ± 0.1
Platelets (10^3/µL)	897.0 ± 47.5	995.4 ± 15.0	943.0 ± 50.2	1,023.1 ± 34.3*	1,066.1 ± 15.5**	986.9 ± 60.1
Leukocytes (10^3/µL)	3.01 ± 0.67	3.73 ± 0.59	3.75 ± 0.40	4.39 ± 0.63	3.80 ± 0.65	3.18 ± 0.49
Segmented neutrophils (%)[c]	19.0 ± 4.4	20.5 ± 2.4	16.0 ± 1.7	14.4 ± 2.1[b]	13.9 ± 1.7	10.4 ± 2.3
Lymphocytes (%)[c]	80.9 ± 4.4	79.4 ± 2.5	84.0 ± 1.7	85.4 ± 2.1[b]	86.1 ± 1.7	89.4 ± 2.3
Monocytes (%)[c]	0.13 ± 0.13	0.13 ± 0.13	0.00 ± 0.00	0.14 ± 0.14[b]	0.00 ± 0.00	0.30 ± 0.16
Eosinophils (%)[c]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00[b]	0.00 ± 0.00	0.00 ± 0.00
Clinical Chemistry						
Urea nitrogen (mg/dL)	26.8 ± 2.3[d]	24.1 ± 0.9[d]	24.6 ± 2.9[e]	21.5 ± 0.5*[f]	24.6 ± 1.9[g]	25.9 ± 1.2[b]
Creatinine (mg/dL)	0.59 ± 0.02	0.55 ± 0.02	0.54 ± 0.02	0.51 ± 0.01**	0.50 ± 0.02**	0.46 ± 0.02***
Total protein (g/dL)	6.2 ± 0.1	6.0 ± 0.0[b]	5.9 ± 0.1*[b]	6.1 ± 0.1[b]	6.3 ± 0.1	6.3 ± 0.1
Alanine aminotransferase (µg/L)	31 ± 4[b]	32 ± 2	47 ± 10	27 ± 4	31 ± 5	26 ± 3
Alkaline phosphatase (µg/L)	124 ± 6	126 ± 6	119 ± 5	115 ± 4	125 ± 5	133 ± 5
Bile acids (µmol/L)	26.9 ± 2.1	21.6 ± 1.3*	21.4 ± 1.1*[b]	19.7 ± 1.2**	22.3 ± 2.5*	22.8 ± 1.2

TABLE C5
Hematology and Clinical Chemistry Data for Mice in the 28-Day Feed Study of Malachite Green Chloride

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Female						
Hematology						
n	8	8	8	8	8	7
Hematocrit (%)	48.3 ± 0.5	48.0 ± 0.2	47.4 ± 0.3	47.4 ± 0.4	46.8 ± 0.4**[b]	46.9 ± 0.3**
Hemoglobin (g/dL)	17.2 ± 0.2	17.1 ± 0.1	16.8 ± 0.1	16.7 ± 0.1*	16.7 ± 0.2*[b]	16.6 ± 0.1**
Erythrocytes (10^6/μL)	10.02 ± 0.10	9.98 ± 0.06	9.79 ± 0.06*	9.74 ± 0.06**	9.61 ± 0.07***[b]	9.53 ± 0.06***
Reticulocytes (%)[c]	2.18 ± 0.12	2.38 ± 0.24	2.94 ± 0.18	3.34 ± 0.38*[b]	3.34 ± 0.22*	4.07 ± 0.53*
Mean cell volume (fL)	48.2 ± 0.1	48.1 ± 0.1	48.4 ± 0.2	48.7 ± 0.2*	48.7 ± 0.2*[b]	49.2 ± 0.2***
Mean cell hemoglobin (pg)	17.2 ± 0.1	17.1 ± 0.1	17.2 ± 0.1	17.2 ± 0.1	17.4 ± 0.1[b]	17.4 ± 0.1
Mean cell hemoglobin concentration (g/dL)	35.6 ± 0.1	35.6 ± 0.1	35.5 ± 0.2	35.3 ± 0.1	35.7 ± 0.1[b]	35.4 ± 0.1
Platelets (10^3/μL)	902.5 ± 19.6	879.9 ± 20.1	886.9 ± 21.5	825.4 ± 33.8*	848.3 ± 14.6	858.9 ± 27.1[h]
Leukocytes (10^3/μL)	5.09 ± 0.41	4.28 ± 0.53	6.45 ± 0.85	4.64 ± 0.77	6.01 ± 1.24	4.96 ± 0.96[h]
Segmented neutrophils (%)[c]	19.63 ± 2.46	21.13 ± 2.99	14.38 ± 2.70	21.63 ± 3.63	19.13 ± 3.16	14.25 ± 1.56[h]
Lymphocytes (%)[c]	79.88 ± 2.33	78.75 ± 3.04	85.50 ± 2.67	78.25 ± 3.60	80.88 ± 3.16	85.50 ± 1.57[h]
Monocytes (%)[c]	0.25 ± 0.16	0.13 ± 0.13	0.13 ± 0.13	0.13 ± 0.13	0.00 ± 0.00	0.25 ± 0.25[h]
Eosinophils (%)[c]	0.25 ± 0.25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00[h]
Clinical Chemistry						
n	8	7	7	7	7	8
Urea nitrogen (mg/dL)	19.8 ± 0.8[b]	20.8 ± 0.5	20.0 ± 0.6[h]	21.1 ± 0.9[i]	20.2 ± 0.5	21.3 ± 0.7
Creatinine (mg/dL)	0.61 ± 0.05	0.63 ± 0.02	0.57 ± 0.02	0.57 ± 0.02	0.47 ± 0.03**	0.50 ± 0.02*[b]
Total protein (g/dL)	6.2 ± 0.1[b]	6.4 ± 0.1	6.2 ± 0.1[h]	6.2 ± 0.1	6.0 ± 0.1	6.1 ± 0.1
Alanine aminotransferase (μg/L)	74 ± 17	51 ± 6	42 ± 7	75 ± 17	55 ± 11	61 ± 18
Alkaline phosphatase (μg/L)	193 ± 8	190 ± 4[h]	181 ± 3	187 ± 5	182 ± 6[h]	182 ± 7
Bile acids (μmol/L)	22.5 ± 2.4[i]	19.8 ± 2.2[f]	17.7 ± 1.6[i]	14.5 ± 1.2*[i]	15.6 ± 4.6*[e]	13.5 ± 1.5**[f]

* Significantly different ($P \leq 0.05$) from the control group by Dunnett's test
** $P \leq 0.01$
*** $P \leq 0.001$
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=7
[c] Significant outliers were not excluded from statistical analyses.
[d] n=4
[e] n=3
[f] n=5
[g] n=2
[h] n=8
[i] n=6

