NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDY OF DIETARY ZINC

(CAS NO. 5263-02-5)

IN SPRAGUE DAWLEY RATS
(Hsd:Sprague Dawley SD)

(FEED STUDY)

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

January 2019

NTP TR 592

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

FOREWORD

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

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

The NTP conducts its studies in compliance with its laboratory health and safety guidelines and FDA Good Laboratory Practice Regulations and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. Studies are subjected to retrospective quality assurance audits before being presented for public review.

NTP Technical Reports are indexed in the NIH/NLM PubMed database and are available free of charge electronically on the NTP website (*http://ntp.niehs.nih.gov*).

NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDY OF DIETARY ZINC

(CAS NO. 5263-02-5)

IN SPRAGUE DAWLEY RATS
(Hsd:Sprague Dawley SD)

(FEED STUDY)

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

January 2019

NTP TR 592

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

CONTRIBUTORS

National Toxicology Program
Evaluated and interpreted results and reported findings

N.R. Catlin, Ph.D., Study Scientist
A.E. Brix, D.V.M., Ph.D., Study Pathologist
Experimental Pathology Laboratories, Inc.
C.R. Blystone, Ph.D.
M.C. Cora, D.V.M.
P.M. Foster, Ph.D.
R.A. Herbert, D.V.M., Ph.D.
M.J. Hooth, Ph.D.
A.P. King-Herbert, D.V.M.
G.E. Kissling, Ph.D.
B.S. McIntyre, Ph.D.
D.E. Malarkey, D.V.M., Ph.D.
S.L. Smith-Roe, Ph.D.
M.D. Stout, Ph.D.
G.S. Travlos, D.V.M.
M.K. Vallant, B.S., M.T.
S. Waidyanatha, Ph.D.
N.J. Walker, Ph.D.
K.L. Witt, M.S.
M.E. Wyde, Ph.D.

Battelle Columbus Operations
Conducted study and evaluated pathology findings

M.R. Hejtmancik, Ph.D., Principal Investigator
D.K. Gerken, D.V.M, Ph.D.
K.A.B. Knostman, D.V.M., Ph.D.

Experimental Pathology Laboratories, Inc.
Provided pathology review

M.H. Hamlin, II, D.V.M., Principal Investigator
A.E. Brix, D.V.M., Ph.D.
C.C. Shackelford, D.V.M., Ph.D.

Dynamac Corporation
Prepared quality assessment audits

S. Brecher, Ph.D., Principal Investigator
S. Iyer, B.S.
V.S. Tharakan, D.V.M.

NTP Pathology Working Group
Evaluated slides and contributed to pathology report on 2-year rats (June 24, 2015)

G.D. Hill, D.V.M., Ph.D., Coordinator
ILS, Inc.
A.E. Brix, D.V.M., Ph.D.
Experimental Pathology Laboratories, Inc.
M.F. Cesta, D.V.M., Ph.D.
National Toxicology Program
S.A. Elmore, D.V.M., M.S.
National Toxicology Program
R.A. Herbert, D.V.M., Ph.D.
National Toxicology Program
T. Osborne, D.V.M., Ph.D.
National Toxicology Program
A.R. Pandiri, D.V.M., Ph.D.
National Toxicology Program
C.C. Shackelford, D.V.M., Ph.D.
Experimental Pathology Laboratories, Inc.
J.M. Ward, D.V.M., Ph.D.
Global Vet Pathology

Social & Scientific Systems, Inc.
Provided statistical analyses

M.V. Smith, Ph.D., Principal Investigator
L.J. Betz, M.S.
S.F. Harris, B.S.

Biotechnical Services, Inc.
Prepared Technical Report

S.R. Gunnels, M.A., Principal Investigator
B.F. Hall, M.S.
L.M. Harper, B.S.
J.I. Powers, M.A.P.
D.C. Serbus, Ph.D.

CONTENTS

ABSTRACT

DIETARY ZINC[a]

CAS No. 5263-02-5[a]

Chemical Formula: $C_2H_6O_{12}Zn_5$ Molecular Weight: ~549.0

Synonym: Zinc hydroxide carbonate

Zinc is a naturally occurring element and is ubiquitous in the environment. Zinc itself is stable in dry air, but exposure to moist air results in the formation of zinc oxide or basic carbonate. Due to the reactivity of zinc metal, it is not found as a free element in nature but as a variety of different compounds including zinc chloride, zinc oxide, and zinc sulfate. Zinc and zinc compounds are used across a wide range of industries that include rubber production, animal feed supplementation, as a fertilizer additive, in cosmetics and drugs, as a paint pigment, in dental cements, as a wood preservative, in batteries, in galvanizing and metal work, in textile production, in television screens and watches, and in smoke bombs. Of the zinc compounds, zinc oxide is the most widely used.

Zinc was nominated by the Agency for Toxic Substances and Disease Registry (ATSDR) for carcinogenicity and genotoxicity testing based on the increasing size of the population exposed to zinc through dietary supplements and the lack of studies examining the carcinogenicity of zinc. There was an additional nomination to investigate the tumorigenicity of zinc deficiency by private individuals as a result of data showing that deficiency of some vitamins and minerals in humans can cause DNA damage. Zinc carbonate basic was selected as the source of dietary zinc due to its use as the source of supplemental zinc in rodent diets. Male and female Hsd:Sprague Dawley SD rats were exposed to dietary zinc in feed for 2 years. Genetic toxicology studies were conducted in rat peripheral blood erythrocytes, peripheral blood leukocytes, and colon epithelial cells.

2-YEAR STUDY IN RATS

Groups of 50 male and 50 female rats were fed diets containing varying levels of dietary zinc [3.5, 7, 38 (control), 250, or 500 ppm] for 104 to 106 weeks. The 3.5 and 7 ppm diets were considered to be zinc deficient, the control diet of 38 ppm was considered to be zinc sufficient, and the 250 and 500 ppm dietary zinc concentrations represented diets with excess zinc. Dietary concentrations of 3.5, 7, 38, 250, and 500 ppm resulted in average daily intakes of 0.1, 0.3, 1.4, 8.7, and 17.6 mg dietary zinc/kg body weight to males and 0.1, 0.3, 1.5, 9.9, and 19.9 mg/kg to females. Ten male and 10 female additional special study rats were exposed to the same concentrations for 53 weeks and used for micronuclei evaluations, comet assays, hematology, and trace metal concentration determinations.

There were no chemical-related effects on survival. However, male rats maintained on the 3.5 ppm zinc-deficient diet had an increased survival rate compared to the controls that was likely due to low survival of the control group as a result of nephropathy.

Mean body weights of 3.5, 7, 250, and 500 ppm males and females were within 10% of those of the controls (38 ppm) at the end of the study. Feed consumption by zinc deficient and zinc excess groups of males and females was generally similar to that by the control groups.

[a] Zinc carbonate basic (CAS No. 5263-02-5) was used as the dietary zinc source, and the formula and molecular weight shown are for zinc carbonate basic.

The incidences of adenoma of the pancreas were increased in 7 and 3.5 ppm males, and the incidence of multiple adenoma was significantly increased in 3.5 ppm males. Compared to the 38 ppm (control) groups, significantly increased incidences of acinar atrophy occurred in the pancreas of 500 ppm males and females.

In the testis of the 3.5 ppm males, the incidence of bilateral germinal epithelium atrophy was significantly increased.

GENETIC TOXICOLOGY

The frequency of micronucleated immature erythrocytes [reticulocytes or polychromatic erythrocytes (PCEs)] was measured in rat peripheral blood samples obtained at five sequential time points (up to 12 months) during the 2-year study. No biologically significant increases in micronucleated red blood cells were observed at any sampling time in either sex. Sporadic alterations in the percentage of PCEs were not considered related to treatment.

In the comet assay, no effects on percent tail DNA in blood leukocytes of male or female rats were observed at 19 days (male rats only), 3 months, or 6 months or in males at 9 months. At 12 months, increases in percent tail DNA were seen in blood leukocytes of male rats in both the excess dietary zinc and zinc-deficient diet groups. In female rats, increases in percent tail DNA were observed in blood leukocytes in the zinc-deficient diet group at 9 and 12 months.

In the colon epithelial cell samples obtained at 12 months, increased levels of DNA damage were observed in male and female rats fed a diet containing excess zinc. In addition, a significant decrease in DNA migration (percent tail DNA) was observed in females maintained for 12 months on the zinc-deficient diets. This decrease is suggestive of DNA cross-linking, a type of DNA damage.

CONCLUSIONS

Under the conditions of this 2-year dietary study, there was *equivocal evidence of carcinogenic activity** of diets deficient in zinc in male Hsd:Sprague Dawley SD rats based on higher incidences of adenoma of the pancreas and increased incidences of animals with multiple pancreatic adenomas. There was *no evidence of carcinogenic activity* of diets deficient in zinc (3.5 or 7 ppm) in female Hsd:Sprague Dawley SD rats. There was *no evidence of carcinogenic activity* of diets containing excess zinc (250 or 500 ppm) in male or female Hsd:Sprague Dawley SD rats.

Exposure to diets containing excess zinc resulted in increased incidences of nonneoplastic lesions of the pancreas in male and female rats. Exposure to diets deficient in zinc resulted in increased incidences of nonneoplastic lesions of the testes in male rats.

---

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 8. A summary of the Peer Review Panel comments and the public discussion on this Technical Report appears on page 10.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Dietary Zinc

	Male Hsd:Sprague Dawley SD Rats	Female Hsd:Sprague Dawley SD Rats
Concentrations in feed	3.5, 7, **38 (control)**, 250, or 500 ppm	3.5, 7, **38 (control)**, 250, or 500 ppm
Average daily doses	0.1, 0.3, **1.4** (control), 8.7, or 17.6 mg/kg	0.1, 0.3, **1.5** (controls), 9.9, or 19.9 mg/kg
Survival rates	31/50, 28/50, **20/50**, 21/50, 21/50	32/50, 34/50, **25/50**, 27/50, 31/50
Body weights	Exposed groups within 10% of the control group at the end of the study	Exposed groups within 10% of the control group at the end of the study
Nonneoplastic effects	Pancreas: acinus, atrophy (3/50, 4/48, **3/49**, 3/48, 13/48) Testis: bilateral, germinal epithelium, atrophy (7/50, 1/50, **0/50**, 0/50, 1/50); germinal epithelium, atrophy (3/50, 0/50, **5/50**, 3/50, 4/50); germinal epithelium, atrophy, includes bilateral (10/50, 1/50, **5/50**, 3/50, 5/50)	Pancreas: acinus, atrophy (4/48, 2/49, **2/50**, 5/49, 10/49)
Neoplastic effects	None	None
Equivocal findings	Pancreas: adenoma (21/50, 19/48, **11/49**, 13/48, 10/48);	None
Level of evidence of carcinogenic activity	Equivocal evidence with a diet deficient in zinc (3.5 and 7 ppm) No evidence with excess zinc in the diet (250 and 500 ppm)	No evidence with a diet deficient in zinc (3.5 and 7 ppm) No evidence with excess zinc in the diet (250 and 500 ppm)

Genetic toxicology

Micronucleated erythrocytes in rat peripheral blood *in vivo*:	Negative in males and females
DNA damage (comet assay) in rats	
Blood in rats fed zinc deficient diets:	Positive in males at 12 months and females at 9 and 12 months. Negative in males at 19 days and 3, 6, and 9 months and females at 3 and 6 months.
Blood in rats fed excess zinc diets:	Positive in males at 12 months. Negative in males at 19 days and 3, 6, and 9 months and females at all time points.
Colon in rats fed zinc deficient diets:	Negative in males and positive in females.
Colon in rats fed excess zinc diets:	Positive in males and females

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

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

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

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

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

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

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

NATIONAL TOXICOLOGY PROGRAM TECHNICAL REPORTS
PEER REVIEW PANEL

The members of the Peer Review Panel who evaluated the draft NTP Technical Report on dietary zinc on July 13, 2017, are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, panel members have five major responsibilities in reviewing the NTP studies:

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

Russell C. Cattley, V.M.D., Ph.D., Chairperson
College of Veterinary Medicine
Auburn University
Auburn, AL

Michael W. Conner, D.V.M., Primary Reviewer
Global Blood Therapeutics, Inc.
San Francisco, CA

Noël Dybdal, D.V.M., Ph.D.
Genentech
San Francisco, CA

Terry Gordon, Ph.D., Primary Reviewer
New York University School of Medicine
New York, NY

Gabriele Ludewig, Ph.D., Primary Reviewer
College of Public Health
University of Iowa
Iowa City, IA

Kristini K. Miles, Ph.D.
Kimberly-Clark Corporation
Roswell, GA

Richard A. Peterson, II, D.V.M., Ph.D., Primary Reviewer
AbbVie
Chicago, IL

SUMMARY OF PEER REVIEW PANEL COMMENTS

On July 13, 2017, the draft Technical Report on the toxicology and carcinogenesis studies of dietary zinc received public review by the National Toxicology Program's Technical Reports Peer Review Panel. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. M. E. Wyde, NIEHS, introduced the draft NTP Technical Report on dietary zinc by noting that zinc is an essential trace element with various other critical biological functions. Dr. Wyde also noted that in many men over 18 and women over 14, zinc intake is below the estimated average requirement and with the popularity of zinc as a dietary supplement, many are also ingesting excess zinc. Dietary zinc deficiency was nominated by private individuals. Excess zinc exposure was nominated by the Agency for Toxic Substances and Disease Registry. Zinc carbonate was selected to be the test article in 2-year feed studies in rats. Genotoxicity testing was also conducted. Dr. Wyde stated that the management of zinc levels was a critical element of the study design, particularly elimination of extraneous sources of zinc.

The proposed conclusions were *equivocal evidence of carcinogenic activity* of diets deficient in zinc in male Hsd:Sprague Dawley SD rats, *no evidence of carcinogenic activity* of diets deficient in zinc in female Hsd:Sprague Dawley SD rats, and *no evidence of carcinogenic activity* of diets containing excess zinc in male or female Hsd:Sprague Dawley SD rats.

Dr. Ludewig, the first reviewer, found the study design and conduct appropriate, with the data well-described and analyzed. She said the study was interesting in that it involved both an excess and a deficiency of dietary zinc. She agreed with the proposed conclusions. She questioned references to *blood levels* of the metals, in that metals are usually measured in plasma or serum, not whole blood. She also requested clarification of LOD and LOQ with respect to measured copper levels. She said she would have added selenium to the analysis and would have liked to see metal determinations in the organs. She also would have liked to have seen a discussion about kinetics with respect to the reference to 'no effects on zinc levels in blood,' especially for the zinc deficient group. She asked for further explanation of the adenomas in the pituitary gland and discussion of clear cell foci in the liver. She found the atrophy of the pancreas in the excess zinc exposure to be surprising, because the pancreas is the major organ to get rid of excess zinc- suggesting that excretion is completely changed in those animals. She requested more discussion of the literature on zinc deficiency and Alzheimer's disease.

Dr. A. E. Brix, NIEHS, EPL, Inc., said there were no good historical controls due to the unique diet the animals were fed, and that the pituitary adenomas in the deficiency diet were probably due to the lower survival in controls than in the other two groups. Regarding the clear cell foci, she noted that typically results are not brought forward into the body of the report if they do not attain statistical significance unless they are very unusual or important. Clear cell foci can be a common background lesion, she added. She said that historical controls are not kept for nonneoplastic lesions, but the incidences of clear cell foci seen in this study are in line with what we would expect to see. Regarding the atrophy of the pancreas, she observed that it is commonly seen in old rats.

Dr. Wyde noted that metal levels were measured in blood, and the statement in the report pointed out by Dr. Ludewig was a mistake and would be corrected. Regarding the copper data, the staff agreed that more discussion should be added to the report. Regarding the zinc excretion, he said the staff would check the literature to see if there would be further information that could be added to the report on that issue.

Dr. Dybdal, the second reviewer, agreed that the study was well-designed. She was impressed with the efforts that were made to manage and track environmental zinc exposure. She felt that the report was well-written, including the presentation of the nonneoplastic lesions. She agreed with the proposed conclusions.

Dr. Miles, the third reviewer, also felt that the study was comprehensive and well-designed. She said that there appeared to be a sex difference. She recommended inclusion, in the introduction, of additional information on phytate. She asked why the colon, rather than the duodenum, was selected for the comet assay, since the duodenum is the area reportedly responsible for the majority of zinc absorption. She agreed with the proposed conclusions.

Dr. Wyde agreed to add more information on phytates to the introduction. He said that several other tissues were considered for the comet assay, but the colon was one of the few that yielded results.

Dr. Peterson, the fourth reviewer, said the study design was excellent, with a single study evaluating both high and low concentrations. He approved of the care taken to avoid environmental contamination and agreed with the proposed conclusions.

Dr. Conner noted that there is a body of literature about zinc deficiency in animals. He recommended some discussion of cell proliferation in the esophagus associated with zinc deficiency in rodents. He found it interesting that no effect on the esophagus had been observed in the NTP study. He discussed the difficulty of removing zinc from the environment and diet, and questioned how successfully it had been done in the study, given the blood levels seen.

Dr. Dybdal recommended caution about the early literature Dr. Conner referenced due to confounding factors.

Dr. Wyde said he was also surprised that nothing was seen in the esophagus.

Dr. Gordon said that there should have been more discussion and emphasis in the report about how the environment and diet were controlled for zinc contamination. Dr. Wyde said that diet batches were analyzed carefully for zinc content throughout the study. He agreed to check the report and add to it as necessary.

Dr. Ludewig felt that the diet had been well described. She said she was surprised by the statement that zinc carbonate had been chosen due to its bioavailability. Having checked the literature, she felt that the choice was justified, but not due to 'greater' bioavailability. Dr. Wyde agreed to change the language in the report to reflect Dr. Ludewig's comment.

Dr. Cattley called for the conclusions to be projected. Dr. Dybdal moved to accept the conclusions as written. Dr. Conner seconded the motion. The panel voted (6 yes) to accept the conclusions as written.

INTRODUCTION

DIETARY ZINC[a]

CAS No. 5263-02-5[a]

Chemical Formula: $C_2H_6O_{12}Zn_5$ Molecular Weight: ~549.0

Synonym: Zinc hydroxide carbonate

For the purposes of this Technical Report, an extensive review of the literature pertaining to zinc was considered beyond the scope of this report and only the studies on oral exposures of zinc in experimental animals and in humans are highlighted here. Several more detailed reviews, as well as a full toxicologic profile for zinc and zinc compounds, can be found in the literature (WHO, 2001; IOM, 2002; ATSDR, 2005).

CHEMICAL AND PHYSICAL PROPERTIES

Zinc, a blue-white metal, is a naturally occurring element and is ubiquitous in the environment. Zinc is stable in dry air, but exposure to moist air results in the formation of zinc oxide or basic carbonate. Due to the reactivity of zinc metal, it is not found as a free element in nature but as a variety of different compounds including zinc chloride, zinc oxide, and zinc sulfate (Goodwin, 1998; WHO, 2001; Peganova and Eder, 2004).

PRODUCTION, USE, AND HUMAN EXPOSURE

Zinc is a natural element of the earth's crust. In the United States, the concentrations of zinc in soils and earth surface materials range from less than 5 to 2,900 mg/kg, with a mean of 60 mg/kg (Shacklette and Boerngen, 1984). The existence of zinc in the environment (air, water, soil) is natural. However, anthropogenic sources of zinc, such as mining and metallurgic operations involving zinc and the use of commercial products containing zinc, result in higher environmental levels of zinc. Of the 1,662 hazardous waste sites that have been proposed for inclusion on the United States Environmental Protection Agency (USEPA) National Priorities List, zinc has been identified in at least 985 sites (ATSDR, 2005). However, the number of these sites that have specifically been evaluated for zinc is unknown. The majority of the zinc produced today comes from the mineral sphalerite (ZnS) (Goodwin, 1998). Smithsonite, also known as zinc carbonate, and hemimorphite are also important sources of zinc from nature (Goodwin, 1998). As of 2015, approximately 850,000 metric tons of zinc were produced in the United States from domestic ores; the world production from mines is approximately 13,400,000 metric tons (USGS, 2016). Zinc is also imported into the United States; in 2013, 713,000 metric tons were imported as refined zinc and 3,000 metric tons were imported as ores and concentrates (USGS, 2016).