TABLE C6
Hematology and Clinical Chemistry Data for Female Mice in the 28-Day Feed Study of Leucomalachite Green[a]

	0 ppm	290 ppm	580 ppm	1,160 ppm
n	7	8	8	8
Hematology				
Hematocrit (%)	47.5 ± 0.4	48.6 ± 0.5	47.8 ± 0.6	47.0 ± 1.3
Hemoglobin (g/dL)	16.4 ± 0.2	16.8 ± 0.2	16.5 ± 0.2	16.3 ± 0.5
Erythrocytes (10^6/μL)	9.73 ± 0.08	9.96 ± 0.11	9.80 ± 0.13	9.56 ± 0.26
Reticulocytes (%)	3.66 ± 0.24[b]	3.98 ± 0.23	3.89 ± 0.24	3.54 ± 0.17
Mean cell volume (fL)	48.9 ± 0.1	48.8 ± 0.2	48.8 ± 0.1	49.2 ± 0.1
Mean cell hemoglobin (pg)	16.9 ± 0.1	16.9 ± 0.1	16.9 ± 0.0	17.1 ± 0.1
Mean cell hemoglobin concentration (g/dL)	34.5 ± 0.1	34.6 ± 0.1	34.5 ± 0.1	34.7 ± 0.2
Platelets (10^3/μL)	770.7 ± 43.7	754.1 ± 95.7	956.8 ± 67.1	953.1 ± 51.9
Leukocytes (10^3/μL)	5.99 ± 0.58	3.86 ± 0.33	4.72 ± 0.69	4.55 ± 0.78
Segmented neutrophils (%)	20.43 ± 1.79[b]	23.63 ± 0.91	21.50 ± 1.32	22.75 ± 0.92
Lymphocytes (%)	78.57 ± 2.03[b]	76.25 ± 0.92	78.50 ± 1.32	77.00 ± 0.91
Clinical Chemistry				
Urea nitrogen (mg/dL)	25.9 ± 1.6	24.0 ± 1.0	23.7 ± 1.2	22.9 ± 1.0[c]
Creatinine (mg/dL)	0.40 ± 0.00[c]	0.40 ± 0.02	0.40 ± 0.02	0.33 ± 0.02[c]
Total protein (g/dL)	5.8 ± 0.1	5.8 ± 0.1	5.4 ± 0.1*	5.4 ± 0.0*
Alanine aminotransferase (μg/L)	45 ± 7	55 ± 14	80 ± 27	74 ± 22
Alkaline phosphatase (μg/L)	181 ± 6	189 ± 7	196 ± 7	198 ± 5

* Significantly different ($P \leq 0.05$) from the control group by Dunnett's test
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=8
[c] n=6

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

TABLE D1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 28-Day Feed Study of Malachite Green Chloride[a]

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Male						
n	8	8	8	8	8	8
Necropsy body wt	217 ± 4	227 ± 3	210 ± 8	229 ± 4	219 ± 3	188 ± 4**
L. Kidney						
Absolute	0.889 ± 0.037	0.934 ± 0.022	0.845 ± 0.047	0.899 ± 0.038	0.892 ± 0.024	0.778 ± 0.036*
Relative	4.10 ± 0.13	4.12 ± 0.04	4.06 ± 0.09	3.93 ± 0.10	4.09 ± 0.13	4.14 ± 0.05
R. Kidney						
Absolute	0.876 ± 0.035	0.912 ± 0.021	0.834 ± 0.052	0.889 ± 0.037	0.894 ± 0.025	0.760 ± 0.032*
Relative	4.04 ± 0.12	4.02 ± 0.06	3.99 ± 0.07	3.89 ± 0.11	4.10 ± 0.13	4.05 ± 0.04
Liver						
Absolute	7.521 ± 0.325	8.055 ± 0.236	7.562 ± 0.478	8.299 ± 0.392	9.072 ± 0.372**	8.082 ± 0.443
Relative	34.66 ± 1.19	35.48 ± 0.60	36.14 ± 0.58	36.28 ± 1.08	41.62 ± 1.91**	42.96 ± 0.93**
Female						
n	8	8	8	8	8	8
Necropsy body wt	145 ± 2	146 ± 2	146 ± 2	149 ± 2	138 ± 1**	119 ± 2**
L. Kidney						
Absolute	0.623 ± 0.020	0.638 ± 0.024	0.627 ± 0.026	0.644 ± 0.020	0.602 ± 0.009	0.523 ± 0.022**
Relative	4.28 ± 0.08	4.36 ± 0.06	4.28 ± 0.08	4.31 ± 0.05	4.38 ± 0.07	4.38 ± 0.13
R. Kidney						
Absolute	0.613 ± 0.020	0.626 ± 0.026	0.617 ± 0.023	0.634 ± 0.025	0.591 ± 0.013	0.512 ± 0.018**
Relative	4.21 ± 0.07	4.27 ± 0.08	4.22 ± 0.09	4.23 ± 0.07	4.29 ± 0.06	4.29 ± 0.05
Liver						
Absolute	4.257 ± 0.104	4.491 ± 0.239	4.457 ± 0.221	4.924 ± 0.148**	4.996 ± 0.089**	4.953 ± 0.116**
Relative	29.30 ± 0.39	30.61 ± 0.88	30.45 ± 1.05	32.97 ± 0.47**	36.28 ± 0.47**	41.62 ± 0.85**

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

TABLE D2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male Rats in the 28-Day Feed Study of Leucomalachite Green[a]

	0 ppm	290 ppm	580 ppm	1,160 ppm
n	8	8	8	8
Necropsy body wt	239 ± 3	234 ± 4	227 ± 3*	219 ± 3**
Kidney				
Absolute	1.817 ± 0.032	1.813 ± 0.052	1.740 ± 0.039	1.785 ± 0.053
Relative	7.6 ± 0.1	7.8 ± 0.1	7.7 ± 0.1	8.1 ± 0.1**
Liver				
Absolute	7.953 ± 0.242	8.619 ± 0.243	8.580 ± 0.291	10.137 ± 0.251**
Relative	33.2 ± 0.4	36.9 ± 0.4**	37.8 ± 0.8**	46.3 ± 0.7**

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

TABLE D3
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 28-Day Feed Study of Malachite Green Chloride[a]