Zinc exposure in humans is mainly through ingestion of food, drinking water, polluted air, and tobacco products and through occupational exposure. Zinc is an essential element in humans and animals due to its requirement for membrane stability, enzyme activities, and in protein and nucleic acid metabolism (WHO, 2001). The National Academy of Sciences has established recommended daily allowances (RDAs) for men and women at 11 mg per day and 8 mg per day, respectively (IOM, 2002). The RDAs increase for women during pregnancy to 11 mg per day for adult women and 12 mg per day for women between ages 14 and 18. These RDAs increase a further 1 mg per day during lactation. For humans, the average intake of zinc from food and water ranges from 5.2 to 16.2 mg per day, and an additional 1 mg per day may be provided through dietary supplements (USEPA, 1980; Pennington *et al.*, 1986). Zinc deficiencies occur when the dietary intake of zinc falls well below these RDAs. A tolerable upper

[a] Zinc carbonate basic (CAS No. 5263-02-5) was used as the dietary zinc source, and the formula and molecular weight shown are for zinc carbonate basic.

intake level of 40 mg per day for adult men and women is based on the potential for elevated zinc intakes to interfere with copper absorption (Simpson *et al*., 2011). Food is a major source of zinc within the general population and animal products tend to have the highest zinc levels of the most commonly consumed foods (NRC, 1979; USEPA, 1987). Meats, poultry, and fish have levels of 24.5 mg zinc/kg on average whereas grains and potatoes contain an average of 7 mg/kg (Mahaffey *et al*., 1975). From 1982 to 1984, the Food and Drug Administration (FDA) conducted an extensive survey of diets in individuals across the United States and estimated the daily zinc intake (mg per day) across eight age and sex groups: 6- to 11-month-old infants, 5.24; 2-year-old children, 7.37; 14- to 16-year-old girls, 9.90; 14- to 16-year-old boys, 15.61; 25- to 30-year-old women, 9.56; 25- to 30-year-old men, 16.15; 60- to 65-year-old women, 8.51; and 60- to 85-year-old men, 12.64 (Pennington *et al*., 1986). These results are comparable to other studies evaluating the daily intake of zinc, including the United States Department of Agriculture's Continuing Survey of Food Intakes by Individuals and the National Health and Nutrition Examination Survey III. Many foods are also fortified with zinc as a public measure to combat zinc deficiencies in the population (Allen, 1998; Rosado, 2003; Hotz *et al*., 2005). In 1987, the amount of zinc (zinc chloride, zinc gluconate, zinc oxide, zinc sulfate) used by food manufacturers in the United States for fortification ranged from 10 to 102,150 kg (Rosado, 2003). In a literature review, the National Research Council concluded that zinc measurements in drinking water were typically below 5 mg/L (NAS, 1977). Dietary supplements are also a source of zinc in the human diet and it can come in many forms, including zinc gluconate, zinc sulfate, and zinc acetate.

Zinc has also been used in the prevention and treatment of the common cold in humans, as lozenges (approximately 13.3 mg of zinc), sprays, or intranasal gels with doses ranging from 80 to 92 mg of zinc acetate or zinc gluconate per day (Hemilä and Chalker, 2015). There is some evidence that zinc may be effective in reducing the duration and severity of symptoms related to the common cold (Kurugöl *et al*., 2007; Science *et al*., 2012; Allan and Arroll, 2014; Hemilä and Chalker, 2015). However, the data supporting this evidence are conflicting, with some studies reporting no therapeutic effect for cold treatment (Caruso *et al*., 2007). The use of zinc in certain preparations for cold treatment has also been associated with olfactory nasal epithelium tissue damage and the development of permanent anosmia (Alexander and Davidson, 2006; Carboni *et al*., 2006; Lim *et al*., 2009). The National Occupational Exposure Survey conducted from 1980 to 1983 estimated that a total of 269 workers (including 22 women) are exposed to elemental zinc and 133,608 workers (including 17,586 women) are exposed to other forms of zinc in the workplace annually (NIOSH, 1990). Occupational groups where the workers are exposed to either elemental zinc or zinc compounds include those in the fabricated metal products industry, in machine operations, janitors and cleaners, and those producing stone, clay, and glass products (NIOSH, 1990).

Zinc is widely used in industry as a protective coating for other metals, such as iron or steel. Methods of protective coating typically include galvanization, zinc plating, and painting with zinc-bearing paints (ATSDR, 2005). As a weak metal, zinc is typically alloyed with other metals (e.g., aluminum, copper, titanium, and magnesium) to increase its strength. Industry consumption of zinc in 2002 was reported as 265,000 metric tons for galvanizing, 103,000 metric tons for zinc-based alloys, and 86,800 metric tons for brass and bronze production (USGS, 2002). It is also used in dental, medical, and household applications. Zinc salts are used in pharmaceuticals as solubilizing agents in drugs such as insulin (*Merck*, 1983; Lloyd, 1984; Lloyd and Showak, 1984). Medically, zinc compounds are administered as supplements in the treatment of zinc deficiency (Keen and Hurley, 1977).

Many of the zinc compounds are used across a wide range of industries that include, but are not limited to rubber production (zinc oxide, zinc chloride, zinc hydroxide), animal feed supplementation (zinc oxide, zinc sulfate), as a fertilizer additive (zinc oxide, zinc sulfate), in cosmetics and drugs as an antifungal (zinc oxide), as a paint pigment (zinc oxide, zinc sulfide, zinc chromate), in dental cements (zinc oxide, zinc chloride, zinc sulfide, zinc phosphate), as a wood preservative (zinc chloride, zinc acetate), in batteries (zinc chloride), in galvanizing and metal work (zinc chloride, zinc sulfate, zinc cyanide, zinc phosphate), in textile production (zinc chloride, zinc acetate), and in television screens and watches (zinc sulfide) (Lewis, 1997; Goodwin, 1998; *Merck*, 2006). Zinc chloride is also a primary ingredient in smoke bombs used for crowd dispersal, in fire-fighting exercises, and by the military (WHO, 2001). Of the zinc compounds, zinc oxide is the most widely used.

REGULATORY STATUS

Zinc compounds, including zinc carbonate, are listed in the "Toxic Chemicals Subject to Section 313 of the Emergency Planning and Community Right-to-Know Act of 1986" (40 CFR, § 372.65). Zinc acetate, zinc oxide, and zinc sulfide are permitted through Federal regulations for use as components of adhesives, coatings

or rubber-packaging materials that are intended for contact with food (21 CFR, § 175.105, § 175.300, § 177.2600). The zinc compounds zinc chloride, zinc oxide, zinc stearate, and zinc sulfate are used as food additives and are Generally Recognized as Safe by the FDA when they are used "in accordance with good manufacturing practices" (21 CFR, § 175.300, § 182.90, § 182.5985, § 182.5991, § 182.5994, § 182.5997). Additionally, the FDA permits zinc oxide for use as a color additive in drugs and cosmetics with certain restrictions (21 CFR, § 73.2991). The Agency for Toxic Substances and Disease Registry (ATSDR, 2005) has derived an oral Minimal Risk Level of 0.3 mg zinc/kg body weight per day, based on a study by Yadrick *et al.* (1989) that demonstrated decreased erythrocyte superoxide dismutase and serum ferritin changes in women who were given supplements containing zinc gluconate for 10 weeks. The USEPA has derived an oral reference dose of 0.3 mg/kg per day for zinc based on a decrease in erythrocyte copper-zinc superoxide dismutase activity in human volunteers (IRIS, 2003).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

The absorption of zinc ($^{65}ZnCl_2$) occurs through both passive diffusion and a carrier-mediated process throughout the entire intestine, with the largest proportion of zinc absorption occurring from the duodenum (Methfessel and Spencer, 1973; Tacnet *et al.*, 1990). The intestinal absorption of low levels of zinc appears to be a carrier mediated process that involves a cysteine-rich intestinal protein (Davies, 1980; Gunshin *et al.*, 1991; Sturniolo *et al.*, 1991; Hempe and Cousins, 1992). Zinc absorption by this process can become saturated when the zinc concentration becomes too high in the intestine due to the limited binding capacity of cysteine-rich intestinal protein for zinc. Two zinc transporter protein families have since been identified and include the ZnT (solute-linked carrier 30) proteins that are responsible for lowering intracellular zinc and the Zip (Zrt- and Irt-like) proteins that promote zinc transport (Cousins *et al.*, 2006). Metallothionein, a metal-binding protein present within the mucosal cells, can be induced by zinc and contributes to zinc homeostasis (Richards and Cousins, 1975). When zinc binds to metallothionein, it remains in the mucosal cell lining until the bound zinc is excreted from the rat through sloughing off of these cells within the gastrointestinal tract (Foulkes and McMullen, 1987). Studies have shown that zinc absorption in rats can be increased when the metallothionein levels are lower (Flanagan *et al.*, 1983). In male Wistar rats fed a diet with 0.81 mg zinc/kg body weight as zinc chloride or zinc carbonate, the amount of labeled zinc absorbed ranged from 40.0% to 48.4% (Galvez-Morros *et al.*, 1992). Zinc absorption rates can be affected by the presence of phytate, the salt from phytic acid and the principal storage form of phosphorous in many plant tissues (Lopez *et al.*, 2002). As a common constituent of plant-derived foods like cereals or legumes, zinc absorption rates can be decreased by diets containing phytate or high amounts of phosphorus due to the binding of zinc and phosphate, which results in the coprecipitation of zinc with calcium phosphate in the intestines (Nelson *et al.*, 1985; Sandström and Sandberg, 1992). This has been demonstrated in rats fed radiolabeled zinc and phytate supplemented diets that excreted significantly more zinc in the feces compared to rats fed the same diet without phytate (Davies and Nightingale, 1975).

The interaction of zinc with other metals, such as copper, cadmium, and cobalt, has also been extensively studied (ATSDR, 2005). Dietary intake of zinc has been shown to interfere with copper absorption; mainly when the levels of zinc are significantly higher than those of copper (Fischer *et al.*, 1981). This is due to their competition for the same metallothionein protein, of which, copper has the higher affinity (Ogiso *et al.*, 1979). *In vitro*, cadmium also competes with zinc for binding to metallothionein (Harford and Sarkar, 1991), and has been demonstrated to interfere with the distribution of tissue zinc and lead to accumulation of zinc in the liver and kidney (Gachot and Poujeol, 1992). In rats, administration of 7 ppm zinc acetate in the diet resulted in a reduction in cadmium-induced carcinogenesis of the prostate gland and the testes (Waalkes and Rehm, 1992). Additionally, zinc chloride had a protective effect against cobalt-induced testis toxicity when the zinc and cobalt were co-administered to mice (Anderson *et al.*, 1993).

Significantly increased levels of zinc were distributed across the heart, spleen, kidneys, liver, bone, and blood from rats that were fed 191 mg zinc/kg body weight per day as zinc acetate over 3 months (Llobet *et al.*, 1988). Of these tissues, the largest increases in zinc were observed in bone (258% of the control) and blood (520% of the control). Mice that were given 76.9 mg/kg per day as zinc sulfate (Schiffer *et al.*, 1991) or 38 mg/kg per day as zinc nitrate (Cooke *et al.*, 1990) for approximately 1 month, also demonstrated elevated zinc levels in the kidneys and the liver. Zinc can be found in the blood as either a diffusible or nondiffusible form (NRC, 1979). Approximately two-thirds of zinc in its diffusible form is freely exchangeable and is loosely bound to albumin (Cousins, 1985). The diffusible form of zinc can also bind to amino acids, particularly histidine and cysteine, and form a complex that can be transported passively across tissue membranes to bind

to proteins such as metallothionein (Henkin, 1974). A small amount of the nondiffusible form of zinc binds tightly with α2μ-globulin in the liver and circulates in the plasma (Henkin, 1974; Cousins, 1985). When zinc is bound to α2μ-globulin, it is not freely exchangeable with other zinc ligands such as zinc-albumin and the zinc-amino acid complexes in the serum.

Rats demonstrated a linear increase in fecal excretion of zinc that was proportional to the amount of zinc supplemented into their diet (32 mg zinc/kg body weight per day as zinc oxide for 7 to 42 days or 50 to 339 mg/kg for 21 days) (Ansari *et al*., 1975, 1976). Diets supplemented with different forms of zinc (zinc chloride, zinc sulfate, zinc phosphate, or zinc citrate) did not result in any differences in fecal excretion, total excretion, or retention of zinc when fed to rats (Seal and Heaton, 1983). Zinc is mainly excreted in the bile of rats as a complex with reduced glutathione (Alexander *et al.*, 1981). Excretion of zinc can be influenced by several factors including the ingestion of a zinc-deficient or malnourished diet, which can increase the urinary excretion of zinc as a result of tissue breakdown and catabolism that occurs during starvation (Spencer *et al*., 1976).

Humans

Several studies measuring the oral absorption rates of several forms of zinc have been performed in humans. In humans, the absorption of zinc from short-term exposures to zinc supplements can range from 8% to 81% and is influenced by the amount of zinc ingested and the amount and type of food ingested (Reinhold *et al*., 1976; Sandström and Cederblad, 1980; Aamodt *et al.*, 1983; Istfan *et al*., 1983; Sandström and Abrahamson, 1989; Hunt *et al*., 1991; Sandström and Sandberg, 1992). High-protein diets have been shown to facilitate zinc absorption (Hunt *et al*., 1991), while the calcium and phosphate present in dairy products decreases zinc absorption and plasma zinc concentration (Pécoud *et al*., 1975). Zinc-deficient individuals have an increased absorption rate of administered zinc (Spencer *et al*., 1985; Johnson *et al*., 1988). As is seen with rats, phytate in the diet can also reduce the reabsorption of zinc that is secreted in the gastrointestinal tract in humans (Sandström and Sandberg, 1992). The absorption of zinc can also be influenced by endogenous substances such as amino acids that, when complexed with zinc, enhance the absorption of zinc from all intestinal segments (Wapnir and Stiel, 1986).

Zinc is one of the most abundant trace metals in humans and is normally found distributed across all tissues. Approximately 90% of the total amount of zinc in the body is in the muscle and bone (approximately 60% and 30%, respectively) (Wastney *et al*., 1986). Considerable concentrations of zinc can be measured within many tissues including the liver, gastrointestinal tract, kidney, skin, lung, brain, heart, pancreas, prostate, retina, and sperm (Forssén, 1972; Llobet *et al*., 1988; He *et al*., 1991). The levels of zinc within these tissues can vary across individuals and the distribution of zinc within some of these tissues appears to be influenced by age (Schroeder *et al*., 1967; Forssén, 1972). For example, zinc concentrations have been shown to increase in the liver, pancreas, and prostate and decrease in the uterus and aorta with age. The National Human Adipose Tissue Survey, conducted in the United States in 1982, measured zinc concentrations in adipose tissue that ranged from 1.1 to 6.0 μg/g (ATSDR, 2005). Similarly, the mean whole blood zinc concentrations for residents of Baajoz, Spain, were reported as 6.95 ± 1.08 mg/L (Moreno *et al*., 1999). The blood and serum levels for adolescents (15 years old) in the Swedish cities of Trollhättan and Uppsala had median zinc concentrations of 0.99 and 6.1 mg/L, respectively (Bárány *et al*., 2002). Peak zinc plasma levels occur 2 to 3 hours following a single oral dose (0.7 mg/kg as zinc sulfate) (Nève *et al*., 1991; Sturniolo *et al*., 1991). The concentration of zinc in fingernails and toenails from populations in the United States, Canada, and Japan were 105, 109, and 94 μg/g, respectively (Takagi *et al*., 1988). These levels were similar to the geometric mean of zinc concentrations in the toenails and scalp hair of children in Germany that had measurements of 129 and 108 mg/kg, respectively (Wilhelm *et al*., 1991). A study measuring total concentrations of zinc in 29 different tissues from human cadavers found the lowest concentration of zinc in males and females occurred in the adipose tissue (1.5 ± 2.2 mg/kg wet weight), while the highest average concentrations were found in the skull for males (54.3 mg/kg wet weight) and in the skeletal muscle of females (59.0 mg/kg wet weight) (Saltzman *et al*., 1990).

During pregnancy, zinc transfer across perfused placentas is slow, with only 3% of the maternal zinc entering the fetal compartment over 2 hours (Beer *et al*., 1992). A study of maternal and cord blood from mothers in Singapore demonstrated that maternal zinc levels (4.97 ± 1.15 mg/L) are normally higher than cord blood levels (1.58 ± 0.45 mg/L) (Ong *et al*., 1993). These maternal and cord blood mean zinc levels are similar to those that were measured in Indian women, which were 6.33 and 2.53 mg/L, respectively (Raghunath *et al*., 2000). Lactation also serves as an exposure route of infants to zinc (Rossowska and Nakamoto, 1992). Zinc levels in human milk have been found to increase and peak in the first 2 days postpartum (12.0 ± 4 mg/L) and then decline during the period of lactation (5.0 ± 1.4 mg/L at 6 days postpartum) to levels that are only 12% of what they were initially (Arnaud and

Favier, 1995; Dórea, 2002). Conversely, lactating Polish women at 10 to 30 days postpartum had mean levels of zinc in blood plasma of 0.76 ± 0.20 mg/L, which increased from the levels that were measured at 0 to 4 days postpartum (0.51 ± 0.13 mg/L).

In humans, ingested zinc is mainly excreted through the intestine and feces (Davies and Nightingale, 1975; Reinhold *et al*., 1976; Wastney *et al*., 1986). Other minor excretion routes include the urine (Wastney *et al*., 1986), saliva, hair, and sweat (Prasad *et al*., 1963; Hambidge *et al*., 1972; Henkin *et al*., 1975; Greger and Sickles, 1979; Rivlin, 1983). The excretion of zinc in the feces and urine reflects zinc intake, with excretion increasing as intake increases (Spencer *et al*., 1985; Wastney *et al*., 1986). Zinc mean concentrations were measured in the feces of urban Hispanics (75 mg/kg wet weight) and rural African Americans (94 mg/kg wet weight) in the United States (ATSDR, 2005), as well as in the ear wax (88 and 103 mg/kg dry weight), blood plasma (0.79 and 1.7 mg/kg dry weight), sweat (0.50 and 1.58 mg/kg dry weight), and skin (15.6 and 1,000 mg/kg dry weight) from two individuals from California (Krishnan and Que Hee, 1992).

TOXICITY

Experimental Animals

The major targets of zinc toxicity in animals include the gastrointestinal and hematopoietic systems. Other targets for zinc toxicity in animals include the liver, the pancreas, and the kidney. The LD_{50}s for several zinc compounds (zinc acetate, zinc nitrate, zinc chloride, and zinc sulfate) range from 186 to 623 mg zinc/kg per day in both rats and mice (Domingo *et al*., 1988). Zinc acetate is the most lethal to rats followed by zinc nitrate, zinc chloride, and then zinc sulfate. In mice, zinc acetate is also the most lethal followed by zinc nitrate, zinc sulfate, and then zinc chloride (Domingo *et al*., 1988).

Mice given 1,110 mg zinc/kg per day as zinc sulfate in the diet for 13 weeks developed ulcers in the forestomach, in contrast to rats that exhibited no gastrointestinal effects following a diet with half that amount (565 mg/kg per day) given over 13 weeks (Maita *et al*., 1981). Gastrointestinal effects were also observed in ferrets that ingested 390 mg/kg per day as zinc oxide for 2 weeks and included intestinal hemorrhaging and a 75% reduction in food intake (Straube *et al*., 1980). These effects were not demonstrated in ferrets from the same study that were fed 195 mg/kg per day for up to 21 days.

The liver may be a target of zinc exposure; however, the studies performed to this date are inconclusive due to many factors that include low numbers of animals or use of an inappropriate animal model. Rats fed 191 mg zinc/kg per day as zinc acetate demonstrated no histopathology of the liver or any changes in serum enzyme levels, which included measurements of serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, and alkaline phosphatase (Llobet *et al*., 1988). Similarly, mink that were fed 327 mg zinc/kg per day as zinc sulfate for 144 days did not display any liver histopathology (Aulerich *et al*., 1991). Hepatic effects (necrotic hepatocytes and large quantities of hemo-siderin in Kupffer cells) were observed in sheep that were administered time-weighted-average doses of 19 mg zinc/kg per day as zinc oxide over 49 to 72 days (Allen *et al*., 1983). Hexobarbital sleeping times were decreased in rats that received 40 mg zinc/kg per day as zinc sulfate, demonstrating a potential induction of microsomal enzymes (Kadiiska *et al*., 1985). Increased serum cholesterol was observed in two separate studies in rats that were administered either 2.8 or 10 mg zinc/kg per day as zinc acetate in the diet over 2 to 7 months (Klevya and Hyg, 1973; Katya-Katya *et al*., 1984). However, other studies have not shown an effect on total cholesterol, high-density lipoprotein (HDL) cholesterol, or serum triglycerides in rats fed 3 or 25 mg zinc/kg per day of an unspecified zinc compound (Fischer *et al*., 1980).

Administration of 1,110 mg zinc/kg per day as zinc sulfate in female mice for 13 weeks resulted in increased absolute and relative kidney weights as well as unspecified regressive kidney lesions; these effects were not seen in the mice and rats from the same studies that received 104 or 565 mg zinc/kg per day, respectively (Maita *et al*., 1981). In another study, epithelial cell damage of the glomerulus and proximal convoluted tubules was observed in rats exposed for 3 months to 191 mg zinc/kg per day as zinc acetate (Llobet *et al*., 1988).

Humans

In humans, zinc deficiency has been associated with several health outcomes. These outcomes include but are not limited to dermatitis, anorexia, growth retardation, inefficient wound healing, hypogonadism and impaired reproductive capacity, and increased incidences of congenital malformations from zinc deficient mothers (Sandstead, 1981; Kumar *et al*., 2007).

Ingestion of zinc sulfate has been associated with gastrointestinal issues, such as vomiting, diarrhea, abdominal cramps, and nausea (Brown *et al*., 1964; Moore, 1978; CDC, 1983; Samman and Roberts, 1987). These gastrointestinal effects have also been seen with

zinc gluconate, which induced severe nausea and vomiting following ingestion of 6.8 mg/kg (Lewis and Kokan, 1998). Ingestion of zinc oxide through exposure to beverages prepared or stored with galvanized metals has been associated with gastrointestinal distress (nausea, vomiting, and diarrhea) (Callender and Gentzkow, 1937; CDC, 1983).