	0 ppm	25 ppm	100 ppm	300 ppm	600 ppm	1,200 ppm
Male						
n	8	8	8	8	8	8
Necropsy body wt	22.4 ± 0.3	21.8 ± 0.2	23.1 ± 0.6	23.1 ± 0.1	21.4 ± 0.3	22.3 ± 0.6
L. Kidney						
Absolute	0.168 ± 0.001	0.166 ± 0.005	0.170 ± 0.016	0.176 ± 0.006	0.148 ± 0.005	0.164 ± 0.014
Relative	7.5 ± 0.2	7.6 ± 0.3	7.3 ± 0.3	7.6 ± 0.3	6.9 ± 0.4	7.3 ± 0.3
R. Kidney						
Absolute	0.180 ± 0.005	0.170 ± 0.006	0.183 ± 0.016	0.188 ± 0.009	0.155 ± 0.008	0.171 ± 0.013
Relative	8.0 ± 0.2	7.8 ± 0.3	7.9 ± 0.3	8.2 ± 0.4	7.2 ± 0.3	7.7 ± 0.2
Liver						
Absolute	0.893 ± 0.035	0.880 ± 0.025	0.932 ± 0.053	0.924 ± 0.013	0.897 ± 0.032	0.920 ± 0.045
Relative	39.8 ± 0.9	40.3 ± 0.9	40.4 ± 0.4	40.1 ± 0.2	41.8 ± 0.5*	41.2 ± 0.4
Female						
n	8	8	8	8	8	8
Necropsy body wt	17.3 ± 0.2	16.8 ± 0.2*	17.2 ± 0.2	16.3 ± 0.2**	16.3 ± 0.1**	15.6 ± 0.2**
L. Kidney						
Absolute	0.128 ± 0.005	0.119 ± 0.003	0.125 ± 0.004	0.122 ± 0.005	0.113 ± 0.003*	0.108 ± 0.004**
Relative	7.4 ± 0.2	7.1 ± 0.2	7.3 ± 0.2	7.5 ± 0.3	7.0 ± 0.2	6.9 ± 0.3
R. Kidney						
Absolute	0.133 ± 0.005	0.128 ± 0.003	0.131 ± 0.004	0.127 ± 0.004	0.119 ± 0.003*	0.116 ± 0.003**
Relative	7.7 ± 0.2	7.7 ± 0.1	7.6 ± 0.2	7.8 ± 0.2	7.3 ± 0.2	7.4 ± 0.2
Liver						
Absolute	0.768 ± 0.036	0.766 ± 0.012	0.803 ± 0.023	0.787 ± 0.027	0.775 ± 0.030	0.734 ± 0.020
Relative	44.4 ± 1.4	45.7 ± 0.6	46.6 ± 0.5	48.2 ± 1.3*	47.6 ± 1.7	47.0 ± 1.2

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

TABLE D4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Female Mice in the 28-Day Feed Study of Leucomalachite Green[a]

	0 ppm	290 ppm	580 ppm	1,160 ppm
n	8	8	8	8
Necropsy body wt	17.6 ± 0.2	17.1 ± 0.2	16.6 ± 0.2**	16.0 ± 0.2**
L. Kidney				
Absolute	0.130 ± 0.003	0.123 ± 0.004	0.113 ± 0.003**	0.109 ± 0.004**
Relative	7.4 ± 0.1	7.2 ± 0.1	6.8 ± 0.1**	6.8 ± 0.1**
R. Kidney				
Absolute	0.138 ± 0.004	0.130 ± 0.004	0.121 ± 0.004**	0.117 ± 0.004**
Relative	7.8 ± 0.2	7.6 ± 0.1	7.3 ± 0.2*	7.3 ± 0.2*
Liver				
Absolute	0.748 ± 0.014	0.724 ± 0.010	0.712 ± 0.018	0.715 ± 0.019
Relative	42.6 ± 0.7	42.5 ± 0.7	43.0 ± 0.6	44.8 ± 0.8*

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

APPENDIX E
GENETIC TOXICOLOGY

TABLE E1
Mutagenicity of Malachite Green Chloride in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Revertants/Plate[b] −S9 Trial 1	−S9 Trial 2	+ hamster S9 10%	+ hamster S9 30%	+ rat S9 10%	+ rat S9 30%
TA102	0	272 ± 2.1	284 ± 1.8	256 ± 2.6	266 ± 2.1	259 ± 2.0	337 ± 4.6
	0.1	282 ± 2.3	280 ± 1.8	250 ± 2.9	255 ± 2.6	257 ± 1.8	342 ± 4.5
	0.3	288 ± 1.8	290 ± 2.3	258 ± 2.7	274 ± 2.7	264 ± 2.2	336 ± 5.5
	1.0	267 ± 2.1	295 ± 2.3	259 ± 1.8	271 ± 2.4	274 ± 2.3	349 ± 4.7
	3.3	277 ± 3.1	300 ± 2.6	249 ± 2.0	282 ± 3.1	280 ± 1.7	357 ± 4.6
	10.0	271 ± 2.3	285 ± 2.4	260 ± 1.5	258 ± 1.9	259 ± 3.2	325 ± 3.4
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control[c]		1,416 ± 14.2	961 ± 2.3	831 ± 4.3	759 ± 4.2	892 ± 3.6	1,027 ± 12.5
TA104	0	279 ± 9.0	343 ± 2.3	280 ± 1.3	284 ± 2.3	276 ± 1.9	284 ± 2.6
	0.1	272 ± 2.0	349 ± 1.5	288 ± 2.8	277 ± 4.1	283 ± 2.1	288 ± 1.5
	0.3	289 ± 0.6	358 ± 2.0	277 ± 2.0	286 ± 1.8	281 ± 4.6	290 ± 1.8
	1.0	292 ± 1.2	364 ± 2.4	286 ± 1.3	289 ± 1.3	278 ± 4.1	286 ± 2.1
	3.3	289 ± 1.5	358 ± 2.6	273 ± 1.8	299 ± 1.8	282 ± 3.2	290 ± 2.6
	10.0	273 ± 1.8	348 ± 2.4	277 ± 2.2	293 ± 1.8	280 ± 1.7	289 ± 1.8
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		818 ± 6.5	1,116 ± 4.9	1,343 ± 17.5	1,396 ± 3.8	1,463 ± 13.7	989 ± 8.0
TA100	0	127 ± 0.9	129 ± 1.2	126 ± 0.3	151 ± 1.5	112 ± 2.0	137 ± 1.5
	0.1	127 ± 1.2	132 ± 1.2	133 ± 2.3	150 ± 1.5	115 ± 3.2	149 ± 1.7
	0.3	127 ± 0.9	131 ± 2.7	134 ± 2.7	147 ± 1.5	108 ± 3.2	154 ± 1.8
	1.0	127 ± 1.7	140 ± 1.9	142 ± 1.5	152 ± 2.7	113 ± 1.8	144 ± 0.9
	3.3	128 ± 1.3	138 ± 1.5	135 ± 3.0	156 ± 1.2	113 ± 1.8	145 ± 2.3
	10.0	129 ± 0.9	132 ± 1.8	125 ± 1.5	153 ± 1.8	114 ± 2.1	136 ± 1.0
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		531 ± 5.2	467 ± 15.2	734 ± 5.4	729 ± 3.5	885 ± 3.8	882 ± 4.6
TA1535	0	17 ± 1.3	17 ± 0.9	17 ± 0.7	14 ± 0.3	17 ± 0.9	20 ± 1.5
	0.1	17 ± 0.9	17 ± 0.7	15 ± 0.9	16 ± 1.2	18 ± 0.9	21 ± 2.1
	0.3	17 ± 1.0	19 ± 0.6	17 ± 1.2	16 ± 0.9	15 ± 1.5	20 ± 1.2
	1.0	17 ± 1.8	18 ± 1.9	16 ± 0.3	17 ± 1.0	15 ± 1.0	18 ± 1.2
	3.3	17 ± 1.7	17 ± 1.5	16 ± 1.2	17 ± 1.5	17 ± 1.2	21 ± 1.7
	10.0	17 ± 0.9	17 ± 0.9	17 ± 1.3	16 ± 1.7	16 ± 0.7	18 ± 2.6
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		563 ± 3.5	230 ± 4.0	221 ± 4.9	180 ± 2.6	274 ± 4.8	238 ± 9.0