Copper deficiencies can result from long-term consumption of zinc. Intermediate (6 to 13 weeks) or long-term exposures to zinc compounds (0.7 to 0.9 mg/kg per day) can lead to minor subclinical changes in copper-sensitive enzymes, such as superoxide dismutase (Fischer *et al.*, 1984; Yadrick *et al.*, 1989; Davis *et al*., 2000; Milne *et al*., 2001). Anemia has been reported in several studies with individuals who have taken zinc supplements over a long period of time (1 to 8 years) (Porter *et al*., 1977; Prasad *et al*., 1978; Patterson *et al*., 1985; Hale *et al.*, 1988; Broun *et al.*, 1990; Stroud, 1991; Summerfield *et al*., 1992; Salzman *et al*., 2002; Willis *et al*., 2005). Female subjects who ingested supplements containing 50 mg zinc per day as zinc gluconate for 10 weeks displayed a significant reduction in erythrocyte superoxide dismutase activity, hematocrit, and serum ferritin when compared to their pretreatment levels (Yadrick *et al.*, 1989). Decreases in erythrocyte superoxide dismutase activity were also reported in males administered 50 mg zinc per day as zinc gluconate for 6 weeks (Fischer *et al.*, 1984). Several other studies with men and women (0.68 to 0.83 mg/kg per day) given zinc supplements for 4 to 6 weeks, reported significant increases and decreases in copper-sensitive enzymes (i.e., erythrocyte superoxide dismutase) (Fischer *et al.*, 1984; Yadrick *et al.*, 1989; Davis *et al*., 2000; Milne *et al*., 2001). This is in contrast to another study that demonstrated no significant changes in hematologic or immunologic parameters or in copper-sensitive enzymes as a result of zinc exposure (0.43 mg supplemental zinc/kg per day) in healthy men (Bonham *et al.*, 2003a,b).

There are several conflicting studies that examine hepatic effects through serum lipid profiles in humans that were exposed to either zinc sulfate or zinc gluconate from 3 to 12 months. Reduced high density lipoprotein (HDL) cholesterol was seen following ingestion of either 2.3 to 4.3 mg/kg per day for 5 to 6 weeks (Hooper *et al*., 1980; Chandra, 1984) or 0.71 mg/kg per day for 12 weeks (Black *et al.*, 1988). Additionally, young women taking 1.6 mg zinc/kg per day for 2 months had decreased HDL cholesterol (Freeland-Graves *et al*., 1980). However, no effects on HDL cholesterol were seen in young men or women taking zinc for 6 weeks (2.0 or 2.4 mg/kg per day, respectively), but low-density lipoprotein (LDL) cholesterol was significantly decreased in the women (Samman and Roberts, 1988). No effects on HDL, LDL, or triglyceride levels were seen in men taking 0.43 mg per day as zinc glycine chelate (Bonham *et al*., 2003b). Black *et al*. (1988) also reported no changes in serum cholesterol, triglyceride, or LDL cholesterol. Chronic exposure (8 years) to 2 mg/kg per day in subjects older than 68 years also had no effects on triglycerides or cholesterol levels (Hale *et al*., 1988).

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Experimental Animals

The role of zinc in reproduction has been extensively studied. In the male, the concentration of zinc is high in multiple species in the adult testis and the prostate gland, which have the highest zinc concentration compared to any other organ (Srivastava and Setty, 1985; Bedwal and Bahuguna, 1994). Zinc deficiency can lead to gonadal dysfunction, decreased testicular weight, and diminished seminiferous tubules (Bedwal and Bahuguna, 1994). These effects are thought to stem from impaired angiotensin converting enzyme activity (Cushman and Cheung, 1971; Jaiswal *et al*., 1984; Bedwal and Bahuguna, 1994), which ultimately leads to decreased steroidogenesis and inhibited spermatogenesis (Barney *et al*., 1968; Reeves and O'Dell, 1981; Bedwal and Bahuguna, 1994). The effects on spermatogenesis in the zinc-deficient rat are frequently manifested as defects in spermatozoa (Pařízek *et al*., 1966; Bedwal and Bahuguna, 1994). Testis atrophy, attributed to low availability or increased urinary excretion of zinc, is frequently observed in zinc deficient states that include sickle cell anemia, chronic alcoholism, idiopathic male sterility, or the toxic effects of di-(2-ethyl hexyl) phthalate or other phthalic acid esters (Cater *et al.*, 1977; Foster *et al.*, 1980; Oishi and Hiraga, 1983; Bedwal and Bahuguna, 1994). In females across multiple species, zinc deficiency, as a result of low levels of zinc in the diet (0 to 2 ppm), can interfere with the synthesis and secretion of follicle-stimulating hormone and luteinizing hormone, contributing to observed abnormal ovarian development (Bedwal and Bahuguna, 1994) and disruption of the estrous cycle in rats (Hurley and Swenerton, 1966; Gombe *et al.*, 1973; Bedwal and Bahuguna, 1994). A reduction in the receptivity of females to mating also appears to be a result of zinc deficiencies (<1 ppm) (Hurley and Swenerton, 1966; Apgar, 1970).

Low levels of dietary zinc (0 to 7.5 ppm) during gestation in rats have been associated with adverse outcomes, including increased fetal resorption rates, reduced or inhibited fetal growth, high incidences of congenital malformations, prolonged gestation, stillbirths, and

difficulty in parturition (Hurley and Swenerton, 1966; Apgar, 1970; Beach *et al*., 1980; Soltan and Jenkins, 1982; Masters and Moir, 1983; Sato *et al*., 1985; Bedwal and Bahuguna, 1994; Khan *et al*., 2001; Johnson *et al*., 2011). Zinc has been shown to be essential to fetal skeletal development in rats, with zinc deficient dams (0 to 1.3 ppm) exhibiting severe anomalies in long bones, vertebrae, and ribs (Hurley and Swenerton, 1966; Hickory *et al*., 1979). Gross congenital malformations induced in the fetuses of zinc deficient mothers fed diets with zinc levels ranging from 0 to 9 ppm encompassed a wide range of organ systems that included skeletal, brain, central nervous system, eye, heart, lung and urogenital systems (Hurley and Swenerton, 1966; Warkany and Petering, 1972; Sandstead, 1973; Beach *et al*., 1980). Excess maternal dietary zinc (0.2% to 0.5% of the diet) has demonstrated similar developmental outcomes to zinc deficiency (reduced fetal growth and weight, increased stillbirths, and increased fetal resorptions); however, congenital malformations were not observed in most studies (Schlicker and Cox, 1968; Cox *et al*., 1969; Ketcheson *et al*., 1969; Mulhern *et al*., 1986; Johnson *et al*., 2011). There are also some indications of fetal endocrine effects as a result of maternal excess zinc exposure (30 mg/kg per day as zinc chloride), with Johnson *et al*. (2011) reporting an increase in anogenital distance in the male offspring.

Humans

As in animals, zinc is an essential component of reproduction and development in humans and has been extensively studied and reviewed (Apgar, 1985; Favier, 1992; Bedwal and Bahuguna, 1994). The importance of zinc in human reproduction is demonstrated by the high levels of zinc in reproductive tissues and in its fluctuations in reproductive processes. Within the testis, the concentration of zinc in seminal plasma is much greater than in blood plasma and is thought to be important for both spermatogenesis and spermatozoa maintenance (Walsh *et al*., 1994). This is demonstrated with studies showing positive correlations between zinc concentrations in blood and seminal plasma and sperm-cell density, as well as lower zinc concentrations in infertile men (Xu *et al*., 1994; Ames and Wakimoto, 2002). In women, plasma zinc concentrations fluctuate depending on menstrual cycle and pregnancy status, and low serum zinc concentrations have been associated with several pregnancy risk factors (Verburg *et al*., 1974; Cherry *et al*., 1981; Apgar, 1985; Bedwal and Bahuguna, 1994).

Epidemiological data in humans support some of the experimental observations in animals where zinc deficiencies have been associated with adverse developmental outcomes. This is supported by the effects seen in pregnant women suffering from acrodermatitis enteropathica. Acrodermatitis enteropathica is an autosomal recessive disease that results in impaired zinc absorption in the mothers, leading to high frequencies of fetal deaths, and congenital abnormalities in the form of neural tube defects (Verburg *et al*., 1974; Hambidge *et al*., 1975; Soltan and Jenkins, 1982; Bedwal and Bahuguna, 1994; Simpson *et al*., 2011; Chaffee and King, 2012). There appears to be a relationship between zinc supplementation during pregnancy and small decreases in preterm birth (Meadows *et al*., 1981; Simmer and Thompson, 1985; Kynast and Saling, 1986; Bedwal and Bahuguna, 1994; Ota *et al*., 2015); however, these data are conflicting as several recent systematic reviews report that prenatal zinc supplementation does not affect fetal growth (Chaffee and King, 2012; Grieger and Clifton, 2015; Ota *et al*., 2015). Further, no significant evidence was found for an association between plasma zinc concentration in pregnant women and fetal growth (Tamura *et al*., 2000). There is no clear evidence for associations between low maternal zinc levels and other fetal outcomes including fetal loss, congenital malformations, intra-uterine growth retardation, prolonged labor, and preterm or postterm deliveries (Bedwal and Bahuguna, 1994; Ianotti *et al*., 2008; Johnson *et al*., 2011; Simpson *et al*., 2011; Grieger and Clifton, 2015; Ota *et al*., 2015).

CARCINOGENICITY

Experimental Animals

The potential carcinogenicity of zinc has been evaluated in only a few animal studies. Several studies have demonstrated early proliferative changes within the esophageal tissue of rats fed zinc-deficient diets ($\leq$ 1 ppm) for 1 to 3 months that included hyperkeratosis, parakeratosis, and expansion of the number of cell layers (Barney *et al*., 1968; Swenerton and Hurley, 1968; Diamond *et al*., 1971). However, aside from the formation of esophageal cancer in one rat (out of 25) on a zinc-deficient diet (2.5 to 3 ppm) in a single study (Newberne *et al*., 1997), these proliferative lesions seen with chronic zinc deficiency fail to progress to carcinogenesis without the addition of a known carcinogen. The majority of these studies investigating the influence of a zinc-deficient diet (2 to 4 ppm) on the promotion of tumorigenesis found nearly 100% gastrointestinal tumor incidences when the zinc-deficient animals were also exposed to a known carcinogen such as N-nitrosomethylbenzylamine (Schrager *et al*., 1986; Barch and Fox, 1987; Fong *et al*., 1996; Newberne, *et al*., 1997). Researchers have also found that when zinc is replenished in zinc-deficient rats, apoptosis of the esophageal epithelial cells is induced, possibly reducing

the increased potential for cancer progression from the early proliferative changes (Fong *et al.*, 2001).

Chronic zinc-deficiency has also been shown to influence the carcinogenicity of oral cadmium when administered to male rats, where rats maintained on a 7 ppm zinc diet showed decreases in the carcinogenic potential of cadmium in the prostate, testes, and hematopoietic system (Waalkes and Rehm, 1992). A 1-year carcinogenicity study with 0, 1,000, or 5,000 ppm zinc sulfate (0, 170, 850 mg/kg per day) in the drinking water or zinc oleate in the diet (5,000 ppm for 3 months, then 2,500 ppm for the next 3 months, followed by 1,250 ppm for the remainder of the study) of mice found no significant differences in the incidences of hepatocellular carcinoma, malignant lymphoma, or lung adenoma (Walters and Roe, 1965). However, the incidences of hepatocellular carcinoma in the zinc sulfate supplemented diet were increased compared to the controls (30.4% vs. 12.5%). In contrast, Halme (1961) found that 10 to 20 mg zinc/L drinking water increased the frequencies of tumors in both tumor-resistant and tumor-susceptible mouse strains, although tumor type and statistics were not reported for this study. Hypertrophy of the adrenal cortex and pancreatic islets was reported, in the absence of corresponding tumors, in a study with mice given 500 mg zinc/L drinking water as zinc sulfate over 14 months (Aughey *et al.*, 1977). These available animal studies have not adequately established a link between long-term exposure to zinc compounds and increased cancer incidence.

Humans

Studies examining the influence of dietary zinc deficiency on cancer outcomes in humans yield conflicting results and appear to depend on several factors, including geographical region. In Eastern European, Asian, and African countries, dietary zinc deficiency has been associated with the high incidences of esophageal cancer through human epidemiological studies (Kmet and Mahboubi, 1972; Lin *et al*., 1976; Van Rensburg, 1981). This association is supported by Abnet *et al*. (2005), who found a strong association between high tissue zinc concentrations measured in Chinese individuals and a reduced risk of developing esophageal squamous cell carcinoma. Studies within the United States have also shown conflicting associations between low nutrition status, including dietary zinc deficiency, and development of cancer (Kaul *et al*., 1986; Lee *et al*., 2005). Mellow *et al*. (1983) found significantly decreased plasma zinc levels [65.7 ± 3.3 µg/dL (mean ± SEM) compared to healthy controls (80.5 ± 2.4 µg/dL)] in individuals who developed squamous cancers of the head and neck. However, an ecological study of Americans between 1970 and 1974 found a reduced risk for breast, colon, and esophageal cancer, and an increased risk of ovarian and prostate cancer (Grant, 2008). A systematic review of 19 epidemiological studies investigating zinc intake and its association with other gastric cancers, such as esophageal, gastric, and colorectal cancer, also showed inconsistent associations (Li *et al*., 2014). Dietary zinc deficiency has also been shown to influence the carcinogenicity of other chemicals in humans, such as methylbenzylnitrosamine, although here the directionality of the influence appears to depend on the carcinogenic agent itself (Lin *et al*., 1976; Yang, 1980; Barch and Fox, 1987).

GENETIC TOXICITY

Metals that display genotoxicity often do so via generation of oxygen radicals. In bacterial assays purported to be sensitive to compounds that induce DNA alterations via oxygen radical generation, however, zinc was inactive. Zinc sulfate was not mutagenic in *Salmonella typhimurium* strain TA102 when tested in the absence of exogenous metabolic activation (S9 mix) (Marzin and Phi, 1985) and zinc chloride demonstrated no ability to induce DNA damage in a DNA repair-deficient strain of *Escherichia coli*, in the absence of S9 mix (Nishioka, 1975).

In mammalian cell systems, the data for zinc are mixed but it appears that in cells or animals exposed to abnormally high or low levels of zinc, genotoxic effects are observed more often than not. In human lymphoblastoid cells and primary human oral keratinocytes, zinc in the form of zinc sulfate or zinc carnosine, when present in excess, induced increases in micronuclei as well as DNA damage measured by the comet assay (Sharif *et al*., 2011, 2012). In both cell lines and both assays, abnormally low levels of zinc were also associated with elevated levels of DNA damage, while normal levels of zinc had no effect on these endpoints.

In additional studies, zinc deficiency was shown to increase DNA damage in rat glioma cells (Ho and Ames, 2002), human prostate gland epithelial cells (Yan *et al*., 2008), and human lung fibroblasts (Ho *et al*., 2003). Oxidative DNA damage was shown to be a contributing factor in the DNA damage observed in zinc deficient human lung fibroblasts (Ho *et al*., 2003).

In vivo, DNA damage measured by the comet assay was increased in peripheral blood cells of Sprague Dawley rats fed a zinc-deficient diet (< 1 ppm zinc) for 3 weeks (Song *et al*., 2009a) and DNA damage was subsequently reduced to near baseline levels when rats were provided a zinc-adequate diet (30 ppm) for 10 days. Evidence of

oxidative damage (induction of the DNA base excision repair enzyme, 8-oxoguanine glycosylase), was seen in rats fed the zinc-deficient diet. In this study, hepatic zinc concentrations were reduced by 30% in the animals fed the zinc-deficient diet; reintroduction of zinc in the diet of these animals restored hepatic zinc to normal levels.

In male Sprague Dawley rats fed a zinc-deficient diet (< 1 ppm zinc) for 28 days, single-strand DNA breaks detected by the alkaline elution assay were significantly increased in liver, but DNA from the spleen was not affected (Castro *et al*., 1992), although both organs were confirmed to be zinc deficient. Serum zinc concentrations in these rats ranged from 30% to 40% of control.

The effect of a zinc-deficient diet on micronucleus levels in polychromatic erythrocytes (PCEs) from bone marrow was evaluated in male Sprague Dawley rats (Kawasaki *et al*., 2013). Significant increases in micronuclei were detected after 4 and 6 weeks of a zinc-deficient diet (approximately 0.5 ppm zinc), but not after 2 weeks. Serum levels of zinc were reduced by approximately 70% in rats fed the zinc-deficient diet. DNA from bone marrow cells was also evaluated for evidence of oxidative damage from rats that received a zinc-deficient diet for 6 weeks, or rats that received two daily intraperitoneal injections of Tempol (100 µmol/kg), a superoxide scavenger, for 10 days before sacrifice at the end of 6 weeks of zinc deficiency. Oxidative damage to DNA was increased in rats fed a zinc-deficient diet and was suppressed by treatment with Tempol.

Chromosomal aberrations were not increased in bone marrow cells of male or female Swiss albino mice fed a diet deficient in zinc (2.8 ppm zinc) for 8 weeks compared to the levels in mice fed a zinc-adequate diet (51.6 ppm zinc) (Özkul *et al*., 1996). Plasma levels of zinc were approximately 50% lower in mice exposed to the zinc-deficient diet. Male C57Bl mice fed a standard diet supplemented with 5 ppm zinc chloride for 1 month showed no increases in chromosomal aberrations in bone marrow cells (Deknudt and Gerber, 1979); zinc levels in tissues or serum were not determined in this study.

The effect of a zinc-deficient diet on DNA damage was examined in nonhuman primates (Olin *et al*., 1993). Female rhesus monkeys were fed low-zinc diets (2 or 4 ppm) or a zinc-adequate diet (50 ppm) 2 weeks before mating and during pregnancy and lactation. Oxidative DNA damage in the form of DNA strand breakage was evaluated in livers of offspring using the alkaline unwinding assay on postnatal day 30. DNA damage was higher in offspring of monkeys fed zinc-deficient diets, and levels of 8-oxo-2′-deoxyguanosine, measured by high-performance liquid chromatography, were also higher in these animals. Plasma levels of zinc were similar among offspring of monkeys fed diets with 2, 4, or 50 ppm zinc.

Comparatively little is known about the effects of excess zinc or zinc deficiency on the genomic integrity of the germ line. Excess dietary zinc in the form of zinc sulfate, at levels approaching the LD_{50}, did not induce sex-linked recessive lethal mutations in germ cells of male *Drosophila melanogaster* (Gocke *et al*., 1981). Furthermore, chromosome structure and number were not altered in metaphase II oocytes of Golden hamsters fed a zinc-deficient diet (2 ppm) for 8 days (two estrus cycles) compared to Golden hamsters fed a zinc-supplemented diet (89.3 ppm), although 8% fewer oocytes were recovered from the animals exposed to low levels of zinc (Watanabe and Endo, 1997). Serum levels of zinc were approximately 50% lower in the animals fed the zinc-deficient diet.

In a small clinical study of nine healthy male subjects, DNA damage in peripheral blood cells was serially measured using the comet assay while subjects were sequentially fed a controlled diet with normal zinc levels (13 days), followed by a diet deficient in zinc (41 days), and then finally a diet with sufficient zinc levels once again (27 days) (Song *et al*., 2009b). While on the zinc-deficient diet, subjects showed increasing but small elevations in DNA damage levels; restoring sufficient zinc to the diet resulted in reductions in DNA damage levels back to baseline after 27 days.

The mechanisms by which excess zinc or zinc deficiency affect genomic integrity have not been thoroughly investigated. Results from one study using a biochemical approach indicated that excess zinc impaired the activity of enzymes in the base excision repair pathway, which is important for removing chemically modified bases, such as oxidized bases, from DNA (Li *et al*., 2009).

In summary, results from *in vitro* studies suggest that an optimal level of zinc protects cells from DNA damage arising from oxidative stress that occurs if zinc levels are too low or too high. Likewise, *in vivo* studies consistently suggest that dietary zinc deficiency may increase DNA damage detected by the comet assay. However, the effects of non-optimal levels of dietary zinc on micronucleus or chromosomal aberration frequencies were variable.

STUDY RATIONALE

Dietary zinc was nominated by the ATSDR for carcinogenicity and genotoxicity testing based on the increasing size of the population exposed to excess zinc through dietary supplements and the lack of studies examining the carcinogenicity of zinc. There was an additional nomination by private individuals to investigate the tumorigenicity of zinc deficiency as a result of data showing that deficiency of some vitamins and minerals can cause DNA damage. The NTP conducted a 2-year study to evaluate chronic toxicity and carcinogenic activity of varying levels of zinc in the diet.

Plasma levels of zinc, in addition to other clinical end-points, were evaluated at multiple timepoints to assess zinc homeostasis during the study.

A synthetic diet (AIN-93M) was used to control zinc exposure. Although zinc oxide and zinc sulfate are the most common forms of zinc in fortified foods or supplements for humans (Allen, 1998; Rosado, 2003), zinc carbonate was selected for study because it is the zinc salt recommended by the American Institute of Nutrition for the AIN-93M rodent diet (Reeves *et al.*, 1993.)

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF DIETARY ZINC

Zinc carbonate basic {$[ZnCO_3]_2 \cdot [Zn(OH)_2]_3$} was obtained from Sigma-Aldrich (St. Louis, MO) in one lot (1217764) that was used in the 2-year study to create dietary levels of zinc. Analyses to determine the identity, purity, and storage stability were conducted by the analytical chemistry laboratory at Research Triangle Institute (RTI) (Research Triangle Park, NC) and the study laboratory at Battelle Columbus (Columbus, OH) and its sister laboratory Battelle Toxicology Northwest (BTNW) (Richland, WA) (Appendix E). Reports on analyses performed in support of the dietary zinc study are on file at the National Institute of Environmental Health Sciences.

Briefly, zinc carbonate basic (lot 1217764) was a fine white powder. The lot was analyzed by RTI using inductively coupled plasma/optical emission spectroscopy (ICP/OES), X-ray diffraction (XRD) spectroscopy, Fourier-transform infrared (FTIR) spectroscopy, qualitative X-ray fluorescence (XRF) spectroscopy, elemental analyses (conducted by Quantitative Technologies, Inc., Whitehouse, NJ), thermogravimetric analysis (TGA), ashing, and ion chromatography (IC) (conducted by Quantitative Technologies, Inc.) for anionic and cationic impurities. The moisture content of lot 1217764 was determined by RTI using weight loss on drying and by Galbraith Laboratories, Inc. (Knoxville, TN), using Karl Fischer titration (Levine *et al*., 2017).