TABLE E1
Mutagenicity of Malachite Green Chloride in *Salmonella typhimurium*

Strain	Dose (µg/plate)	Revertants/Plate −S9 Trial 1	−S9 Trial 2	+ hamster S9 10%	+ hamster S9 30%	+ rat S9 10%	+ rat S9 30%
TA97	0	118 ± 1.5	123 ± 1.5	127 ± 2.1	130 ± 1.7	153 ± 4.1	128 ± 2.1
	0.1	128 ± 0.7	121 ± 2.1	122 ± 1.2	125 ± 1.9	158 ± 3.5	125 ± 1.3
	0.3	126 ± 1.2	125 ± 0.9	124 ± 2.0	128 ± 1.2	146 ± 3.2	124 ± 1.5
	1.0	126 ± 1.0	127 ± 1.7	127 ± 1.2	123 ± 0.6	157 ± 2.0	134 ± 2.3
	3.3	124 ± 0.7	120 ± 2.4	125 ± 2.7	125 ± 2.6	137 ± 4.4	134 ± 0.6
	10.0	118 ± 1.3	126 ± 1.2	128 ± 1.9	128 ± 0.9	134 ± 3.8	127 ± 1.2
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		387 ± 3.5	251 ± 5.9	472 ± 6.7	781 ± 4.6	533 ± 22.3	462 ± 7.2
TA98	0	46 ± 0.3	21 ± 0.6	34 ± 1.8	29 ± 0.6	37 ± 1.0	35 ± 2.0
	0.1	45 ± 2.1	25 ± 0.7	34 ± 1.2	27 ± 0.9	37 ± 3.3	32 ± 0.6
	0.3	44 ± 1.5	25 ± 2.0	36 ± 1.8	29 ± 0.3	39 ± 3.2	34 ± 1.5
	1.0	45 ± 1.0	28 ± 1.2	38 ± 1.2	31 ± 0.6	39 ± 1.2	36 ± 1.5
	3.3	47 ± 0.9	24 ± 2.1	38 ± 0.3	34 ± 1.5	41 ± 1.5	36 ± 1.8
	10.0	45 ± 1.3	20 ± 0.9	34 ± 1.5	33 ± 1.8	46 ± 0.7	36 ± 0.9
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		285 ± 3.8	344 ± 9.6	983 ± 6.7	829 ± 2.6	360 ± 3.5	442 ± 3.8

[a] Study was performed at Environmental Health Research and Testing, Inc. The detailed protocol is presented by Zeiger *et al.* (1992). 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA97), 4-nitro-*o*-phenylenediamine (TA98), mitomycin-C (TA102), and methyl methanesulfonate (TA104). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE E2
Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Rats Treated with Malachite Green Chloride by Intraperitoneal Injection[a]

Compound	Dose (mg/kg)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	P Value[c]
Phosphate-buffered saline[d]		5	1.00 ± 0.52	
Malachite green chloride	1.094	5	1.00 ± 0.42	0.500
	2.188	5	1.10 ± 0.43	0.414
	4.375	5	2.50 ± 0.45	0.006
	8.750	5	1.50 ± 0.32	0.159
			P=0.051[e]	
Cyclophosphamide[f]	7.5	5	9.60 ± 0.58	0.000

[a] Study was performed at Integrated Laboratory Systems, Inc. The detailed protocol is presented by Shelby *et al*. (1993). PCE=polychromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the solvent control. Dosed group values are significant at P≤0.006; positive control value is significant at P≤0.05 (ILS, 1990)
[d] Solvent control
[e] Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test; significant at P≤0.025 (ILS, 1990)
[f] Positive control

TABLE E3
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Mice Following Administration of Malachite Green Chloride in Feed for 28 Days[a]

Compound	Dose (ppm)	Number of Mice with Erythrocytes Scored	Micronucleated Cells/1,000 Cells[b]	
			PCEs	NCEs
Male				
Vehicle control	0	8	3.31 ± 0.68	1.81 ± 0.29
Malachite green chloride	25	8	2.75 ± 0.36	1.81 ± 0.39
	100	8	2.44 ± 0.44	1.94 ± 0.31
	300	8	1.50 ± 0.20	1.88 ± 0.25
	600	8	2.25 ± 0.22	1.31 ± 0.15
	1,200	8	2.00 ± 0.43	1.38 ± 0.26
			P=0.973[c]	P=0.937
Female				
Vehicle control	0	8	2.63 ± 0.42	1.94 ± 0.36
Malachite green chloride	25	8	2.06 ± 0.48	1.94 ± 0.38
	100	8	2.31 ± 0.34	3.00 ± 0.54
	300	8	2.69 ± 0.62	3.19 ± 0.45
	600	8	3.00 ± 0.23	3.13 ± 0.39
	1200	8	1.67 ± 0.22	2.50 ± 0.38
			P=0.879	P=0.193

[a] Study was performed at Integrated Laboratory Systems, Inc. The detailed protocol is presented by MacGregor *et al*. (1990). PCE=polychromatic erythrocyte; NCE=normochromatic erythrocyte
[b] Mean ± standard error
[c] Significance of micronucleated cells/1,000 cells tested by the one-tailed trend test; significant at $P \leq 0.025$ (ILS, 1990)

TABLE E4
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Female Mice Following Administration of Leucomalachite Green in Feed for 28 Days[a]