Analyses by ICP-OES showed the zinc (Zn) content to be 56.6%, which is slightly lower than the theoretical value of 59.6% based on the molecular formula of zinc carbonate basic. Analysis by ICP-OES indicated levels mostly below the limits of quantitation of the analytical method (< 0.01%) except for calcium (0.0916% w/w) and magnesium (1.32% w/w). Elemental analyses for carbon and hydrogen yielded 3.58% (4.38% theoretical) and 1.07% (1.10% theoretical) weight percentages, respectively. Weight loss on drying determined a water content of 0.30% and Karl Fisher titration indicated 2.52% water. TGA measurements indicated that the test article was not hydrated and also suggested the presence of nonvolatile components such as zinc oxide (ZnO). Measured concentrations of possible anionic and cationic impurities were negligible and in agreement with values reported on the vendor's certificate of analysis.

Due to lack of reference cards for zinc carbonate basic in the database, spectra for the test article were compared with reference cards for other zinc and zinc carbonate compounds in the database including ZnO (CAS No. 1314-13-2), zinc hydroxide [$Zn(OH)_2$] (CAS No. 20427-58-1), zinc carbonate hydroxide hydrate [$Zn_4(CO_3)_2(OH)_6 \cdot H_2O$] (CAS No. 12539-71-8), hydrozincite [$Zn_5(CO_3)_2(OH)_6$] (CAS No. 12122-17-1), and smithsonite ($ZnCO_3$), and no match was found. However, the XRD pattern of the test article contained peaks matching the reference card of ZnO, suggesting the presence of ZnO as a minor component. ZnO and zinc carbonate hydroxide with the same nominal formula as the zinc carbonate basic test article {$[ZnCO_3]_2 \cdot [Zn(OH)_2]_3$} (CAS No. 3486-35-9) were procured and analyzed by XRD. The peak diffraction angles and relative peak height distributions of the test article and zinc carbonate hydroxide generally corresponded with each other, suggesting that the compounds may have been equivalent. [Note: At the time of these analyses zinc carbonate basic and zinc carbonate hydroxide had independent CAS Registry numbers. However, currently these chemicals share the same CAS number (5263-02-5), suggesting that zinc carbonate basic and zinc carbonate hydroxide may be the same.] Each overlapping peak for the zinc oxide spectra was paralleled with an increase in the peak abundance of the zinc carbonate basic test article relative to procured zinc carbonate hydroxide. Taken collectively, XRD analyses suggest that the test chemical is structurally similar or equivalent to the procured zinc carbonate hydroxide, but with zinc oxide as a minor component. In addition, FTIR spectroscopy for the test article closely matched the reference spectrum of zinc carbonate basic with a ZnO signature.

Based on the data collected using multiple techniques, the test article seemed to be composed predominantly of zinc carbonate basic with some ZnO present. Weight percentages for two major components of the test article, zinc (56.6%) and carbon (3.58%), were found to be somewhat lower than the theoretical values (59.6% and 4.38%, respectively), suggesting that zinc compounds

other than zinc carbonate basic may have been present in the test article. Heavy metal levels (e.g., arsenic, cadmium, chromium, mercury, lead, and thallium) were determined to be below the limit of quantitation of 0.01%. Taken together, regardless of the structure, the test article was suitable for use as the zinc source in dietary zinc deficiency and excess toxicity studies with the percent of zinc at 56.6.

Stability studies of the bulk chemical were performed by RTI using ICP/OES. These studies indicated that the test material was stable as a bulk chemical for 15 days when stored in capped plastic bottles at temperatures up to 60° C. To ensure stability, the bulk chemical was stored at room temperature in capped amber glass bottles. Periodic reanalyses of the bulk chemical were performed by the study laboratory at least every 6 months during the 2-year study with inductively coupled plasma/atomic emission spectroscopy (ICP/AES), and no degradation of the bulk chemical was detected.

The test article (stored at ambient temperature) and the frozen (nominal −20° C) reference sample were analyzed by BTNW using ICP/AES and by H&M Analytical Services, Inc. (Allentown, NJ), using developmental XRD. Results of these analyses indicated that the bulk test article and frozen reference samples of the same lot were consistent with each other during the course of the study.

Background Zinc Content of Base Diet

Aliquots of four batches (with nine manufacture dates from June 29, 2009, to April 11, 2011) of the base diet (AIN-93M Modified Low Zinc Feed; Zeigler Brothers, Inc., Gardners, PA), were analyzed by BTNW to prescreen for possible background zinc in the blank vehicle using ICP/AES. All batches of the zinc-deficient base diet were determined to contain less than 1 mg Zn/kg diet (< 1 ppm) and were considered acceptable to be used for formulation preparations.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared monthly by mixing zinc carbonate basic with AIN-93M Modified Low Zinc Feed. The theoretical value of zinc content (59.6%) was used to calculate the amount of zinc in the dose formulations, therefore the doses used in this study are approximately 3% lower than what is stated. A premix was prepared by hand and then blended with additional feed in a Patterson-Kelly twin-shell blender for approximately 15 minutes. Formulations were stored in sealed plastic bag-lined buckets at room temperature for up to 42 days. The 38 ppm formulation was used as the control formulation for the 2-year study.

Homogeneity studies of 3.5, 7, 38, 250, 500, and 1,000 ppm formulations and of 3.5, 38 (control), and 500 ppm dose formulations were performed by RTI and BTNW, respectively. These studies were conducted with ICP/AES or ICP/OES and measured Zn in digested samples of the formulations. ICP/OES was also used in stability studies of 3.5 and 7 ppm dose formulations that were performed by RTI. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in sealed plastic bags under freezer, refrigerated, and room temperature conditions; stability was also confirmed for at least 7 days under simulated animal room conditions.

Periodic analyses of the dose formulations of zinc carbonate basic were conducted by BTNW using ICP/AES. During the 2-year study, the dose formulations were analyzed every 2 to 3 months and animal room samples were also analyzed (Table E2). Of the dose formulations analyzed and used during the study, 102 of 110 were within 10% of the target concentrations; all 20 animal room samples were within 10% of the target concentrations.

Animal Source

Male and female Hsd:Sprague Dawley SD rats were obtained from Harlan, Inc. (Indianapolis, IN), now Envigo (Livermore, CA), for the 2-year study.

Animal Welfare

Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. The rat study was conducted in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. The study was approved by the Battelle Columbus Operations Animal Care and Use Committee and conducted in accordance with all relevant NIH and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines.

2-Year Study

Study Design

Groups of 50 male and 50 female rats were fed diets containing 38 (control), 3.5, 7, 250, or 500 ppm dietary

zinc for 104 to 106 weeks. Generally, in the literature, a zinc-deficient diet in laboratory animals ranged from 0 to 9 ppm [2.34 ± 2.37 (mean ± SD), n=24], a zinc-adequate diet ranged from 9 to 100 ppm (56 ± 25.8, n=16), and a zinc-excess diet ranged from 1,000 to 5,000 ppm (3,000 ± 1,732, n=4).

Historically, the NTP has used a diet (NTP 2000) with measured mean zinc levels from 24 lots ranging from 43.3 to 78.5 ppm, resulting in a mean zinc level of 53.6 ± 8.3 ppm. Additionally, a study with zinc carbonate (Swenerton and Hurley, 1968) demonstrated no significant difference in growth rates in rats fed control diets with zinc levels ranging from 40 to 100 ppm. In the present study, 38 ppm was chosen as the control level of dietary zinc because it is considered adequate for normal growth and survival in rats.

Due to the critical role zinc plays in maintenance of life and the importance as illustrated in the literature with animals fed extremely zinc-deficient or excess zinc diets failing to survive, groups receiving no (< 1 ppm) or higher levels (> 1,000 ppm) of supplemental zinc were not included in this study. This allowed for the chronic evaluation of the influence of varying nutritional intakes of zinc below and above the optimal physiological range. As a result, the exposure concentrations for the zinc-deficient diets (3.5 and 7 ppm) were based on literature indicating a minimum dietary zinc requirement for survival. The exposure concentrations for excess dietary zinc (250 and 500 ppm) were selected to examine the effects of excess zinc in the diet below levels that would result in interference with other essential metals (i.e., copper and iron) and were not in excess of reported LD_{50} values of 186 to 623 mg zinc/kg body weight per day (approximately equivalent to 3,162 and 10,591 ppm, respectively) for several zinc compounds in rodents (Domingo *et al*., 1988). Ten male and 10 female special study rats were exposed to the same concentrations for 53 weeks and used for micronuclei evaluations, comet assays, hematology, and trace metal concentrations.

Rats were quarantined for 9 days before the beginning of the study. Five male and five female rats were randomly selected for parasite evaluation and gross observation for evidence of disease. The animals were 5 to 6 weeks old at the beginning of the study. The health of the animals was monitored during the study according to the protocols of the NTP Sentinel Animal Program (Appendix H). All test results were negative.

Male rats were housed two per cage and female rats were housed four per cage. Rats were fed AIN-93M, a purified low-zinc rodent maintenance diet, with modified levels of zinc for the duration of the study. To accommodate the low zinc concentration needed for the study, egg white solids were used as the major protein source instead of casein. Information on feed composition and contaminants is provided in Appendix G. Feed and water were available *ad libitum*. Feed consumption was measured over a 7-day period and recorded for the first 14 weeks and at approximately 4-week intervals thereafter. Racks were changed and rotated every 2 weeks. Further details of animal maintenance are given in Table 1.

Clinical Examinations, Trace Metal Analysis, and Pathology

All animals were observed twice daily. Clinical findings were recorded every 4 weeks starting on study day 29 and at the end of the study. The animals were weighed initially, weekly for the first 14 weeks, at 4-week intervals thereafter, and at the end of the study.

On study day 19 and after 3, 6, 9, and 12 months of exposure, blood was collected from the retroorbital plexus of special study rats for hematology and trace metal concentrations. Prior to sample collection, each rat's eyes were wiped with a paper towel soaked with deionized water. Rats were anesthetized with a CO_2/O_2 mixture, and blood for hematology and trace metal concentrations was collected into tubes containing K_3EDTA and K_2EDTA, respectively. In order to prevent environmental zinc contamination, the sample tubes remained unopened until the time of sample collection. The following hematologic parameters were measured using an Advia 120 analyzer (Bayer Diagnostics Division, Tarrytown, NY, or Siemans Healthcare Diagnostics, Tarrytown, NY): hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; leukocyte count and differentials. A manual hematocrit was performed and the erythrocyte and platelet morphology was assessed on blood smears. Blood samples for trace metal concentrations were frozen at −70° C and within 48 hours of collection, shipped frozen on dry ice to RTI for analysis. Samples were thawed, digested, and analyzed using ICP/OES on an Optima 4300DV (PerkinElmer, Waltham, MA). Concentrations of zinc, copper, and iron were determined at wavelengths of 213.857, 327.393, and 259.939 nm, respectively (Appendix D).

Complete necropsies and microscopic examinations were performed on all core study rats. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin (except eyes were first fixed in Davidson's solution and testes, epididymides,

and vaginal tunics were first fixed in modified Davidson's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin for microscopic examination. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The report, slides, paraffin blocks, residual wet tissues, and pathology data were sent to the NTP Archives for inventory, slide/block match, wet tissue audit, and storage. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment (QA) laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year study, a QA pathologist evaluated slides from all tumors and the pancreas and pituitary gland from all animals, as well as the adrenal medulla, liver, spleen, and testis of males.

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

TABLE 1
Experimental Design and Materials and Methods in the 2-Year Feed Study of Dietary Zinc

Study Laboratory
Battelle Columbus Operations (Columbus, OH)

Strain and Species
Sprague Dawley (Hsd:Sprague Dawley SD®) rats

Animal Source
Harlan, Inc. (Indianapolis, IN), now Envigo (Livermore, CA)

Time Held Before Study
9 days

Average Age When Study Began
5 to 6 weeks

Date of First Exposure
September 3 (males) or 4 (females), 2009

Duration of Exposure
104 to 105 weeks (females); 105 to 106 weeks (males)

Necropsy Dates
August 29 to September 2 (females) or September 6 to 9 (males), 2011

Average Age at Necropsy
109 to 111 weeks

Size of Study Groups
50 males and 50 females (core study)
10 males and 10 females (special study)

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

Animals per Cage
2 (males) or 4 (females)

Diet
Irradiated AIN-93M Modified Low Zinc Feed (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*

Water
Deionized tap water (Columbus, OH, municipal supply) via 16-ounce glass bottles (Wheaton Science Products, Millville, NJ) with screw caps and Teflon®-coated septa (Qorpak, Bridgeville, PA, and VWR, West Chester, PA) and stainless steel, double-ball bearing sipper tubes (Ancare Corp., Bellmore, NY), available *ad libitum*

Cages
Polycarbonate (Lab Products, Inc., Seaford, DE), changed twice weekly, rotated every 2 weeks

Bedding
Irradiated Sani-Chips (P.J. Murphy Forest Products Corporation, Montville, NJ), changed twice weekly

TABLE 1
Experimental Design and Materials and Methods in the 2-Year Feed Study of Dietary Zinc

Rack Filters
Spun-bonded polyester (Snow Filtration Company, Cincinnati, OH), changed every 2 weeks

Racks
Stainless steel (Lab Products, Inc., Seaford, DE), changed and rotated every 2 weeks

Animal Room Environment
Temperature: 72° ± 3° F
Relative humidity: 50% ± 15%
Room fluorescent light: 12 hours/day
Room air changes: at least 10/hour

Exposure Concentrations
3.5, 7, 38 (control), 250, or 500 ppm in feed, available *ad libitum*

Type and Frequency of Observation
Observed twice daily; animals were weighed initially, weekly for the next 14 weeks, every 4 weeks thereafter, and at the end of the study; clinical findings were recorded on study day 29, at 4-week intervals thereafter, and at the end of the study. Feed consumption was determined over a 7-day period for the first 14 weeks of the study, and at approximately 4-week intervals thereafter.

Method of Euthanasia
Core study rats: carbon dioxide asphyxiation
Special study rats: carbon dioxide/oxygen anesthesia, exsanguination at 12 months

Necropsy
Necropsies were performed on all core study rats.

Hematology
Blood was collected from the retroorbital plexus of special study rats on day 19 and after 3, 6, 9, and 12 months of exposure. The following parameters were measured: hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials. Manual hematocrit and blood smear evaluation were also performed.

Histopathology
Complete histopathology was performed on all core study rats. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, eye, Harderian gland, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, seminal vesicle, skin, spleen, stomach (forestomach and glandular), testis (with epididymis), thymus, thyroid gland, trachea, urinary bladder, and uterus.

Trace Metal Concentrations
Blood was collected from the retroorbital plexus of special study rats on day 19 and after 3, 6, 9, and 12 months of exposure for determination of zinc, copper, and iron concentrations.

STATISTICAL METHODS

Because the goal of this study was to examine the effects of diets either deficient in zinc or containing excess zinc, two sets of statistical analyses were conducted. One set of analyses tested the effects of the zinc deficient diet by comparing the diet containing sufficient zinc (38 ppm), assigned as the control group, to 7 ppm and 3.5 ppm and testing for trends with increasing deficiency across these three dose groups. The second set of analyses tested effects of diets containing excess zinc by comparing the diet containing sufficient zinc (38 ppm), assigned as the control group, to 250 ppm and 500 ppm and testing for trends with increasing excess across these three dose groups.

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Special study animals euthanized at 12 months and animals found dead of other than natural causes were censored; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions are presented in Tables A1, A3, B1, and B3 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A2 and B2) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., mesentery, pleura, peripheral nerve, skeletal muscle, tongue, tooth, and Zymbal's gland) before microscopic evaluation, the denominators consist of the number of animals that had a gross abnormality. When neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed. Tables A2 and B2 also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm. This survival-adjusted rate (based on the Poly-3 method described below) accounts for differential mortality by assigning a reduced risk of neoplasm, proportional to the third power of the fraction of time on study, only to site-specific, lesion-free animals that do not reach terminal euthanasia.

Analysis of Neoplasm and Nonneoplastic Lesion Incidences

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

This method yields a lesion prevalence rate that depends only upon the choice of a shape parameter for a Weibull hazard function describing cumulative lesion incidence over time (Bailer and Portier, 1988). Unless otherwise specified, a value of k=3 was used in the analysis of site-specific lesions. This value was recommended by Bailer and Portier (1988) following an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control F344 rats and B6C3F1/N mice (Portier *et al.*, 1986). Bailer and Portier (1988) showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range from 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams (1993).

Tests of significance included pairwise comparisons of each dosed group with controls and a test for an overall dose-related trend. Continuity-corrected Poly-3 tests were used in the analysis of lesion incidence, and reported P values are one sided. The significance of lower incidences or decreasing trends in lesions is represented as 1–P with the letter N added (e.g., P=0.99 is presented as P=0.01N).

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables.

Body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology and trace metal concentration data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) (as modified by Williams, 1986) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1957) were examined by NTP personnel, and implausible values were eliminated from the analysis.

QUALITY ASSURANCE METHODS

This 2-year study was conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the study were submitted to the NTP Archives, the study was audited retrospectively by an independent QA contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Technical Report.

GENETIC TOXICOLOGY

The genetic toxicity of dietary zinc was assessed by testing the ability of the chemical to increase the frequency of micronucleated erythrocytes in rat peripheral blood and to induce DNA damage in blood and colon epithelial cells. Micronuclei (literally "small nuclei" or Howell-Jolly bodies) are biomarkers of induced structural or numerical chromosomal alterations and are formed when acentric fragments or whole chromosomes fail to incorporate into either of two daughter nuclei during cell division (Schmid, 1975; Heddle *et al.*, 1983). The alkaline (pH > 13) comet assay (OECD, 2014) (also known as the single cell gel electrophoresis assay) detects DNA damage in any of a variety of eukaryotic cell types (Tice *et al.*, 2000; Collins, 2004; Brendler-Schwaab *et al.*, 2005; Burlinson *et al.*, 2007); cell division is not required. The type of DNA damage detected includes nicks, adducts, strand breaks, and abasic sites that are converted to DNA strand breaks after treatment of cells in an alkali (pH > 13) solution. Transient DNA strand breaks generated by the process of DNA excision repair may also be detected. DNA damage caused by crosslinking agents has been detected as a reduction of DNA migration (Pfuhler and Wolf, 1996; Hartmann *et al.*, 2003). The fate of the DNA damage detected by the comet assay is varied; most of the damage is rapidly repaired resulting in no sustained impact on the tissue but some may result in cell death or may be incorrectly passaged by the repair machinery and result in a fixed mutation or chromosomal alteration. The protocols for these studies and the results are given in Appendix C.

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

DNA reactivity carries the potential for carcinogenicity. In this study with dietary zinc, no bacterial mutagenicity studies were conducted. Instead, genotoxicity, in the form of chromosomal damage (micronuclei) and DNA damage (measured using the comet assay), was evaluated in special study rats after 12 months of exposure via feed.

The predictivity for rodent carcinogenicity of clearly positive results in long-term peripheral blood micronucleus tests is high; a weak response in one sex only or negative results in both sexes of one species in this assay do not correlate well with either negative or positive results in rodent carcinogenicity studies (Witt *et al.*, 2000). The NTP has not yet conducted an evaluation of the relationship between DNA damage assessed in the comet assay with rodent carcinogenicity, although others have demonstrated a correlation (Sasaki *et al.*, 2000). However, because of the theoretical and observed associations between induced genetic damage and adverse effects in somatic and germ cells, the determination of *in vivo* genetic effects is important to overall understanding of the risks associated with exposure to a particular chemical.

RESULTS

2-YEAR STUDY

Survival

Estimates of 2-year survival probabilities for male and female rats are shown in Table 2 and in the Kaplan-Meier survival curves (Figure 1). Survival of 3.5 ppm males was significantly greater than that of controls. Almost half of the early deaths in control males were due to nephropathy. There were no significant differences in survival between controls and other exposed groups of males or females.

Body Weights, Feed and Compound Consumption, and Clinical Findings

Mean body weights of 3.5 ppm males were 10% to 20% less than those of the controls between days 15 and 148; after that, mean body weights of 3.5 ppm males stayed within 10% of controls for the remainder of the study (Figure 2 and Table 3). Mean body weights of all other groups of males were within 10% of controls throughout the study. In females, similar decreases in mean body weights occurred in the 3.5 ppm group, but the decreases were less severe and of shorter duration than those seen in males (Figure 2 and Table 4). Mean body weights of 7 and 500 ppm females were within 10% of controls throughout the study; 250 ppm females had a terminal mean body weight 10% higher than that of the controls. Feed consumption by zinc deficient and zinc excess groups of males and females was generally similar to that by the control groups throughout the study (Tables F1 through F4) with the exception of the 3.5 ppm males where feed consumption was as much as 25% less than that by controls by week 3 of the study and slowly recovered to control levels by study week 21. As mentioned in the Materials and Methods, dietary concentrations were approximately 3% lower than the stated doses of 3.5, 7, 38 (control), 250, and 500 ppm because the theoretical value of zinc content (59.6%) was used instead of the measured zinc content in zinc carbonate to calculate the amount of zinc in the dose formulations. This resulted in average daily doses of 0.1, 0.3, 1.4 (control), 8.7, and 17.6 mg dietary zinc/kg body weight to males, and 0.1, 0.3, 1.5 (control), 9.9, and 19.9 mg/kg to females. No clinical observations related to deficient or excess dietary zinc exposure were observed in males or females.

TABLE 2
Survival of Rats in the 2-Year Feed Study of Dietary Zinc

	3.5 ppm	7 ppm	38 ppm (Control)	250 ppm	500 ppm
Male					
Animals initially in study	60	60	60	60	60
Special study animals[a]	10	9	10	10	10
Moribund	12	10	11	15	7
Natural deaths	7	13	19	14	22
Animals surviving to study termination	31	28[d]	20[e]	21	21[d]
Percent probability of survival at end of study[b]	62	53	36	42	40
Mean survival (days)[c]	638	623	611	610	614
Survival analysis[f]	P=0.009N	P=0.144N	P=0.008N		
Survival analysis[g]			P=0.650N	P=0.755N	P=0.670N
Female					
Animals initially in study	60	60	60	60	60
Special study animals[a]	10	9	9	9	9
Moribund	11	12	21	19	14
Natural deaths	7	5	5	5	6
Animals surviving to study termination	32	34	25	27	31
Percent probability of survival at end of study	64	67	50	53	62
Mean survival (days)	621	634	607	598	606
Survival analysis	P=0.171N	P=0.085N	P=0.134N		
Survival analysis			P=0.346N	P=0.920N	P=0.369N

[a] Euthanized at 12 months; censored in the survival analyses
[b] Kaplan-Meier determinations
[c] Mean of all deaths (uncensored, censored, and terminal euthanasia)
[d] Includes one animal that died during the last week of the study
[e] Includes two animals that died during the last week of the study
[f] The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the 3.5 and 7 ppm exposure group columns. A negative trend or lower mortality in an exposure group is indicated by **N.**
[g] The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the 250 and 500 ppm exposure group columns. A negative trend or lower mortality in an exposure group is indicated by **N.**

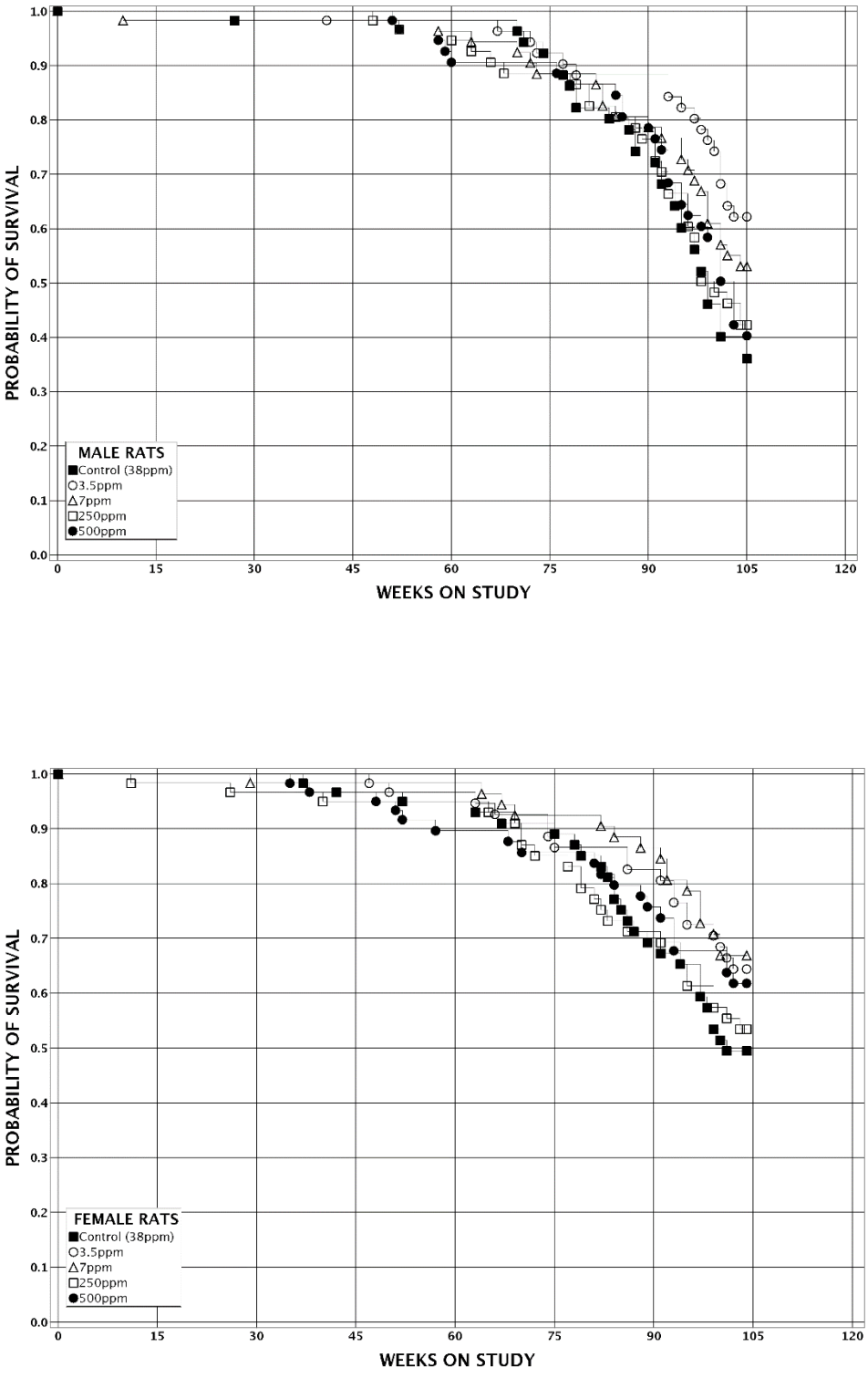
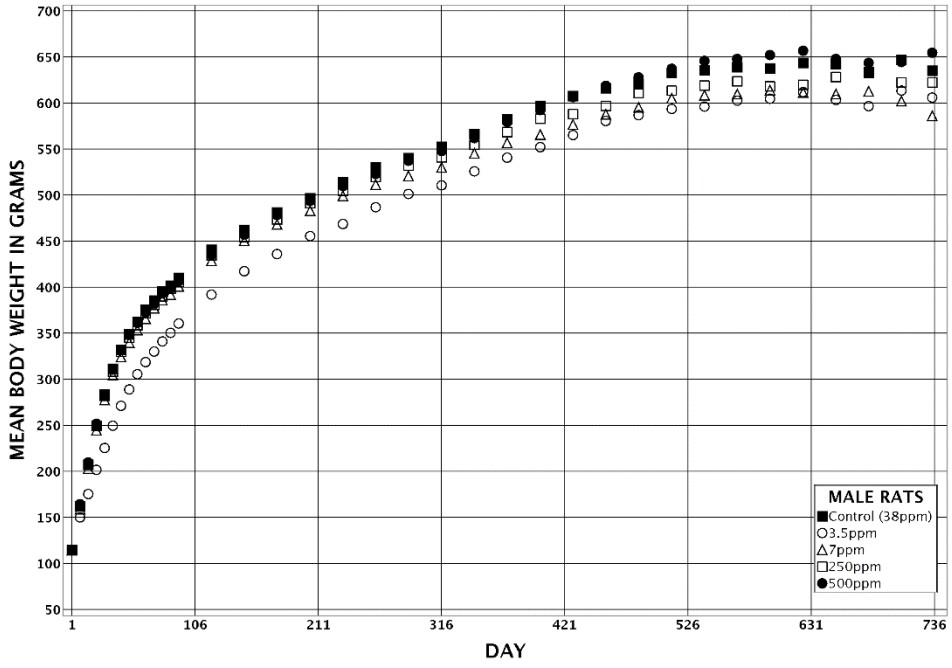


FIGURE 1
Kaplan-Meier Survival Curves for Rats Exposed to Dietary Zinc for 2 Years



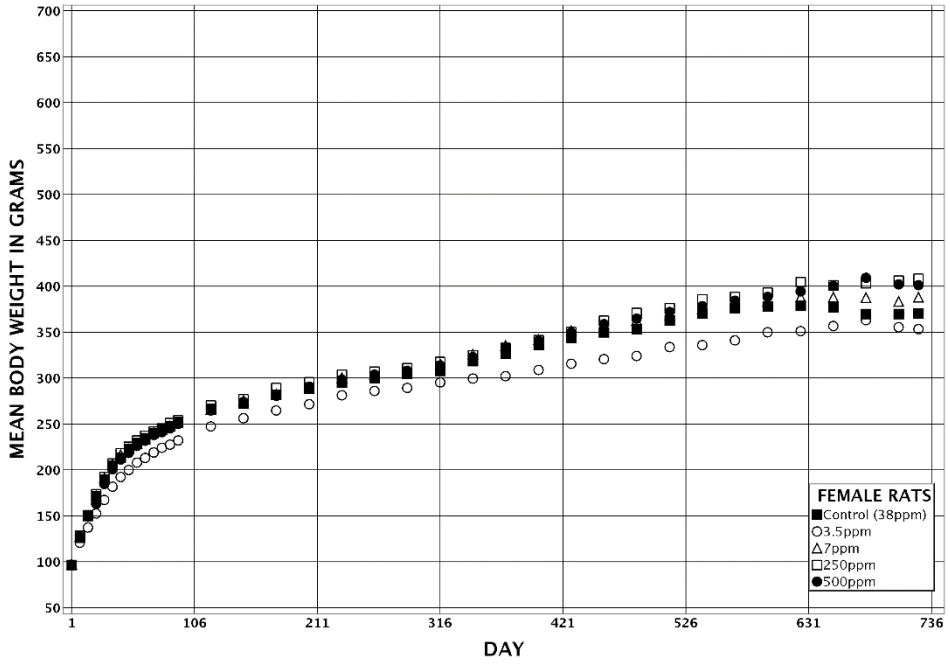


FIGURE 2
Growth Curves for Rats Exposed to Dietary Zinc for 2 Years

TABLE 3
Mean Body Weights and Survival of Male Rats in the 2-Year Feed Study of Dietary Zinc

Day	3.5 ppm			7 ppm			38 ppm (Control)		250 ppm			500 ppm		
	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
1	115	100	60	115	100	60	115	60	115	100	60	115	100	60
8	150	93	60	159	98	60	162	60	163	100	60	164	101	60
15	176	85	60	203	98	60	207	60	208	100	60	210	101	60
22	202	81	60	245	98	60	250	60	250	100	60	252	101	60
29	225	80	60	278	98	60	283	60	282	100	60	284	100	60
36	250	80	60	304	98	60	311	60	309	99	60	311	100	60
43	271	82	60	324	98	60	332	60	330	99	60	332	100	60
50	289	83	60	340	97	60	349	60	345	99	60	349	100	60
57	306	84	60	354	97	60	363	60	359	99	60	362	100	60
64	319	85	60	366	97	60	376	60	372	99	60	373	99	60
71	330	86	60	377	98	59	386	60	381	99	60	382	99	60
78	341	86	60	386	98	59	395	60	390	99	60	393	99	60
85	350	87	60	392	98	59	402	60	398	99	60	399	99	60
92	361	88	60	401	98	59	410	60	406	99	60	406	99	60
120	392	89	60	429	97	59	441	60	435	99	60	435	99	60
148	417	90	60	450	97	59	462	60	454	98	60	457	99	60
176	436	91	60	468	97	59	481	60	474	98	60	479	99	60
204	455	92	60	483	97	59	497	59	492	99	60	494	99	60
232	469	91	60	499	97	59	514	59	505	98	60	510	99	60
260	487	92	60	512	97	59	530	59	520	98	60	523	99	60
288	502	93	59	521	96	59	540	59	532	99	60	538	100	60
316	511	93	59	530	96	59	552	59	541	98	60	548	99	60
344	526	93	59	545	96	59	567	59	555	98	59	562	99	60
372[a]	541	93	49	557	96	50	583	49	569	98	48	580	100	48
400	552	93	49	566	95	50	597	49	583	98	48	593	99	48
428	565	93	49	577	95	49	608	49	588	97	47	606	100	45
456	581	94	49	588	95	48	616	49	597	97	46	619	100	45
484	587	95	48	595	96	48	621	49	611	98	44	628	101	45
512	594	94	46	605	96	45	633	47	614	97	44	637	101	45
540	596	94	45	609	96	45	636	44	619	97	44	646	102	44
568	603	94	44	610	95	44	639	41	624	98	41	648	101	43
596	605	95	44	614	96	41	638	40	618	97	40	652	102	42
624	612	95	44	611	95	41	644	37	620	96	38	657	102	40
652	604	94	42	610	95	39	642	33	628	98	33	648	101	34
680	597	94	40	613	97	35	633	28	634	100	26	644	102	31
708	614	95	32	602	93	28	647	20	623	96	24	644	100	25
Mean for Weeks														
1-13	256	85		296	98		302		300	99		302	100	
14-52	456	91		484	97		499		491	98		495	99	
53-102	589	94		597	95		626		610	97		631	101	

[a] Special study animals were removed during week 53.

TABLE 4
Mean Body Weights and Survival of Female Rats in the 2-Year Feed Study of Dietary Zinc

Day	3.5 ppm			7 ppm			38 ppm (Control)		250 ppm			500 ppm		
	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
1	97	100	60	97	101	60	97	60	97	100	60	97	100	60
8	121	96	60	128	101	60	126	60	129	102	60	128	101	60
15	137	92	60	150	100	60	150	60	151	101	60	149	99	60
22	153	89	60	172	100	60	171	60	174	101	60	163	95	60
29	168	88	60	191	101	60	190	60	192	101	60	185	97	60
36	182	89	60	206	101	60	205	60	207	101	60	201	98	60
43	192	90	60	216	101	60	214	60	218	102	60	211	99	60
50	200	90	60	223	100	60	223	60	226	101	60	219	98	60
57	208	91	60	229	100	60	230	60	232	101	60	226	99	60
64	213	91	60	233	99	60	235	60	237	101	60	232	99	60
71	219	91	60	241	100	60	240	60	242	101	59	238	99	60
78	224	92	60	244	100	60	244	60	246	101	59	241	99	60
85	228	92	60	248	100	60	248	60	252	101	59	246	99	60
92	232	92	60	251	100	60	253	60	254	101	59	250	99	60
120	247	93	60	266	100	60	267	60	270	101	59	265	99	60
148	256	94	60	276	101	60	273	60	277	102	59	274	101	60
176	265	94	60	283	100	60	282	60	290	103	59	281	100	60
204	272	94	60	289	100	59	290	60	296	102	58	291	100	60
232	281	95	60	300	102	59	295	60	304	103	58	299	101	60
260	286	95	60	304	101	59	300	59	307	102	58	304	101	59
288	290	95	60	306	100	59	305	59	311	102	57	308	101	58
316	296	96	60	316	102	59	308	58	318	103	57	314	102	58
344	300	94	59	326	102	59	319	58	325	102	57	323	101	57
372[a]	302	93	48	336	103	50	327	48	333	102	48	334	102	46
400	309	92	48	342	102	50	336	48	342	102	48	341	101	45
428	316	92	48	352	102	50	344	48	351	102	48	350	102	45
456	321	92	47	356	102	49	350	47	363	104	47	359	103	45
484	324	92	46	363	103	47	354	46	371	105	46	365	103	44
512	334	92	46	369	102	47	363	46	376	104	43	372	103	43
540	336	91	43	376	101	47	371	44	386	104	42	379	102	43
568	341	91	43	379	101	47	376	42	389	103	38	384	102	41
596	350	93	43	384	102	45	378	38	393	104	37	389	103	40
624	351	93	41	388	102	44	379	35	405	107	36	395	104	38
652	357	95	38	388	103	41	377	34	401	106	35	400	106	34
680	363	98	36	388	105	37	370	30	404	109	31	410	111	34
708	355	96	33	383	104	34	370	25	406	110	28	402	109	32
Mean for Weeks														
1-13	180	91		198	100		198		200	101		195	98	
14-52	273	94		292	101		289		295	102		291	101	
53-102	335	93		370	102		361		378	105		375	104	

[a] Special study animals were removed during week 53.

Hematology and Trace Metals

Whole blood was collected from special study rats for hematology and trace metal concentrations on day 19, and at 3, 6, 9, and 12 months. On day 19, and at 3 and 6 months, the hematocrit, hemoglobin concentration, mean cell volume, or mean cell hemoglobin were significantly decreased in 500 ppm males, depending on the time point (Table D1). These mild decreases may have been due to mild alterations in erythropoiesis, and were ameliorated by 9 months. Other erythron changes were very mild or inconsistent, may have been due to biological variation, and were not considered toxicologically relevant. At 12 months, leukocyte and lymphocyte counts were decreased in 3.5 and 7 ppm males, and the reason for these decreases was not certain. Several other statistically significant changes were observed in the leukon, but they were mild, inconsistent, and not considered toxicologically relevant.

On day 19, zinc concentrations were significantly increased in 250 and 500 ppm males and females, while copper concentrations were decreased to below the limit of detection in 250 and 500 ppm males and 500 ppm females (Tables 5 and D2). Aside from these changes in zinc and copper concentrations at the first time point measured (day 19), there were no changes in blood zinc concentrations as a result of dietary modulation of zinc levels in the current study when measured for up to a year. Iron concentrations were inconsistently significantly altered in females at several time points. At day 19, iron concentration was minimally increased in 250 ppm females; iron concentrations were mildly decreased in 500 ppm females at months 3 and 6. These changes in iron concentrations were not considered to be biologically relevant.

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms and/or nonneoplastic lesions of the pancreas, testis, pituitary gland, skin, and thyroid gland. Summaries of the incidences of neoplasms and nonneoplastic lesions and statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group are presented in Appendix A for male rats and Appendix B for female rats.

Pancreas: Incidences of acinar adenoma were increased, but not significantly, in 7 and 3.5 ppm males (Tables 6, A1 and A2). The increases in acinar adenoma in these groups were primarily due to increases in the number of animals with multiple acinar adenomas (significant increase at 3.5 ppm). One 38 ppm control male had an acinar carcinoma in addition to multiple acinar adenomas. Acinar hyperplasia was increased in the 3.5 ppm and 500 ppm males, but neither increase was significant. Significantly increased incidences of acinar atrophy occurred in 500 ppm males and females (Tables 6, A3, and B3). Pancreatic acinar adenomas were generally 5 mm or greater in diameter and were composed of proliferative, enlarged acini that caused compression on surrounding adjacent lobules (Plates 1 and 2). Adenomas were occasionally encapsulated, and islet cells were usually not present in acinar adenomas. Areas of acinar hyper-plasia were generally smaller than adenomas, and tended to cause less compression. It was sometimes difficult to distinguish between areas of hyperplasia and adenomas, and the two lesions were considered to represent two different points on a single spectrum of change. Atrophy of pancreatic acini was characterized by small, shrunken ducts surrounded by a few depleted acinar cells, interstitial fibrous connective tissue, and mononuclear cell infiltrates, all of which resulted in overall focal reduction in acinar tissue (Plate 3).

Testis: The incidence of bilateral germinal epithelium atrophy was significantly increased in 3.5 ppm males (Tables 7 and A3). The combined incidence of unilateral and bilateral germinal epithelium atrophy was higher in the 3.5 ppm group, but not significantly. In addition to germinal epithelium atrophy, there was one occurrence of bilateral germ cell degeneration in the testis in each of the 3.5, 250, and 500 ppm groups. Germinal epithelium atrophy was characterized by thinning of the germinal epithelium layer due to reduced numbers of germ cells, with most affected tubules being smaller than normal and lined by only Sertoli cells. Germ cell degeneration was characterized by shrunken germ cells with nuclear condensation and brightly eosinophilic cytoplasm, and occasional vacuolization and phagocytosis of germ cells by Sertoli cells.