Compound	Dose (ppm)	Number of Mice with Erythrocytes Scored	Micronucleated Cells/1,000 Cells[b]	
			PCEs	NCEs
Vehicle control	0	7	3.07 ± 0.41	2.14 ± 0.26
Leucomalachite green	290	8	3.63 ± 0.38	3.69 ± 0.33*
	580	8	3.50 ± 0.41	4.19 ± 0.50*
	1,160	8	2.00 ± 0.37	3.44 ± 0.53
			P=0.984[c]	P=0.067

* Significantly different from the vehicle control group (P≤0.008; ILS, 1990)
[a] Study was performed at Integrated Laboratory Systems, Inc. The detailed protocol is presented by MacGregor *et al*. (1990). PCE=polychromatic erythrocyte; NCE=normochromatic erythrocyte
[b] Mean ± standard error
[c] Significance of micronucleated cells/1,000 cells tested by the one-tailed trend test; significant at P≤0.025 (ILS, 1990)

APPENDIX F
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION

Malachite Green Chloride

Malachite green chloride was obtained from Chemsyn Science Laboratories (Lenexa, KS) in one lot (CSL-96-645-88-23). Identity and purity analyses were conducted by the manufacturer and the study laboratory. Reports on analyses performed in support of the malachite green chloride studies are on file at the National Center for Toxicological Research (NCTR).

Lot CSL-96-645-88-23, a green solid, was identified as malachite green chloride by the study laboratory using ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy and high-performance liquid chromatography (HPLC)/mass spectrometry (MS) by system A (Table F1). The supplier also identified the chemical as malachite green chloride with ^{1}H-nuclear magnetic resonance and ultraviolet/visible spectroscopy. All spectra were consistent with the structure of malachite green chloride. The nuclear magnetic resonance spectrum is presented in Figure F1.

The purity of malachite green chloride was determined with HPLC (system B) by the manufacturer, heavy metal and HPLC (system C) analyses by the study laboratory, and elemental and heavy metal analyses by Galbraith Laboratories, Inc. (Knoxville, TN). Heavy metal analyses by the study laboratory were conducted with inductively coupled plasma/atomic emission spectroscopy, normalized against standards provided by the National Institute of Standards and Technology (Gaithersburg, MD).

Elemental analyses for carbon, hydrogen, nitrogen, and chlorine (total halogens) were in agreement with the theoretical values for malachite green chloride. Results of heavy metal analyses by Galbraith Laboratories, Inc., indicated less than 20 ppm calculated as lead. Results of heavy metal analyses by the study laboratory indicated the following concentrations: tin, 25.0 ppm; zinc, 23.1 ppm, aluminum, 3.69 ppm; iron, 2.25 ppm; copper, 1.62 ppm; and magnesium, 1.17 ppm. HPLC by system B indicated one major peak and four impurities with a combined area of approximately 5.1% of the total peak area. HPLC by system C indicated one major peak and seven impurities with a combined area of approximately 4.7% of the total peak area; two of these impurities were identified as leucomalachite green and the desmethyl analogue of malachite green, present at approximately 1% each, based on peak areas, retention times, and spectral characteristics. The overall purity was determined to be at least 95%.

Reports on liquid chromatography-electrospray ionization (ESI)/MS, ESI/MS, and HPLC/ESI/MS analyses performed in support of the malachite green chloride studies are on file at the NCTR.

The bulk chemical was stored in the original amber bottle in the dark at room temperature. Analyses performed after the completion of the 28-day studies indicated no degradation of the bulk chemical; the stability of malachite green chloride was monitored at 6-month intervals over a 2-year period using HPLC with post-column oxidation.

Leucomalachite Green

Leucomalachite green was obtained from Chemsyn Science Laboratories in one lot (CSL-95-583-08-09). Identity, purity, and stability analyses were conducted by the manufacturer and the study laboratory. Reports on analyses performed in support of the leucomalachite green studies are on file at the NCTR.

Lot CSL-95-583-08-09, a faint green solid, was identified as leucomalachite green by the supplier using ^{1}H-nuclear magnetic resonance, infrared, and ultraviolet/visible spectroscopy and by the study laboratory using ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy. All spectra were consistent with the structure of leucomalachite green. The infrared and nuclear magnetic resonance spectra are presented in Figures F2 and F3.

The purity of leucomalachite green was determined by elemental analyses (performed by Oneida Research Services, Inc., Whitesboro, NY), heavy metal analyses (performed by Galbraith Laboratories, Inc.), and HPLC systems D (supplier) and E (study laboratory).

Elemental analyses for carbon, hydrogen, and nitrogen were in agreement with the theoretical values for leucomalachite green. Results of heavy metal analyses indicated less than 0.30 ppm lead and less than 1.0 ppm heavy metals calculated as lead. HPLC by system D indicated one major peak and two impurities with a combined area of 0.24% of the total peak area. HPLC by system E indicated one major peak and two impurities at each absorbance. One impurity was identified as malachite green based on retention time and spectral characteristics.. The overall purity of lot CSL-95-583-08-09 was determined to greater than 99%.

Reports on liquid chromatography-atmospheric pressure chemical ionization/MS, direct exposure probe/electron ionization/MS, and HPLC/electrospray ionization/MS analyses performed in support of the leucomalachite green studies are on file at the NCTR.

The bulk chemical was stored in the original amber bottle with a double wrapping of Parafilm around the cap; the bottle was placed inside a plastic bag inside another plastic bag filled with a silica gel desiccant and stored at −20° C, protected from light. Analyses performed after the completion of the 28-day studies indicated no degradation of the bulk chemical; the stability of leucomalachite green was monitored at 6-month intervals over a 2-year period using HPLC.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations for malachite green chloride were prepared on 6 days by dissolving the chemical in water and then mixing it with feed (Table F2). The 25 and 600 ppm dose formulations were prepared three times and the 100, 300, and 1,200 ppm dose formulations were prepared twice. The dose formulations for leucomalachite green were prepared by mixing the chemical with feed (Table F2). A premix was prepared by hand and blended with additional feed. The 96 and 290 ppm dose formulations for leucomalachite green were prepared once and the 580 and 1,160 ppm dose formulations were prepared twice. Dose formulations for each chemical were mixed in a Patterson-Kelly twin-shell blender with the intensifier bar on for 20 minutes. Dose formulations were stored in stainless steel feed cans at 4° ± 2° C for up to 92 days (malachite green chloride) or 95 days (leucomalachite green).

Homogeneity and stability studies of the 25 ppm malachite green chloride dose formulations were performed by the study laboratory using HPLC by system F. Homogeneity was confirmed, and stability was confirmed for 92 days for dose formulations stored protected from light at 4° C and for 10 days for dose formulations stored at room temperature, either protected from light or open to air and light. Homogeneity and stability studies of the 96 ppm leucomalachite green dose formulations were performed by the study laboratory with HPLC by system E. Homogeneity was confirmed, and stability was confirmed for 95 days for dose formulations stored protected from light at up to 8° C, and for 32 days for dose formulations stored at room temperature either protected from light or open to air.