Other Tissues: Several lesions occurred with statistically significant differences from controls that were not considered biologically relevant or related to exposure. There was a positive trend in the incidences of pituitary gland adenoma of the pars distalis in the male zinc deficient groups [38 ppm (control), 2/50; 7 ppm, 4/50; 3.5 ppm, 8/50; Tables A1 and A2]. In light of the low survival in the control group, the commonality of pituitary gland adenoma in aged rats, and the fact that the incidences of this neoplasm in the zinc excess groups (250 ppm, 7/50; 500 ppm, 6/49) were not significantly different from that in the control rats, this was considered biological variability, and not due to zinc deficiency in the diet. Significantly decreased incidences of epithelial neoplasms of the skin occurred in 3.5 ppm males [38 ppm (control), 5/50

TABLE 5
Zinc and Copper Concentrations in Blood of Rats in the 2-Year Feed Study of Dietary Zinc[a]

	3.5 ppm	7 ppm	38 ppm (Control)	250 ppm	500 ppm
Male					
n	10	9	10	10	10
Zinc (µg/mL)					
Day 19	6.235 ± 0.557	6.541 ± 0.387[b]	6.668 ± 0.392	8.662 ± 0.403**	9.840 ± 0.382**
Month 3	6.287 ± 0.263	6.766 ± 0.342	6.630 ± 0.148	6.887 ± 0.162	6.659 ± 0.156
Month 6	6.151 ± 0.199	6.148 ± 0.220	6.860 ± 0.366	6.536 ± 0.143	6.302 ± 0.178
Month 9	5.822 ± 0.154	6.041 ± 0.250	6.028 ± 0.197	6.078 ± 0.128	6.057 ± 0.136
Month 12	5.273 ± 0.132	5.217 ± 0.112	5.379 ± 0.158	5.604 ± 0.251	5.722 ± 0.150
Copper (µg/mL)					
Day 19	0.452 ± 0.026	0.441 ± 0.021[b]	0.438 ± 0.056	ND	ND
Month 3	0.604 ± 0.030	0.628 ± 0.022	0.634 ± 0.024	0.584 ± 0.021	0.553 ± 0.038
Month 6	0.543 ± 0.042	0.527 ± 0.065	0.532 ± 0.042	0.480 ± 0.034[c]	0.440 ± 0.035
Month 9	0.831 ± 0.040	0.844 ± 0.066	0.795 ± 0.041	0.862 ± 0.088	0.712 ± 0.040
Month 12	0.745 ± 0.031	0.675 ± 0.023	0.710 ± 0.025	0.705 ± 0.054	0.714 ± 0.028
Female					
n	10	10	10	9	10
Zinc (µg/mL)					
Day 19	3.928 ± 0.169	4.502 ± 0.131	4.458 ± 0.188	6.358 ± 0.580[b]**	7.754 ± 0.202**
Month 3	5.187 ± 0.176	5.301 ± 0.153	5.505 ± 0.138	5.120 ± 0.057	5.448 ± 0.185
Month 6	4.982 ± 0.124	5.052 ± 0.133	4.864 ± 0.072	4.903 ± 0.203	4.608 ± 0.091
Month 9	4.638 ± 0.213	4.998 ± 0.120[c]	5.021 ± 0.149	5.136 ± 0.131	5.200 ± 0.173[c]
Month 12	4.595 ± 0.377	4.537 ± 0.146[c]	4.668 ± 0.197[c]	4.380 ± 0.209	4.733 ± 0.143[c]
Copper (µg/mL)					
Day 19	0.590 ± 0.034	0.669 ± 0.022	0.663 ± 0.029	0.331 ± 0.044[b]**	0.204 ± 0.018**
Month 3	0.655 ± 0.030	0.733 ± 0.023	0.769 ± 0.049	0.733 ± 0.041	0.697 ± 0.073
Month 6	0.184 ± 0.020	0.236 ± 0.069	0.289 ± 0.040	0.432 ± 0.079	0.539 ± 0.070**
Month 9	0.690 ± 0.031	0.743 ± 0.041[c]	0.791 ± 0.037	0.713 ± 0.019	0.685 ± 0.059[c]
Month 12	0.382 ± 0.040	0.399 ± 0.032[c]	0.457 ± 0.042[c]	0.417 ± 0.065	0.447 ± 0.061[c]

** Significantly different ($P \leq 0.01$) from the control group by Shirley's test
[a] Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=10
[c] n=9
ND=All values below the limit of detection at this dose level

TABLE 6
Incidences of Neoplasms and Nonneoplastic Lesions of the Exocrine Pancreas in Rats in the 2-Year Feed Study of Dietary Zinc

	3.5 ppm	7 ppm	38 ppm (Control)	250 ppm	500 ppm
Male					
Number Examined Microscopically	50	48	49	48	48
Acinus, Atrophy[a]	3 (1.3)[b]	4 (1.3)	3 (2.3)	3 (1.3)	13** (1.5)
Acinus, Hyperplasia	32 (2.3)	23 (2.7)	23 (2.0)	21 (2.5)	28 (2.3)
Acinus, Adenoma, Multiple	13*	10	5	8	4
Acinus, Adenoma (includes multiple)					
Overall rate[c]	21/50 (42%)	19/48 (40%)	11/49 (22%)	13/48 (27%)	10/48 (21%)
Adjusted rate[d]	46.5%	46.5%	28.4%	34.7%	26.1%
Terminal rate[e]	14/31 (45%)	16/27 (59%)	8/18 (44%)	11/21 (52%)	9/20 (45%)
First incidence (days)	467	701	652	648	729
Poly-3 test[f]	P=0.065	P=0.069	P=0.064		
Poly-3 test[g]			P=0.462N	P=0.363	P=0.512N
Acinus, Carcinoma	0	0	1	0	0
Acinus, Adenoma or Carcinoma					
Overall rate	21/50 (42%)	19/48 (40%)	11/49 (22%)	13/48 (27%)	10/48 (21%)
Adjusted rate	46.5%	46.5%	28.4%	34.7%	26.1%
Terminal rate	14/31 (45%)	16/27 (59%)	8/18 (44%)	11/21 (52%)	9/20 (45%)
First incidence (days)	467	701	652	648	729
Poly-3 test	P=0.065	P=0.069	P=0.064		
Poly-3 test			P=0.462N	P=0.363	P=0.512N
Female					
Number Examined Microscopically	48	49	50	49	49
Acinus, Atrophy	4 (1.0)	2 (1.0)	2 (1.5)	5 (1.2)	10* (1.4)
Acinus, Hyperplasia	1 (1.0)	5 (1.2)	2 (1.0)	0	1 (1.0)
Acinus, Adenoma	0	0	1	0	0

* Significantly different ($P \leq 0.05$) from the control group by the Poly-3 test
** $P \leq 0.01$
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Number of animals with neoplasm per number of animals with pancreas examined microscopically
[d] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[e] Observed incidence at terminal euthanasia
[f] Beneath the control incidence is the P value associated with the trend test between the control group and the 3.5 and 7 ppm exposure groups. Beneath the 3.5 or 7 ppm exposure group incidence are the P values corresponding to pairwise comparisons between the controls and that exposure group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal euthanasia.
[g] Beneath the control incidence is the P value associated with the trend test between the control group and the 250 and 500 ppm exposure groups. Beneath the 250 or 500 ppm exposure group incidence are the P values corresponding to pairwise comparisons between the controls and that exposure group. A negative trend or a lower incidence in an exposure group is indicated by **N.**

TABLE 7
Incidences of Nonneoplastic Lesions of the Testis in Male Rats in the 2-Year Feed Study of Dietary Zinc

	3.5 ppm	7 ppm	38 ppm (Control)	250 ppm	500 ppm
Number Examined Microscopically	50	50	50	50	50
Bilateral, Germinal Epithelium, Atrophy[a]	7* (2.4)[b]	1 (3.0)	0	0	1 (3.0)
Germinal Epithelium, Atrophy	3 (2.3)	0	5 (2.0)	3 (2.7)	4 (2.8)
Germinal Epithelium, Atrophy (includes bilateral)	10 (2.4)	1 (3.0)	5 (2.0)	3 (2.7)	5 (2.8)
Bilateral, Germ Cell, Degeneration	1 (2.0)	0	0	1 (2.0)	1 (2.0)

* Significantly different (P≤0.05) from the control group by the Poly-3 test
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

7 ppm, 3/50; 3.5 ppm, 0/50; Tables A1 and A2]; and significantly decreased incidences of thyroid gland C-cell hyperplasia occurred in 500 ppm females [38 ppm (control), 18/50; 250 ppm, 14/50; 500 ppm, 5/48; Table B3]. The biological significance of these decreased incidences is unknown, but they were not considered related to exposure to dietary zinc.

GENETIC TOXICOLOGY

The percentage of micronucleated immature erythrocytes [also known as reticulocytes or polychromatic erythrocytes (PCEs)] was measured in peripheral blood of rats during the first year of the 2-year study (Tables C1 through C4). Although data on micronucleus frequencies were also collected for the mature erythrocyte population automatically, this cell population is not appropriate for evaluating micronucleus induction in rats due to the rat spleen's ability to efficiently sequester and destroy damaged reticulocytes soon after they emerge from the bone marrow. Therefore, evaluation of the effects of the deficient and excess zinc diets on chromosome integrity was limited to the immature erythrocyte population.

At the first sample time, 19 days after the study began, female rats maintained on the zinc-deficient diet showed a statistically significant (P≤0.025) increase in micronucleated reticulocytes at both dose levels (3.5 and 7 ppm), although the trend test was not significant (P>0.025). However, the mean micronucleated reticulocyte values observed for these two treatment groups were well within the laboratory historical control range. In addition, no increases were seen at any subsequent sampling time, and for all these reasons, the increased frequencies of micronucleated reticulocytes seen on day 19 in female rats were not judged to be biologically significant. No increases in micronucleated red blood cells were observed at any other sampling time for up to 12 months in either sex.

The percentage of PCEs among total erythrocytes was calculated at each sample time for each sex, and minor, statistically significant, sporadic alterations showing no pattern over time or association with specific diet were observed: zinc-deficient male rats at 19 days; both zinc-deficient and zinc-excess groups of female rats at 6 months. These were considered normal fluctuations and all values were within historical control ranges.

In blood leukocytes (Table C5) and colonic epithelium (Table C6), indications of effects on DNA integrity were observed. In blood leukocyte samples obtained from male rats at 12 months, significant increases in percent tail DNA were observed in both the zinc-deficient and the zinc-excess groups. No significant changes in percent tail DNA were observed in peripheral blood samples at any of the earlier sampling times in either dietary group of male rats. Increased levels of DNA damage were also observed in blood leukocytes of female rats fed the zinc-deficient diet at both the 9- and 12-month sampling times. No significant changes in percent tail DNA were observed in female blood samples at any other sampling times in either dietary group. In colon cell samples obtained after 12 months

of exposure, a significant increase in percent tail DNA was observed in male rats (trend, P=0.019) fed a diet with excess zinc, and a small, but not significant, decrease in percent tail DNA was observed in males maintained on the zinc-deficient diets. A significant increase in percent tail DNA was seen in female rats fed a diet supplemented with excess zinc, and a significant decrease in percent tail DNA was observed in females maintained on the zinc-deficient diets. This same pattern of DNA damage was seen in the male rat colon cell samples, although the decreases in males fed a zinc-deficient diet were not statistically significant.

Overall, indications of increased levels of DNA damage related to excess dietary levels of zinc were seen in blood leukocytes (males only) and colonic epithelial cell samples of male and female rats. In addition, colon cell samples for rats maintained on a zinc-deficient diet showed a significant decrease in DNA migration at 12 months. This reduction in DNA migration could indicate the presence of damage in the form of DNA crosslinking (Hartmann *et al*., 2003). Experiments with known DNA crosslinkers have shown that these chemicals impede DNA migration compared to control exposures (Pfuhler and Wolf, 1996).

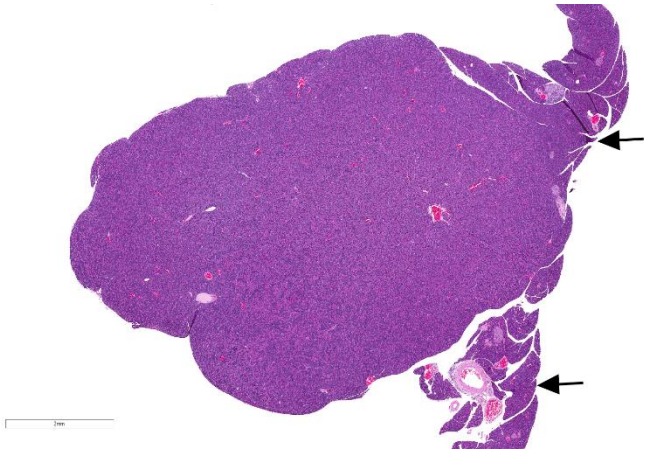


PLATE 1
Large acinus adenoma from the pancreas of a male rat exposed to 3.5 ppm dietary zinc for 2 years. The adenoma comprises most of the pancreatic tissue observed. A small amount of normal pancreas is present in the upper and lower right hand corners (arrows). H&E

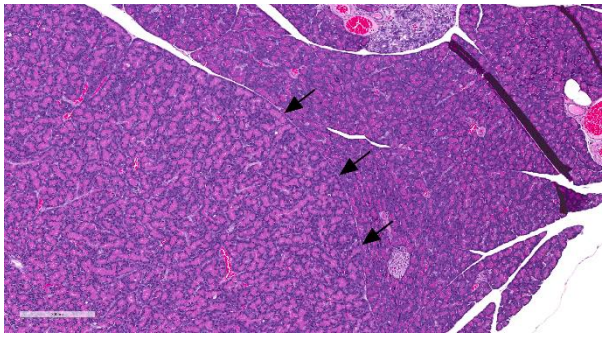


PLATE 2
Higher magnification of Plate 1 showing the transition (arrows) between the adenoma (left hand side) and the normal pancreas (right hand side). H&E

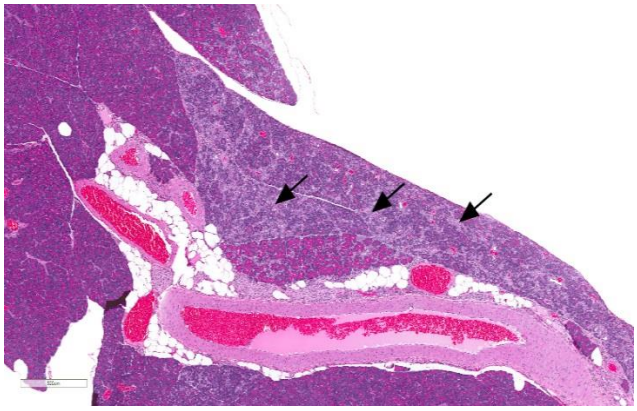


PLATE 3
Mild acinus atrophy in the pancreas of a male rat exposed to 500 ppm dietary zinc for 2 years. Increased pale space (arrows) is apparent between remaining acini in the affected area and contains fibrous connective tissue and inflammatory cells. H&E

DISCUSSION AND CONCLUSIONS

Zinc is a natural component of the Earth's crust that is an essential element required by humans and animals for multiple biological processes. It is also used extensively for many applications in industry. Dietary zinc was nominated by the Agency for Toxic Substances and Disease Registry (ATSDR) for carcinogenesis and genetic toxicity studies based on the increasing size of the population exposed to zinc through dietary supplements and due to a lack of carcinogenicity data in the literature. There was an additional nomination by private individuals to investigate the tumorigenicity of zinc deficiency due to data demonstrating DNA damage as a result of some vitamin and mineral deficiencies. Zinc carbonate was selected as the source of dietary zinc due to its degree of availability and its use as a source of supplemental zinc in rodent diets and vitamin supplements (Fairweather-Tait *et al*., 1992; Kincaid, 1979; Della Lucia *et al*., 2014). The current Technical Report presents the findings and conclusions of the 2-year feed study of rats exposed to varying levels of dietary zinc.

Generally, in the literature, a zinc-deficient diet in laboratory animals ranged from 0 to 9 ppm [2.34 ± 2.37 (mean ± SD)] a zinc-adequate diet ranged from 9 to 100 ppm (56 ± 25.8), and a zinc-excess diet ranged from 1,000 to 5,000 ppm (3,000 ± 1,732). In the current study, doses were chosen to allow for the chronic evaluation of the influence of varying nutritional intakes of zinc below and above the optimal physiological range. The control group of 38 ppm was chosen as a dietary level of zinc that is considered adequate for normal growth and survival in rats. The exposure concentrations for the zinc-deficient diets (3.5 and 7 ppm) were based on literature indicating a minimum dietary zinc requirement for survival. The exposure concentrations for excess zinc in the diet (250 and 500 ppm) were selected to examine the effects of excess zinc in the diet below levels that would result in interference with other essential metals (i.e., copper and iron) and were not in excess of reported LD_{50} values of 186 to 623 mg zinc/kg body weight per day (approximately equivalent to 3,162 and 10,591 ppm, respectively) for several zinc compounds in rodents (Domingo *et al*., 1988).

In the current 2-year study, there were no chemical-related effects on survival. Male rats maintained on the 3.5 ppm zinc-deficient diet had an increased survival rate compared to the controls, but this is likely due to low survival of the control group as a result of nephropathy.

Aside from the first time point measured (day 19), there were no changes in blood zinc concentrations as a result of dietary modulation of zinc levels in the current study when measured for up to a year. This is likely a result of the tight regulation of zinc serum and tissue levels in animals and humans and the many factors that can contribute to zinc homeostasis. Metallothionein-bound zinc has been shown to fluctuate depending on the zinc status of animals, indicating a role for metallothionein in the sequestration of excess zinc or as a reservoir in cases of zinc deficiency (Kelly *et al.*, 1996). The increased toxicity of zinc in metallothionein-null mice provides evidence for this hypothesis (Kelly *et al.*, 1996). Additionally, studies in animals have indicated that adjustments are made in zinc absorption and endogenous intestinal excretion to maintain zinc homeostasis (Miller, 1969; Evans *et al*., 1973; Giugliano and Millward, 1984; King *et al*., 2000). These alterations in excretion have also been demonstrated in humans, where large decreases in plasma zinc concentration as a result of low zinc in the diet can be prevented through rapid decreases of up to 75% in endogenous fecal and urinary zinc excretion (King *et al*., 2000). Following excess dietary zinc intakes in humans, it was also observed that net zinc absorption reaches a plateau with doses of 20 mg/day (Hess *et al.*, 2007). Combined with evidence that plasma or serum zinc concentrations are only useful as biomarkers following severely deficient zinc diets or during extreme zinc supplementation (Hess *et al.*, 2007), these literature studies provide evidence that blood zinc concentrations may have remained unchanged in the present study due to failure to overwhelm normal homeostatic control of zinc. The present study is the longest to date that examines plasma levels of zinc with varying levels of zinc in the diet and, though it does not demonstrate dramatic changes in plasma zinc levels when measured for up to a year, it does provide long-term perspective on the complex homeostatic control of zinc levels following varying levels of zinc in the diet. Overall, it is clear that the regulation of zinc is complex and given the evidence shown in the current study, it is likely that the low

incidences of nonneoplastic and neoplastic lesions may be due to the tight regulation of zinc homeostasis itself.

Zinc has been shown in the literature to interact with both copper and iron, resulting in changes in hematologic parameters (Magee and Matrone, 1960; Van Campen and Scaife, 1967). Zinc, copper, and iron plasma concentrations were repetitively measured in special study rats throughout the first year of the current study (day 19, and 3, 6, 9, and 12 months) to monitor zinc blood concentration changes and examine the potential influence of zinc on these other essential metals. No changes were observed in whole blood levels of males or females fed zinc-deficient diets (3.5 and 7 ppm) in comparison to the zinc sufficient controls (38 ppm). However, early increases (day 19) in zinc blood concentrations were observed with concurrent decreases (below the limit of detection) in copper blood concentrations in males and females provided diets containing excessive amounts of zinc (250 and 500 ppm); blood concentrations of both zinc and copper returned to concentrations similar to those of controls by 3 months. These early alterations in zinc and copper concentrations indicate initial disturbances in the normal homeostatic regulation of zinc. Return of near-normal zinc and copper concentrations following an initial increase after 4 weeks of exposure has also been demonstrated as an adaptive response to long-term high zinc intake in the serum, liver, and kidney of rats (Reeves, 1995). Similarly, rats fed 7.5 to 240 mg zinc (as zinc sulfate)/kg for 5 weeks had significant increases in serum zinc concentrations (189 ± 10 μg/dL compared to 128 ± 8 μg/dL in controls) alongside decreases in serum copper (45 ± 10 μg/dL compared to 114 ± 9 μg/dL in controls) in the top exposure group (Fischer *et al*., 1981). Inhibition of intestinal copper absorption induced by ingestion of high levels of zinc can be attributed to the competitive interaction of zinc and copper for metallothionein within the intestinal lumen (Ogiso *et al*., 1979; Wapnir and Balkman, 1991). This effect has also been demonstrated in humans, where copper deficiency has been observed in susceptible individuals who are exposed to high levels of zinc for treatment of malnourishment or sickle cell anemia (Underwood, 1977; Prasad *et al*., 1978). Though most of the studies in the literature support an inverse relationship between zinc and copper levels, conflicting data demonstrate that different levels of dietary zinc (up to 180 mg/kg) and copper do not significantly influence absorption of each other when fed to rats over a period of a week (Oestreicher and Cousins, 1985). Likewise, in humans, plasma copper levels were not significantly decreased following 100 to 220 mg of zinc sulfate per day for durations of either 6 weeks or 6 months (Henkin *et al.*, 1976; Samman and Roberts, 1987). There are several possible reasons for these differences in the literature, and the influence of zinc on copper metabolism can be altered by many factors, including the ratio of zinc to copper in the diet, absorption and excretion of zinc in the regulation of zinc homeostasis, individual susceptibilities and species differences, the source of zinc in the diet, and the duration of dietary zinc exposure (Johnson and Flagg, 1986). These factors may also play a role in the apparent recovery of the zinc-to-copper ratio presented in the current studies following the initial day 19 measurements.

In males fed diets that were deficient in dietary zinc (3.5 and 7 ppm), there were higher incidences of pancreatic acinar cell adenoma (P=0.065 and P=0.069, respectively). The number of males with multiple adenomas was significantly increased at 3.5 ppm. These neoplasms in males were accompanied by a higher incidence of pancreatic acinar cell hyperplasia in the 3.5 ppm group. Additionally, there was a single pancreatic acinar cell carcinoma in the 38 ppm male control group. Because this study was conducted on a controlled diet, historical controls are not available. The available historical control report (NTP, 2016) for the HSD:Sprague Dawley SD rat had 0/50 and 5/50 incidences of adenoma in the males compared to the current control incidence of 11/49. Acinar hyperplasia, acinar adenoma, and acinar carcinoma are proliferative lesions of the exocrine pancreas and represent a continuum of effect (Boorman and Eustis, 1984). Though the increased incidences were not significant in the current study, the higher incidence of acinar cell hyperplasia provides supporting evidence when combined with increased incidences of acinar cell adenoma. Taken together, the higher incidences of adenoma and statistically increased incidence of multiple adenomas was considered to be equivocal evidence of carcinogenic activity.