Periodic analyses of the dose formulations of malachite green chloride were conducted by the study laboratory using HPLC by system F (Table F3). Analyses of the dose formulations of malachite green chloride were

conducted on one batch each of the 25, 100, and 1,200 ppm dose formulations, on both batches of the 300 ppm dose formulations, and on all three of the 600 ppm dose formulations. During the 28-day studies, seven of eight dose formulations analyzed for rats and mice were within 10% of the target concentrations, with no value greater than 103% of the target concentration (Table F3). The formulation that was not within 10% of the target concentration was diluted with feed and remixed to provide a lower (300 ppm) concentration; the remix was analyzed and found to be within 10% of the target concentration. Periodic analyses of the dose formulations of leucomalachite green were conducted by the study laboratory using HPLC by system E. During the 28-day studies all dose formulations were analyzed. All dose formulations for rats and mice were within 10% of the target concentrations, with no value greater than 104% of the target concentration (Table F4).

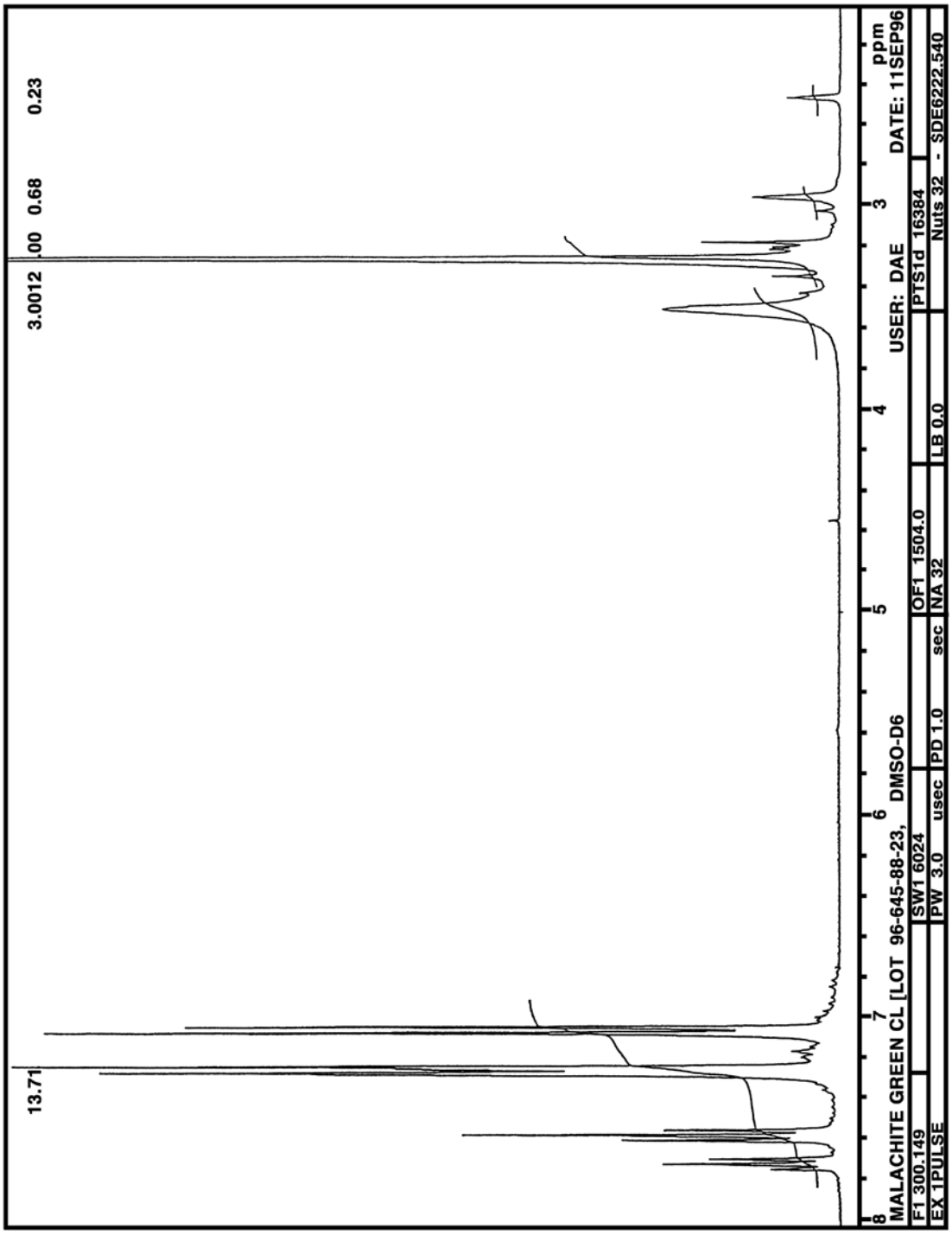

Figure F1
Nuclear Magnetic Resonance Spectrum of Malachite Green Chloride

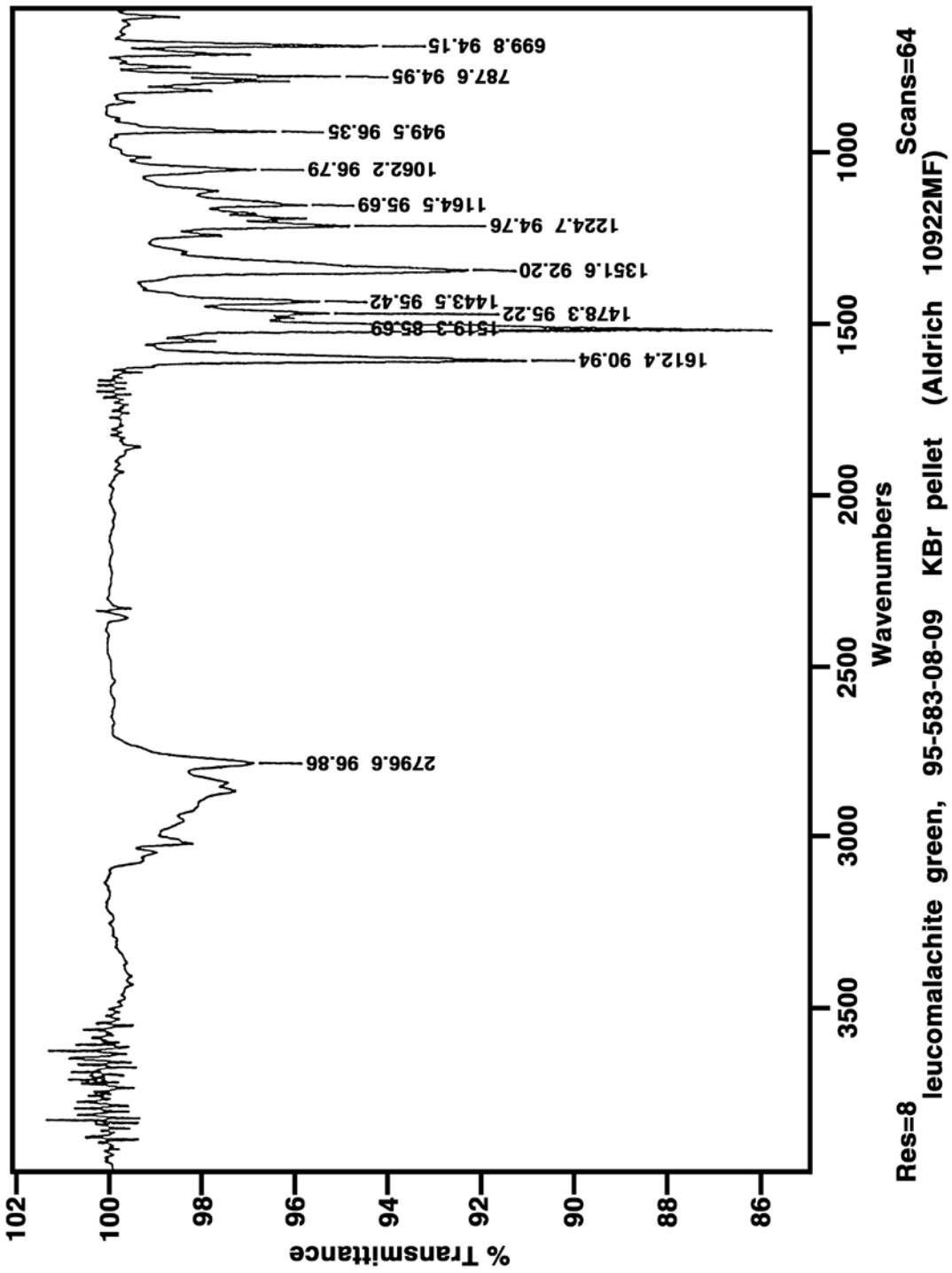

FIGURE F2
Infrared Absorption Spectrum of Leucomalachite Green

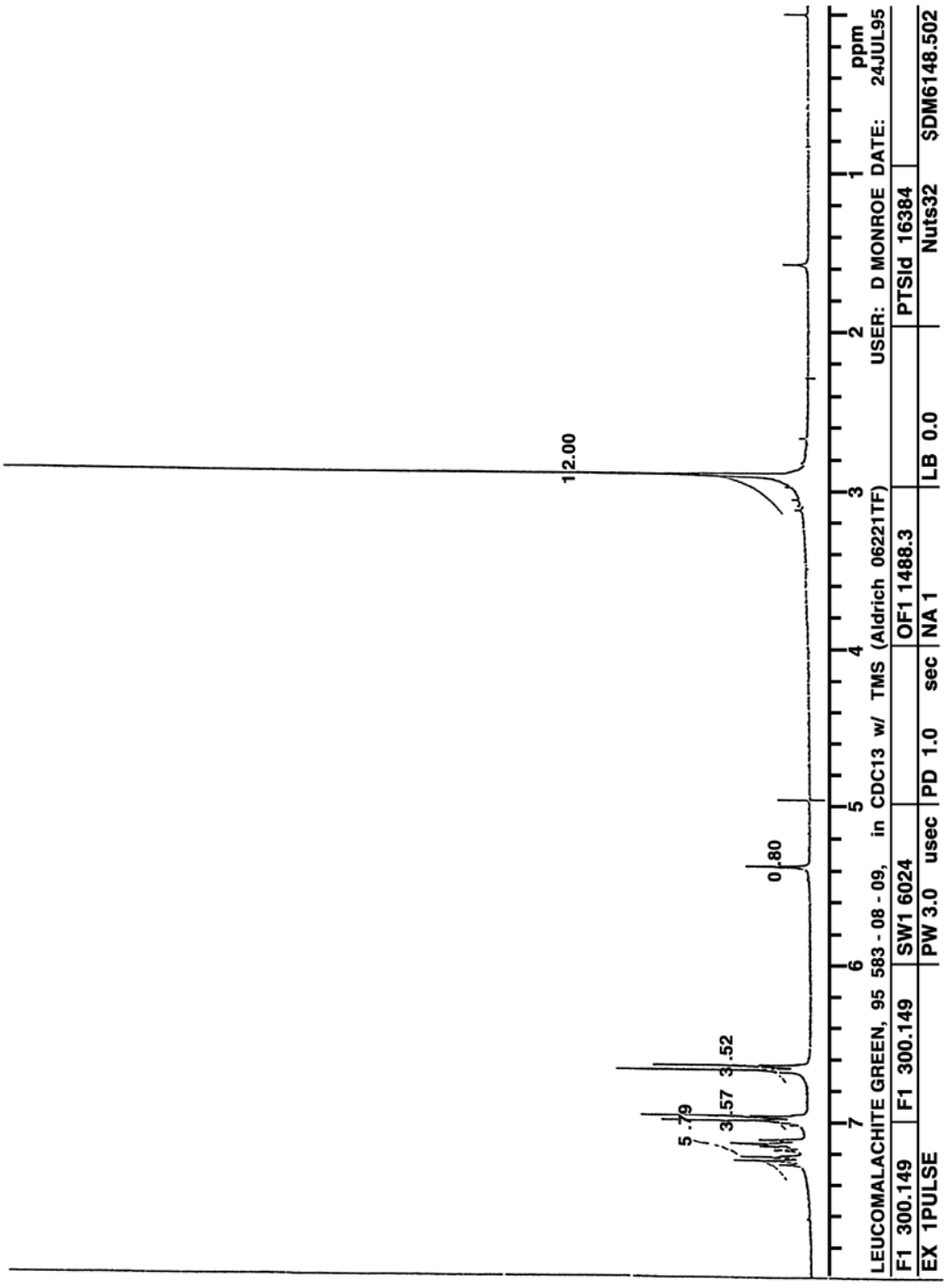

FIGURE F3
Nuclear Magnetic Resonance Spectrum of Leucomalachite Green

TABLE F1
High-Performance Liquid Chromatography Systems Used in the Feed Studies of Malachite Green Chloride and Leucomalachite Green[a]