In the current study, the incidences of acinar atrophy of the pancreas were significantly increased in males and females fed a diet with excess zinc (500 ppm). Atrophy of the pancreatic acini was characterized by small, shrunken ducts surrounded by a few depleted acinar cells, interstitial fibrous connective tissue, and mononuclear cell infiltrates, all of which resulted in an overall focal reduction in acinar tissue. In the literature, zinc toxicity from high levels of zinc has been associated with pancreatic lesions in pigs, including epithelial cell necrosis, diffuse acinar atrophy, and marked interstitial fibrosis (Gabrielson *et al*., 1996). The majority of zinc excretion occurs through the pancreas and as a result, the pancreas has also been identified as a target organ of zinc toxicity in mice through an unknown mechanism (Sutomo *et al*., 1992). Deficiencies of other metals such as copper have also demonstrated pancreatic effects. Rats administered copper chelators through the diet to

induce copper deficiency developed total acinar cell atrophy and fatty infiltration of the pancreatic acinar tissue (Smith *et al*., 1982). Additionally, high levels of dietary zinc have been shown to interact with the absorption of copper (Ogiso *et al*., 1979), an interaction that is supported by data from the current study in which male and female rats in the zinc excess 500 ppm dose groups had decreased blood concentrations of copper.

Dietary zinc exposure for 2 years had some effects on the male reproductive system as indicated by a significantly increased incidence of bilateral germinal epithelium atrophy in the testis of males fed the zinc-deficient diet (3.5 ppm). However, overall combined incidences of unilateral and bilateral germinal epithelium atrophy were not significantly increased due to higher incidences of unilateral germinal epithelium atrophy in the controls. Germinal epithelium atrophy was characterized by a thin germinal epithelium layer as a result of reduced numbers of germ cells, and most of the affected tubules were reduced in size and lined only by Sertoli cells. Although there were not pronounced effects of zinc deficiency in the current study, the importance of zinc in reproduction is illustrated through numerous studies in both animals and humans that have demonstrated decreased testosterone, reduced gonadal growth, and testicular damage (increased apoptosis and atrophy) due to zinc deficiency (Millar *et al*., 1958; Underwood and Somers, 1969; Prasad, 1976; Bedwal and Bahuguna, 1994, Merrells *et al*., 2009; Kumari *et al*., 2011; Sankako *et al*., 2012). Kumari *et al*. (2011) have shown that severe testicular degeneration and a significant loss of germ and somatic cells can occur as early as 4 weeks in rats fed a zinc-deficient diet (1 ppm). This testicular atrophy induced by zinc deficiency has also been shown to be irreversible, with no recovery after zinc was reintroduced into the diet (Millar *et al*., 1958; Barney *et al*., 1968). Testicular atrophy has also been observed frequently in humans with zinc deficient states, which include sickle cell anemia, chronic alcoholism, and idiopathic male sterility (Bedwal and Bahuguna, 1994). The testicular atrophy and arrested spermatogenesis have been attributed to defective cholesterol metabolism and thus low serum testosterone levels that result from zinc deficiency (Reeves and O'Dell, 1981; Bedwal and Bahuguna, 1994). Germinal epithelium atrophy can also be attributed to an ageing effect in rats, and the increased incidence of this lesion in the 3.5 ppm group may be a result of increased survival. However, given the association between zinc deficiency and testicular damage in the literature, it is likely that the testicular effects seen here are due to zinc deficiency.

Both zinc deficiency and excess have been reported in the literature to increase DNA damage in human and rodent cells *in vitro* (Ho and Ames, 2002; Ho *et al*., 2003; Yan *et al*., 2008; Li *et al*., 2009; Sharif *et al*., 2011, 2012). Published data have also shown increases in micronuclei and DNA damage in rats fed a zinc-deficient diet (< 1 ppm) for 3 to 6 weeks, but the dietary zinc levels in these short-term studies were markedly less than the levels in the current NTP 2-year bioassay, which may account for the early detection of genetic damage (Castro *et al*., 1992; Song *et al*., 2009a; Kawasaki *et al*., 2013). In the current NTP rodent study, neither deficiency nor excess zinc in the diet for up to 12 months induced micronuclei in red blood cells, but results from NTP comet assays showed evidence of DNA damage in both leukocytes and colon epithelial cells in male and female rats after long-term exposure. In leukocytes, increases in DNA damage were observed primarily in the male and female rats fed a zinc-deficient diet after 9 or 12 months of exposure. In male rats fed a diet with excess zinc, DNA damage was increased in leukocytes at the 12-month sample time. In colon cells analyzed after 12 months of exposure, evidence of DNA damage was seen in the rats on zinc-deficient diets as well as the rats maintained on a diet with excess zinc. The types of damage observed in the two sets of rats may have been different, however. In this study, a zinc-deficient diet was associated with a significant decrease in DNA migration in the comet assay in colon samples compared to the control diet, an observation consistent with DNA cross-linking (Pfuhler and Wolf, 1996), which has been shown to reduce the ability of DNA to migrate in the comet assay. Additionally, excess zinc in the diet was associated with significant increases in migration compared to the control diet, consistent with DNA fragmentation. Despite the evidence for DNA damage seen in colon epithelial cells of male and female rats exposed to high or low levels of dietary zinc, no preneoplastic lesions or neoplasms were observed in the colon. It is not known whether the observed DNA damage was induced in stem cells in the base of the colonic crypts or in epithelial cells that were fully differentiated. If the latter, then no opportunity would exist for development and expansion of a mutated clone to eventually produce a tumor. Interpretation of the DNA damage observations in colon cells may be further complicated by actions of the gut microbiome, but at this time, information on how this interaction might affect the consequences of zinc levels in the diet is not available. However, the indications of DNA damage associated with non-optimal zinc levels in a variety of *in vitro* and *in vivo* studies may serve as an alert to the potential for DNA damage in other cell types, which might give rise to adverse health consequences, especially if zinc levels remain altered for long periods of time.

CONCLUSIONS

Under the conditions of this 2-year dietary study, there was *equivocal evidence of carcinogenic activity** of diets deficient in zinc in male Hsd:Sprague Dawley SD rats based on higher incidences of adenoma of the pancreas and increased incidences of animals with multiple pancreatic adenomas. There was *no evidence of carcinogenic activity* of diets deficient in zinc (3.5 or 7 ppm) in female Hsd:Sprague Dawley SD rats. There was *no evidence of carcinogenic activity* of diets containing excess zinc (250 or 500 ppm) in male or female Hsd:Sprague Dawley SD rats.

Exposure to diets containing excess zinc resulted in increased incidences of nonneoplastic lesions of the pancreas in male and female rats. Exposure to diets deficient in zinc resulted in increased incidences of nonneoplastic lesions of the testes in male rats.

---

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 8. A summary of the Peer Review Panel comments and the public discussion on this Technical Report appears on page 10.

REFERENCES

Aamodt, R.L., Rumble, W.F., and Henkin, R.I. (1983). Zinc absorption in humans: Effects of age, sex, and food. In *Nutritional Bioavailability of Zinc* (G.E. Inglett, Ed.), pp. 61-82. The American Chemical Society, Washington, DC.

Abnet, C.C., Lai, B., Qiao, Y.-L., Vogt, S., Luo, X.-M., Taylor, P.R., Dong, Z.-W., Mark, S.D., and Dawsey, S.M. (2005). Zinc concentration in esophageal biopsy specimens measured by X-ray fluorescence and esophageal cancer risk. *J. Natl. Cancer Inst.* **97**, 301-306.

Agency for Toxic Substances and Disease Registry (ATSDR) (2005). Toxicological profile for zinc. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA. <http://www.atsdr.cdc.gov/toxprofiles>

Alexander, J., Aaseth, J., and Refsvik, T. (1981). Excretion of zinc in rat bile-a role of glutathione. *Acta Pharmacol. Toxicol.* **49**, 190-194.

Alexander, T.H., and Davidson, T.M. (2006). Intranasal zinc and anosmia: The zinc-induced anosmia syndrome. *Laryngoscope* **116**, 217-220.

Allan, G.M., and Arroll, B. (2014). Prevention and treatment of the common cold: Making sense of the evidence. *CMAJ* **186**, 190-199.

Allen, J.G., Masters, H.G., Peet, R.L., Mullins, K.R., Lewis, R.D., Skirrow, S.Z., and Fry, J. (1983). Zinc toxicity in ruminants. *J. Comp. Pathol*. **93**, 363-377.

Allen, L.H. (1998). Zinc and micronutrient supplements for children. *Am. J. Clin. Nutr*. **68**, 495S-498S.

Ames, B.N., and Wakimoto, P. (2002). Are vitamin and mineral deficiencies a major cancer risk? *Nat. Rev. Cancer* **2**, 694-704.

Anderson, M.B., Lepak, K., Farinas, V., and George, W.J. (1993). Protective action of zinc against cobalt-induced testicular damage in the mouse. *Reprod. Toxicol*. **7**, 49-54.

Ansari, M.S., Miller, W.J., Lassiter, J.W., Neathery, M.W., and Gentry, R.P. (1975). Effects of high but nontoxic dietary zinc on zinc metabolism and adaptations in rats. *Proc. Soc. Exp. Biol. Med*. **150**, 534-536.

Ansari, M.S., Miller, W.J., Neathery, M.W., Lassiter, J.W., Gentry, R.P., and Kincaid, R.L. (1976). Zinc metabolism and homeostasis in rats fed a wide range of high dietary zinc levels. *Proc. Soc. Exp. Biol. Med*. **152**, 192-194.

Apgar, J. (1970). Effect of zinc deficiency on maintenance of pregnancy in the rat. *J. Nutr*. **100**, 470-476.

Apgar, J. (1985). Zinc and reproduction. *Annu. Rev. Nutr*. **5**, 43-68.

Arnaud, J., and Favier, A. (1995). Copper, iron, manganese and zinc contents in human colostrum and transitory milk of French women. *Sci. Total Environ*. **159**, 9-15.

Aughey, E., Grant, L., Furman, B.L., and Dryden, W.F. (1977). The effects of oral zinc supplementation in the mouse. *J. Comp. Path*. **87**, 1-14.

Aulerich, R.J., Bursian, S.J., Poppenga, R.H., Braselton, W.E., and Mullaney, T.P. (1991). Toleration of high concentrations of dietary zinc by mink. *J. Vet. Diagn. Invest.* **3**, 232-237.

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

Bárány, E., Bergdahl, I.A., Bratteby, L.-E., Lundh, T., Samuelson, G., Schütz, A., Skerfving, S., and Oskarsson, A. (2002). Relationships between trace element concentrations in human blood and serum. *Toxicol. Lett.* **134**, 177-184.

Barch, D.H., and Fox, C.C. (1987). Dietary zinc deficiency increases the methylbenzylnitrosamine-induced formation of O^6-methylguanine in the esophageal DNA of the rat. *Carcinogenesis* **8**, 1461-1464.

Barney, G.H., Orgebin-Crist, M.C., and Macapinlac, M.P. (1968). Genesis of esophageal parakeratosis and histologic changes in the testes of the zinc-deficient rat and their reversal by zinc repletion. *J. Nutr.* **95**, 526-534.

Beach, R.S., Gershwin, M.E., and Hurley, L.S. (1980). Growth and development in postnatally zinc deprived mice. *J. Nutr.* **110**, 201-211.

Bedwal, R.S., and Bahuguna, A. (1994). Zinc, copper and selenium in reproduction. *Experientia* **50**, 626-640.

Beer, W.H., Johnson, R.F., Guentzel, M.N., Lozano, J., Henderson, G.I., and Schenker, S. (1992). Human placental transfer of zinc: Normal characteristics and role of ethanol. *Alcohol Clin. Exp. Res*. **16**, 98-105.

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

Black, M.R., Medeiros, D.M., Brunett, E., and Welk, R. (1988). Zinc supplements and serum lipids in young adult white males. *Am. J. Clin. Nutr*. **47**, 970-975.

Bonham, M., O'Connor, J.M., Alexander, H.D., Coulter, J., Walsh, P.M., McAnena, L.B., Downes, C.S., Hannigan, B.M., and Strain, J.J. (2003a). Zinc supplementation has no effect on circulating levels of peripheral blood leucocytes and lymphocyte subsets in healthy adult men. *Br. J. Nutr*. **89**, 695-703.

Bonham, M., O'Connor, J.M., McAnena, L.B., Walsh, P.M., Downes, C.S, Hannigan, B.M., and Strain, J.J. (2003b). Zinc supplementation has no effect on lipoprotein metabolism, hemostasis, and putative indices of copper status in healthy men. *Biol. Trace Elem. Res*. **93**, 75-86.

Boorman, G.A., and Eustis, S.L. (1984). Proliferative lesions of the exocrine pancreas. *Environ. Health Perspect.* **56**, 213-217.

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

Brendler-Schwaab, S., Hartmann, A., Pfuhler, S., and Speit, G. (2005). The *in vivo* comet assay: Use and status in genotoxicity testing. *Mutagenesis* **20**, 245-254.

Broun, E.R., Greist, A., Tricot, G., and Hoffman, R. (1990). Excessive zinc ingestion: A reversible cause of sideroblastic anemia and bone marrow depression. *JAMA* **264**, 1441-1443.

Brown, M.A., Thom, J.V., Orth, G.L., Cova, P., and Juarez, J. (1964). Food poisoning involving zinc contamination. *Arch. Environ. Health* **8**, 657-660.

Burlinson, B., Tice, R.R., Speit, G., Agurell, E., Brendler-Schwaab, S.Y., Collins, A.R., Escobar, P., Honma, M., Kumaravel, T.S., Nakajima, M., Sasaki, Y.F., Thybaud, V., Uno, Y., Vasquez, M., and Hartmann, A. (2007). Fourth International Work Group on Genotoxicity Testing: Results of the *in vivo* comet assay workgroup. *Mutat. Res*. **627**, 31-35.

Callender, G.R., and Gentzkow, C.J. (1937). Acute poisoning by the zinc and antimony content of limeade prepared in a galvanized iron can. *Mil. Surg.* **80**, 67-71.

Cameron, C. (2005). Water-based fluids approach synthetic capabilities. *Offshore* **65**, 44.

Carboni, A.A., Cullen, K.J., and Lavelle, W.G. (2006). The effects of zinc on the olfactory neuroepithelium and olfactory bulbs of the Sprague-Dawley rat after oral administration of zinc-gluconate trihydrate. *Am. J. Rhinol*. **20**, 262-268.

Cao, Z., Zhang, Z., Wang, F., and Wang, G. (2009). Synthesis and UV shielding properties of zinc oxide ultrafine particles modified with silica and trimethyl siloxane. *Colloids and Surfaces A: Physiochem. Eng. Aspects* **340**, 161-167.

Caruso, T.J., Prober, C.G., and Gwaltney, J.M., Jr. (2007). Treatment of naturally acquired common colds with zinc: A structured review. *Clin. Infect. Dis*. **45**, 569-574.

Castro, C.E., Kaspin, L.C., Chen, S.-S., and Nolker, S.G. (1992). Zinc deficiency increases the frequency of single-strand DNA breaks in rat liver. *Nutr. Res*. **12**, 721-736.

Cater, B.R., Cook, M.W., Gangolli, S.D., and Grasso, P. (1977). Studies on dibutyl phthalate-induced testicular atrophy in rats: Effect on zinc metabolism. *Toxicol. Appl. Pharmacol*. **41**, 609-618.

Centers for Disease Control (CDC) (1983). Illness associated with elevated levels of zinc in fruit punch-New Mexico. *Morb. Mortal. Wkly. Rep*. **32**, 257-258. <http://www.cdc.gov/mmwr/preview/mmwrhtml/00000082.htm>

Chaffee, B.W., and King, J.C. (2012). Effect of zinc supplementation on pregnancy and infant outcomes: A systematic review. *Paediatr. Perinat. Epidemiol.* **26**, 118-137.

Chandra, R.K. (1984). Excessive intake of zinc impairs immune responses. *JAMA* **252**, 1443-1446.

Cherry, F.F., Bennet, E.A., Bazzano, G.S., Johnson, L.K., Fosmire, G.J., and Batson, H.K. (1981). Plasma zinc in hypertension/toxemia and other reproductive variables in adolescent pregnancy. *Am. J. Clin. Nutr*. **34**, 2367-2375.

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

Code of Federal Regulations (CFR) **21**, § 73.2991.

Code of Federal Regulations (CFR) **21**, § 175.105.

Code of Federal Regulations (CFR) **21**, § 175.300.

Code of Federal Regulations (CFR) **21**, § 177.2600.

Code of Federal Regulations (CFR) **21**, § 182.5985.

Code of Federal Regulations (CFR) **21**, § 182.5991.

Code of Federal Regulations (CFR) **21**, § 182.5994.

Code of Federal Regulations (CFR) **21**, § 182.5997.

Code of Federal Regulations (CFR) **21**, § 182.90.

Code of Federal Regulations (CFR) **40**, § 372.65.

Collins, A.R. (2004). The comet assay for DNA damage and repair. Principles, applications and limitations. *Mol. Biotechnol*. **26**, 249-261.

Cooke, J.A., Andrews, S.M., and Johnson, M.S. (1990). The accumulation of lead, zinc, cadmium and fluoride in the wood mouse (*Apodemus sylvaticus L.). Water Air Soil Pollut.* **51**, 55-63.

Cousins, R.J. (1985). Absorption, transport, and hepatic metabolism of copper and zinc: Special reference to metallothionein and ceruloplasmin. *Physiol. Rev*. **65**, 238-309.

Cousins, R.J., Liuzzi, J.P., and Lichten, L.A. (2006). Mammalian zinc transport, trafficking, and signals. *J. Biol. Chem*. **281**, 24,085-24,089.

Cox, D.H., Schlicker, S.A., and Chu, R.C. (1969). Excess dietary zinc for the maternal rat, and zinc, iron, copper, calcium, and magnesium content and enzyme activity in maternal and fetal tissues. *J. Nutr*. **98**, 459-466.

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

Crawford, B.D. (1985). Perspectives on the somatic mutation model of carcinogenesis. In *Advances in Modern Environmental Toxicology. Mechanisms and Toxicity of Chemical Carcinogens and Mutagens* (M.A. Mehlman, W.G. Flamm, and R.J. Lorentzen, Eds.), pp. 13-59. Princeton Scientific Publishing Co., Inc., Princeton, NJ.

Cushman, D.W., and Cheung, H.S. (1971). Concentrations of angiotensin-converting enzyme in tissues of the rat. *Biochem. Biophys. Acta* **250**, 261-265.

Davies, N.T. (1980). Studies on the absorption of zinc by rat intestine. *Br. J. Nutr*. **43**, 189-203.

Davies, N.T., and Nightingale R. (1975). The effects of phytate on intestinal absorption and secretion of zinc, and whole body retention of Zn, copper, iron and manganese in rats. *Br. J. Nutr*. **34**, 243-258.

Davis, C.D., Milne, D.B., and Nielsen, F.H. (2000). Changes in dietary zinc and copper affect zinc-status indicators of postmenopausal women, notably, extracellular superoxide dismutase and amyloid precursor proteins. *Am. J. Clin. Nutr*. **71**, 781-788.

Deknudt, G., and Gerber, G.B. (1979). Chromosomal aberrations in bone-marrow cells of mice given a normal or a calcium-deficient diet supplemented with various heavy metals. *Mutat. Res.* **68**, 163-168.

Della Lucia, C.M., Santos. L.L., Rodrigues, K.C., Rodrigues, V.C., Martino, H.S., and Sant'Ana, H.M. (2014). Bioavailability of zinc in Wistar rats fed with rice fortified with zinc oxide. *Nutrients* **6**, 2279-2289.

Dertinger, S.D., Camphausen, K., MacGregor, J.T., Bishop, M.E., Torous, D.K., Avlasevich, S., Cairns, S., Tometsko, C.R., Menard, C., Muanza, T., Chen, Y., Miller, R.K., Cederbrant, K., Sandelin, K., Pontén, I., and Bolcsfoldi, G. (2004). Three-color labeling method for flow cytometric measurement of cytogenetic damage in rodent and human blood. *Environ. Mol. Mutagen.* **44**, 427-435.

Diamond, I., Swenerton, H., and Hurley, L.S. (1971). Testicular and esophageal lesions in zinc-deficient rats and their reversibility. *J. Nutr*. **101**, 77-84.

Dixon, W.J., and Massey, F.J., Jr. (1957). *Introduction to Statistical Analysis*, 2nd ed., pp. 276-278, 412. McGraw-Hill Book Company, Inc., New York.

Domingo, J.L., Llobet, J.M., Paternain, J.L., and Corbella, J. (1988). Acute zinc intoxication: Comparison of the antidotal efficacy of several chelating agents. *Vet. Hum. Toxicol*. **30**, 224-228.

Dórea, J.G. (2002). Zinc and copper in breast-milk and home-prepared milk fed to urban infants from low-income families. *J. Trace Elem. Exp. Med*. **15**, 123-129.

Du, S., Liu, H., and Chen, Y. (2009). Large-scale preparation of porous ultrathin Ga-doped ZnO nano-needles from 3D basic zinc carbonate superstructures. *Nanotechnology* **20**. DOI:10.1088/0957-4484/20/8/085611

Dunn, O.J. (1964). Multiple comparisons using rank sums. *Technometrics* **6**, 241-252.

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

Evans, G.W., Grace, C.I., and Hahn, C. (1973). Homeostatic regulation of zinc absorption in the rat. *Proc. Soc. Exp. Biol. Med.*, **143**, 723-725.

Fairweather-Tait, S.J., Fox, T.E., Wharf, S.G., and Ghani, N.A., (1992). A preliminary study of the bioavailability of iron- and zinc-glycine chelates. *Food. Addit. Contam*. **96**, 97-101.

Favier, A.E. (1992). The role of zinc in reproduction: Hormonal mechanisms. *Biol. Trace Elem. Res*. **32**, 363-382.