Detection System	Column	Solvent System
System A Ultraviolet light (260 nm) and evaporative light scattering detection coupled with mass spectrometry	VYDAC C_{18} Pharmaceutical, 250 mm × 4.6 mm, 5-µm particle size (Vydac, Hesperia, CA)	A) Water and B) methanol with 0.1% formic acid; 50% A:50% B for 10 minutes, then linear gradient to 5% A:95% B in 5 minutes at a flow rate of 500 µL/minute
System B Ultraviolet (254 nm)	Inertsil 5 ODS 2, 150 mm × 4.6 mm, 5-µm particle size (GL Sciences, Japan)	A) Methanol:50 mM phosphate, pH 3.0; and B) gradient from 50% B to 80% A:20% B in 25 minutes at a flow rate of 1.0 mL/minute
System C Ultraviolet/visible photodiode array (scanning from 230 nm to 750 nm), with monitoring at 254 nm and 618 nm	Spherisorb S5 Nitrile (250 mm × 4.6 mm) (Waters Corp., Milford, MA)	A) Acetonitrile:0.05 M ammonium acetate buffer, pH 4.6 (40:60) and B) acetonitrile:0.05 M ammonium acetate buffer, pH 4.6 (60:40); 100% A for 5 minutes, then linear gradient to 100% B from 5 to 10 minutes at a flow rate of 1.5 mL/minute
System D Ultraviolet (254 nm)	Phenomenex Partisil 5 ODS (3), 150 mm × 4.6 mm, 5-µm particle size (Phenomenex, Torrance, CA)	A) Acetonitrile and B) 25 mM phosphate, pH 3.0 (75% A:25% B); flow rate 1.0 mL/minute
System E Ultraviolet/visible photodiode array and evaporative light scattering detection, with monitoring at 254 nm and 618 nm	Supelco Cyano, 200 mm × 4.6 mm, 5-µm particle size (Supelco, Inc., Bellefonte, PA)	A) Acetonitrile and B) 0.05 M ammonium acetate buffer, pH 4.6 (60% A:40% B); flow rate 1.0 mL/minute
System F Visible (620 nm)	Spherisorb Cyano (250 mm × 4.6 mm), 5-µm particle size (Waters Corp.)	A) Acetonitrile and B) 0.05 M ammonium acetate buffer, pH 4.5 (70% A:30% B); flow rate 1.0 mL/minute

[a] Chromatographs were manufactured by Varex (Hopkins, MN) (system A) and Varian, Inc. (Palo Alto, CA).

TABLE F2
Preparation and Storage of Dose Formulations in the Feed Studies of Malachite Green Chloride and Leucomalachite Green

Malachite Green Chloride	Leucomalachite Green
Preparation	
Malachite green chloride was dissolved in deionized, distilled water and mixed with feed and then blended in a Patterson-Kelly twin-shell blender with the intensifier bar on for 20 minutes. Three batches each of the 25 and 600 ppm dose formulations and two each of the 100, 300, and 1,200 ppm dose formulations were prepared.	Leucomalachite green was ground into a fine powder with a mortar and pestle. A premix of feed and leucomalachite green was prepared by hand and then layered into the remaining feed and blended in a Patterson-Kelly twin-shell blender with the intensifier bar on for 20 minutes. One batch each of the 96 and 290 ppm dose formulations and two batches each of the 580 and 1,160 ppm dose formulations were prepared.
Chemical Lot Number	
CSL-96-645-88-23	CSL-95-583-08-09
Maximum Storage Time	
92 days	95 days
Storage Conditions	
Stored in stainless steel feed cans at 4° ± 2° C	Stored in stainless steel feed cans at 4° ± 2° C
Study Laboratory	
National Center for Toxicological Research (Jefferson, AR)	National Center for Toxicological Research (Jefferson, AR)

TABLE F3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 28-Day Feed Studies of Malachite Green Chloride

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
3 November 1996	3 November 1996	25	24.7	−1
8 November 1996	8 November 1996	100	100	0
		300	303	+1
20 November 1996	20 November 1996	600	599	0
		1,200	1,241	+3
7 January 1997	7 January 1997	600	532[b]	−11
26 February 1997	26 February 1997	300	293	−2
		600	554[c]	−8

[a] Results of triplicate analyses
[b] Remixed
[c] Results of remix

TABLE F4
Results of Analyses of Dose Formulations Administered to Male Rats and Female Mice in the 28-Day Feed Study of Leucomalachite Green

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
11 June 1996	11 June 1996	96	95	−1
		580	576	−1
5 July 1996	5 July 1996	290	301	+4
		580	573	−1
		1,160	1,133	−2
3 September 1996	3 September 1996	1,160	1,109	−4

[a] Results of triplicate analyses

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Chemical	TOX No.
Hexachloro-1,3-butadiene	1
n-Hexane	2
Acetone	3
1,2-Dichloroethane	4
Cobalt Sulfate Heptahydrate	5
Pentachlorobenzene	6
1,2,4,5-Tetrachlorobenzene	7
D & C Yellow No. 11	8
o-Cresol, *m*-Cresol, and *p*-Cresol	9
Ethylbenzene	10
Antimony Potassium Tartrate	11
Castor Oil	12
Trinitrofluorenone	13
p-Chloro-α,α,α-trifluorotoluene	14
t-Butyl Perbenzoate	15
Glyphosate	16
Black Newsprint Ink	17
Methyl Ethyl Ketone Peroxide	18
Formic Acid	19
Diethanolamine	20
2-Hydroxy-4-methoxybenzophenone	21
N, N-Dimethylformamide	22
o-Nitrotoluene, *m*-Nitrotoluene, and *p*-Nitrotoluene	23
1,6-Hexanediamine	24
Glutaraldehyde	25
Ethylene Glycol Ethers	26
Riddelliine	27
Tetrachlorophthalic Anhydride	28
Cupric Sulfate	29
Dibutyl Phthalate	30
Isoprene	31
Methylene Bis(thiocyanate)	32
2-Chloronitrobenzene and 4-Chloronitrobenzene	33
1-Nitropyrene	34
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Sodium Selenate and Sodium Selenite	38
Cadmium Oxide	39
β-Bromo-β-nitrostyrene	40
1,1,1-Trichloroethane	41
1,3-Diphenylguanidine	42
o-, *m*-, and *p*-Chloroaniline	43
o-Nitrotoluene and *o*-Toluidine Hydrochloride	44
Halogenated Ethanes	45
Methapyrilene Hydrochloride	46
Methacrylonitrile	47
1,1,2,2-Tetrachloroethane	49
Cyclohexanone Oxime	50
Methyl Ethyl Ketoxime	51
Urethane	52
t-Butyl Alcohol	53
1,4-Butanediol	54
trans-1,2-Dichloroethylene	55
Carisoprodol	56
Benzyltrimethylammonium Chloride	57
60-Hz Magnetic Fields	58
Chloral Hydrate	59
Benzophenone	61
3,3′,4,4′-Tetrachloroazobenzene	65
3,3′,4,4′-Tetrachloroazoxybenzene	66
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