Fischer, P.W.F., Giroux, A., Belonje, B., and Shah, B.G. (1980). The effect of dietary copper and zinc on cholesterol metabolism. *Am. J. Clin. Nutr.* **33**, 1019-1025.

Fischer, P.W.F., Giroux, A., and L'Abbé, M.R. (1981). The effect of dietary zinc on intestinal copper absorption. *Am. J. Clin. Nutr*. **34**, 1670-1675.

Fischer, P.W.F., Giroux, A., and L'Abbé, M.R. (1984). Effect of zinc supplementation on copper status in adult man. *Am. J. Clin. Nutr.* **40**, 743-746.

Flanagan, P.R., Haist, J., and Valberg, L.S. (1983). Zinc absorption, intraluminal zinc and intestinal metallothionein levels in zinc-deficient and zinc-replete rodents. *J. Nutr*. **113**, 962-972.

Fong, L.Y.Y, Li, J.-X., Farber, J.L., and Magee, P.N. (1996). Cell proliferation and esophageal carcinogenesis in the zinc-deficient rat. *Carcinogenesis* **17**, 1841-1848.

Fong, L.Y.Y., Nguyen, V.T., and Farber, J.L. (2001). Esophageal cancer prevention in zinc-deficient rats: Rapid induction of apoptosis by replenishing zinc. *J. Natl. Cancer Inst.* **93**, 1525-1533.

Forssén, A. (1972). Inorganic elements in the human body. I. Occurrence of Ba, Br, Ca, Cd, Cs, Cu, K, Mn, Ni, Sn, Sr, Y, and Zn in the human body. *Ann. Med. Exp. Biol. Fenn*. **50**, 99-162.

Foster, P.M.D., Thomas, L.V., Cook, M.W., and Gangolli, S.D. (1980). Study of the testicular effects and the changes in zinc excretion produced by some *n*-alkyl phthalates in the rat. *Toxicol. Appl. Pharmacol*. **54**, 392-398.

Foulkes, E.C., and McMullen, D.M. (1987). Kinetics of transepithelial movement of heavy metals in rat jejunum. *Am. J. Physiol*. **253**, G134-G138.

Freeland-Graves, J.H, Han, W.-H., Friedman, B.J., and Shorey, R.L. (1980). Effect of dietary Zn/Cu ratios on cholesterol and HDL cholesterol levels in women. *Nutr. Rep. Int.* **22**, 285-293.

Gabrielson, K.L., Remillard, R.L., and Huso, D.L. (1996). Zinc toxicity with pancreatic acinar necrosis in piglets receiving total parenteral nutrition. *Vet. Pathol*. **33**, 692-696.

Gachot, B., and Poujeol, P. (1992). Effects of cadmium and copper on zinc transport kinetics by isolated renal proximal cells. *Biol. Trace Elem. Res*. **35**, 93-103.

Galvez-Morros, M., Garcia-Martinez, O., Wright, A.J.A., and Southon, S. (1992). Bioavailability in the rat of zinc and iron from the basic salts $Zn_5(OH)_8Cl_2 \cdot H_2O$, $Fe(OH)SO_4$ and $Fe_4(OH)_{11}NO_3 \cdot 2H_2O$. *Food Chem.* **43**, 377-381.

Ghanayem, B.I., Witt, K.L., Kissling, G.E., Tice, R.R., and Recio, L. (2005). Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: Evidence consistent with a glycidamide-mediated effect. *Mutat. Res.* **578**, 284-297.

Giugliano, R., and Millward, D.J. (1984). Growth and zinc homeostasis in the severely Zn-deficient rat. *Br. J. Nutr*. **52**, 545-560.

Gocke, E., King, M.-T., Eckhardt, K., and Wild, D. (1981). Mutagenicity of cosmetics ingredients licensed by the European Communities. *Mutat. Res*. **90**, 91-109.

Gombe, S., Apgar, J., and Hansel, W. (1973). Effect of zinc deficiency and restricted food intake on plasma and pituitary LH and hypothalamic LRF in female rats. *Biol. Reprod*. **9**, 415-419.

Goodwin, F.E. (1998). Zinc compounds. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th ed. (J. Kroschwitz and M. Howe-Grant, Eds.), Vol. 25, pp. 840-853. John Wiley & Sons, New York.

Grant, W.B. (2008). An ecological study of cancer mortality rates including indices for dietary iron and zinc. *Anticancer Res*. **28**, 1955-1964.

Greger, J.L., and Sickles, V.S. (1979). Saliva zinc levels: Potential indicators of zinc status. *Am. J. Clin. Nutr*. **32**, 1859-1866.

Grieger, J.A., and Clifton, V.L. (2015). A review of the impact of dietary intakes in human pregnancy on infant birthweight. *Nutrients* **7**, 153-178.

Gunshin, H., Noguchi, T., and Naito, H. (1991). Effect of calcium on the zinc uptake by brush-border membrane vesicles isolated from the rat small intestine. *Agric. Biol. Chem*. **55**, 2813-2816.

Hale, W.E., May, F.E., Thomas, R.G., Moore, M.T., and Stewart, R.B. (1988). Effect of zinc supplementation on the development of cardiovascular disease in the elderly. *J. Nutr. Elder*. **8**, 49-57.

Halme, E. (1961). On the carcinogenic effect of drinking water containing zinc. *Vitalstoffe* **6**, 59-66.

Hambidge, K.M., Hambidge, C., Jacobs, M., and Baum, J.D. (1972). Low levels of zinc in hair, anorexia, poor growth and hypogeusia in children. *Pediatr. Res*. **6**, 868-874.

Hambidge, K.M., Neldner, K.H., and Walravens, P.A. (1975). Zinc, acrodermatitis enteropathica, and congenital malformations. *Lancet* **March 8**, 577-578.

Harford, C., and Sarkar, B. (1991). Induction of metallothionein by simultaneous administration of cadmium (II) and zinc (II). *Biochem. Biophys. Res. Comm*. **177**, 224-228.

Hartmann, A., Agurell, E., Beevers, C., Brendler-Schwaab, S., Burlinson, B., Clay, P., Collins, A., Smith, A., Speit, G., Thybaud, V., and Tice, R.R. (2003). Recommendations for conducting the *in vivo* alkaline Comet assay. *Mutagenesis*, **18**, 45-51.

He, L.-S., Yan, X.-S., and Wu, D.-C. (1991). Age-dependent variation of zinc-65 metabolism in LACA mice. *Int. J. Radiat. Biol*. **60**, 907-916.

Heddle, J.A., Hite, M., Kirkhart, B., Mavournin, K., MacGregor, J.T., Newell, G.W., and Salamone, M.F. (1983). The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat. Res*. **123**, 61-118.

Hemilä, H., and Chalker, E. (2015). The effectiveness of high dose zinc acetate lozenges on various common cold symptoms: A meta-analysis. *BMC Fam. Pract*. **16**. doi: 10.1186/s12875-015-0237-6.

Hempe, J.M., and Cousins, R.J. (1992). Cysteine-rich intestinal protein and intestinal metallothionein: An inverse relationship as a conceptual model for zinc absorption in rats. *J. Nutr*. **122**, 89-95.

Henkin, R.I. (1974). Metal-albumin-amino acid interactions: Chemical and physiological interrelationships. *Adv. Exp. Med. Biol*. **48**, 299-328.

Henkin, R.I., Mueller, C.W., and Wolf, R.O. (1975). Estimation of zinc concentration of parotid saliva by flameless atomic absorption spectrophotometry in normal subjects and in patients with idiopathic hypogeusia. *J. Lab. Clin. Med*. **86**, 175-180.

Henkin, R.I., Schecter, P.J., Friedewald, W.T., Demets, D.L., and Raff, M. (1976). A double blind study of the effects of zinc sulfate on taste and smell dysfunction. *Am. J. Med. Sci*. **272**, 285-299.

Herd, D.B. (1997). Mineral supplementation of beef cows in Texas. Texas A&M University.

Hess, S.Y., Peerson, J.M, King, J.C., and Brown, K.H. (2007). Use of serum zinc concentration as an indicator of population zinc status. *Food Nutr. Bull*. **28** (Suppl.), S403-S429.

Hickory, W., Nanda, R., and Catalanotto, F.A. (1979). Fetal skeletal malformations associated with moderate zinc deficiency during pregnancy. *J. Nutr*. **109**, 883-891.

Ho, E., and Ames, B.N. (2002). Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFKB, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. *Proc. Natl. Acad. Sci. USA* **99**, 16,770-16,775.

Ho, E., Courtemanche, C., and Ames, B.N. (2003). Zinc deficiency induces oxidative DNA damage and increases p53 expression in human lung fibroblasts. *J. Nutr.* **133**, 2543-2548.

Hooper, P.L., Visconti, L., Garry, P.J., and Johnson, G.E. (1980). Zinc lowers high-density lipoprotein-cholesterol levels. *JAMA* **244**, 1960-1961.

Hotz, C., DeHaene, J., Woodhouse, L.R., Villalpando, S., Rivera, J.A., and King, J.C. (2005). Zinc absorption from zinc oxide, zinc sulfate, zinc oxide + EDTA, or sodium-zinc EDTA does not differ when added as fortificants to maize tortillas. *J. Nutr*. **135**, 1102-1105.

Hunt, J.R., Lykken, G.I., and Mullen, L.K. (1991). Moderate and high amounts of protein from casein enhance human absorption of zinc from whole-wheat or white rolls. *Nutr. Res*. **11**, 413-418.

Hurley, L.S., and Swenerton, H. (1966). Congenital malformations resulting from zinc deficiency in rats. *Proc. Soc. Exp. Biol. Med*. **123**, 692-696.

Ianotti, L.L., Zavaleta, N., León, Z., Shankar, A.H., and Caulfield, L.E. (2008). Maternal zinc supplementation and growth in Peruvian infants. *Am. J. Clin. Nutr*. **88**, 154-160.

Institute of Medicine (IOM) (2002). Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. A Report of the Panel on Micronutrients, Subcommittees on Upper Reference Levels of Nutrients and of Interpretation and Uses of Dietary Reference Intakes, and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 442-501. CASRN 7440-66-6. Food and Nutrition Board, Institute of Medicine. National Academies Press, Washington, DC.

Integrated Risk Information System (IRIS) (2003). Zinc and compounds. United States Environmental Protection Agency, Washington, DC <https://cfpub.epa.gov./ncea/iris2/ChemicalLanding.cfm?substance_number=426> Accessed April 21, 2016.

Istfan, N.W., Janghorbani, M., and Young, V.R. (1983). Absorption of stable^{70} Zn in healthy young men in relation to zinc intake. *Am. J. Clin. Nutr*. **38**, 187-194.

Jaiswal, A., Joshi, P., Kumar, M.V., Panda, J.N., and Singh, L.N. (1984). Angiotensin converting enzyme in the testis and epididymis of mammals. *Andrologia* **16**, 410-416.

Johnson, F.O., Gilbreath, E.T., Ogden, L., Graham, T.C., and Gorham, S. (2011). Reproductive and developmental toxicities of zinc supplemented rats. *Reprod. Toxicol*. **31**, 134-143.

Johnson, M.A., and Flagg, E.W. (1986). Effects of sucrose and cornstarch on the development of copper deficiency in rats fed high levels of zinc. *Nutr. Res*. **6**, 1307-1319.

Johnson, P.E., Hunt, J.R., and Ralston, N.V.C. (1988). The effect of past and current dietary Zn intake on Zn absorption and endogenous excretion in the rat. *J. Nutr.* **118**, 1205-1209.

Jonckheere, A.R. (1954). A distribution-free *k*-sample test against ordered alternatives. *Biometrika* **41**, 133-145.

Kadiiska, M., Stoytchev, T., and Serbinova, E. (1985). Effect of some heavy metal salts on hepatic monooxygenases after subchronic exposure. *Arch. Toxicol. Suppl*. **8**, 313-315.

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

Katya-Katya, M., Ensminger A., Méjean, L., and Debry, G. (1984). The effect of zinc supplementation on plasma cholesterol levels. *Nutr. Res*. **4**, 633-638.

Kaul, L., Nidiry, J.J., Charles-Marcel, Z., Enterline, J.P., and Brown, C.O. (1986). Diet and esophageal cancer: A case-control study. *Nutr. Res*. **6**, 905-912.

Kawasaki, I., Suzuki, Y., and Yanagisawa, H. (2013). Zinc deficiency enhances the induction of micronuclei and 8-hydroxy-2′-deoxyguanosine via superoxide radical in bone marrow of zinc-deficient rats. *Biol. Trace Elem. Res*. **154**, 120-126.

Keen, C.L., and Hurley, L.S. (1977). Zinc absorption through skin: Correction of zinc deficiency in the rat. *Am. J. Clin. Nutr*. **30**, 528-530.

Kelly, E.J., Quaife, C.J., Froelick, G.J., and Palmiter, R.D. (1996). Metallothionein I and II protect against zinc deficiency and zinc toxicity in mice. *J. Nutr*. **126**, 1782-1790.

Ketcheson, M.R., Barron, G.P., and Cox, D.H. (1969). Relationship of maternal dietary zinc during gestation and lactation to development and zinc, iron and copper content of the postnatal rat. *J. Nutr*. **98**, 303-311.

Khan, A.T., Atkinson, A., Graham, T.C., Green, M., Ali, S., Thompson, S.J., and Shireen, K.F. (2001). Effects of low levels of zinc on reproductive performance of rats. Published abstract. College of Veterinary Medicine, Nursing and Allied Health and Department of Food and Nutritional Sciences, Tuskegee University, Tuskegee, AL.

Kincaid, R.L. (1979). Biological availability of zinc from inorganic sources with excess dietary calcium. *J. Dairy Sci*. **62**, 1081-1085.

King, J.C., Shames, D.M., and Woodhouse, L.R. (2000). Zinc homeostasis in humans. *J. Nutr*. **130**, 1360S-1366S.

Kissling, G.E., Dertinger, S.D., Hayashi, M., and MacGregor, J.T. (2007). Sensitivity of the erythrocyte micronucleus assay: Dependence on number of cells scored and inter-animal variability. *Mutat. Res.* **634**, 235-240.

Klevya, L.M., and Hyg, S.D. (1973). Hypercholesterolemia in rats produced by an increase in the ratio of zinc to copper ingested. *Am. J. Clin. Nutr*. **26**, 1060-1068.

Kmet, J., and Mahboubi, E. (1972). Esophageal cancer in the Caspian littoral of Iran: Initial studies. *Science* **175**, 846-853.

Koga, N., and Tanaka, H. (2005). Thermal decomposition of copper (II) and zinc carbonate hydroxides by means of TG-MS: Quantitative analyses of evolved gases. *J. Therm. Anal. Calorim*. **82**, 725-729.

Krishnan, U., and Que Hee, S.S. (1992). Ear wax: A new biological monitoring medium for metals? *Bull. Environ. Contam. Toxicol*. **48**, 481-486.

Kumar, V., Abbas, A.K., Fausto, N., and Mitchell, R.N., Eds. (2007). *Robbins Basic Pathology*, 8th ed., p. 315. Saunders Elsevier, Philadelphia.

Kumari, D., Nair, N., and Bedwal, R.S. (2011). Effect of dietary zinc deficiency on testes of Wistar rats: Morphometric and cell quantification studies. *J. Trace Elem. Med. Biol*. **25**, 47-53.

Kurugöl, Z., Bayram, N., and Atik, T. (2007). Effect of zinc sulfate on common cold in children: Randomized, double blind study. *Pediatr. Int*. **49**, 842-847.

Kynast, G., and Saling, E. (1986). Effect of oral zinc application during pregnancy. *Gynecol. Obstet. Invest*. **21**, 117-123.

Lee, D.-H., Anderson, K.E., Folsom, A.R., and Jacobs, D.R., Jr. (2005). Heme iron, zinc and upper digestive tract cancer: The Iowa Women's Health Study. *Int. J. Cancer* **117**, 643-647.

Levine, K.E., Collins, B.J., Stout, M.D., Wyde, M., Afton, S.E., Essader, A.S., Ennis, T.J., Amato, K.E., McWilliams, A.C., Fletcher, B.L., Fernando, R.A., Harrington, J.M., Catlin, N., Robinson, V.G., and Waidyanatha, S. (2017). Characterization of zinc carbonate basic for use as a source of zinc in a rodent study investigating the effects of dietary zinc deficiency or excess. *Anal. Lett*. In press.

Lewis, M.R., and Kokan, L. (1998). Zinc gluconate. *J. Toxicol. Clin. Toxicol*. **36**, 99-101.

Lewis, R.J. (1997). *Hawley's Condensed Chemical Dictionary*, pp. 1194-1202. John Wiley & Sons, Inc., New York.

Li, H., Swiercz, R., and Englander, E.W. (2009). Elevated metals compromise repair of oxidative DNA damage via the base excision repair pathway: Implications of pathologic iron overload in the brain on integrity of neuronal DNA. *J. Neurochem.* **110**, 1774-1783.

Li, P., Xu, J., Shi, Y., Ye, Y., Chen, K., Yang, J., and Wu, Y. (2014). Association between zinc intake and risk of digestive tract cancers: A systematic review and meta-analysis. *Clin. Nutr.* **33**, 415-420.

Lim, J.H., Davis, G.E., Wang, Z., Li, V., Wu, Y., Rue, T.C., and Storm, D.R. (2009). Zicam-induced damage to mouse and human nasal tissue. *PLoS ONE* **4**. doi: 10.1371/journal.pone.0007647.

Lin, H.J., Chan, W.C., Fong, L.Y.Y., and Newberne, P.M. (1976). Zinc levels in serum, hair and tumors from patients with esophageal cancer. *Nutr. Rep. Int.* **15**, 635-643.

Llobet, J.M., Domingo, J.L., Colomina, M.T., Mayayo, E., and Corbella, J. (1988). Subchronic oral toxicity of zinc in rats. *Bull. Environ. Contam. Toxicol*. **41**, 36-43.

Lloyd, T.B. (1984). Zinc compounds. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 3rd ed. (M. Grayson, Ed.), Vol. 24, pp. 851-863. John Wiley and Sons, New York.

Lloyd, T.B., and Showak, W. (1984). Zinc and zinc alloys. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 3rd ed. (M. Grayson, Ed.), Vol. 24, pp. 835-836. John Wiley and Sons, New York.

Lopez, H.W., Leenhardt, F., Coudray, C., and Rémésy, C. (2002), Minerals and phytic acid interactions: Is it a real problem for human nutrition? *Int. J. Food Sci. Technol*., **37**, 727-739.

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

MacGregor, J.T., Bishop, M.E., McNamee, J.P., Hayashi, M., Asano, N., Wakata, A., Nakajima, M., Saito, J., Aidoo, A., Moore, M.M., and Dertinger, S.D. (2006). Flow cytometric analysis of micronuclei in peripheral blood reticulocytes: II. An efficient method of monitoring chromosomal damage in the rat. *Toxicol. Sci*. **94**, 92-107.

Magee, A.C., and Matrone, G. (1960). Studies on growth, copper metabolism and iron metabolism of rats fed high levels of zinc. *J. Nutr*. **72**, 233-242.

Mahaffey, K.R., Corneliussen, P.E., Jelinek, C.F., and Fiorino, J.A. (1975). Heavy metal exposure from foods. *Environ. Health Perspect.* **12**, 63-69.

Maita, K., Hirano, M., Mitsumori, K., Takahashi, K., and Shirasu, Y. (1981). Subacute toxicity studies with zinc sulfate in mice and rats. *J. Pestic. Sci*. **6**, 327-336.

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

Marzin, D.R., and Phi, H.V. (1985). Study of the mutagenicity of metal derivatives with *Salmonella typhimurium* TA102. *Mutat. Res.* **155**, 49-51.

Masters, D.G., and Moir, R.J. (1983). Effect of zinc deficiency on the pregnant ewe and developing foetus. *Br. J. Nutr.* **49**, 365-372.

Meadows, N.J., Ruse, W., Smith, M.F., Day, J., Keeling, P.W.N., Scopes, J.W., and Thompson, R.P.H. (1981). Zinc and small babies. *Lancet* **November 21**, 1135-1137.

Mellow, M.H., Layne, E.A., Lipman, T.O., Kaushik, M., Hostetler, C., and Smith, J.C. (1983). Plasma zinc and vitamin A in human squamous carcinoma of the esophagus. *Cancer* **51**, 1615-1620.

The Merck Index (1983). 10th ed. (M. Windholz, Ed.), pp. 1455-1458. Merck & Co., Inc., Rahway, NJ.

The Merck Index (2006). 14th ed. (M.J. O'Neil, Ed.), pp. 1747-1750. Merck & Co., Inc., Whitehouse Station, NJ.

Merrells, K.J., Blewett, H., Jamieson, J.A., Taylor, C.G., and Suh, M. (2009). Relationship between abnormal sperm morphology induced by dietary zinc deficiency and lipid composition in testes of growing rats. *Br. J. Nutr*. **102**, 226-232.

Methfessel, A.H., and Spencer, H. (1973). Zinc metabolism in the rat. I. Intestinal absorption of zinc. *J. Appl. Physiol*. **34**, 58-62.

Millar, M.J., Fischer, M.I., Elcoate, P.V., and Mawson, C.A. (1958). The effects of dietary zinc deficiency on the reproductive system of male rats. *Can. J. Biochem. Physiol*. **36**, 557-569.

Miller, J.A., and Miller, E.C. (1977). Ultimate chemical carcinogens as reactive mutagenic electrophiles. In *Origins of Human Cancer* (H.H. Hiatt, J.D. Watson, and J.A. Winsten, Eds.), pp. 605-627. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Miller, W.J. (1969). Absorption, tissue distribution, endogenous excretion, and homeostatic control of zinc in ruminants. *Am J. Clin. Nutr*. **22**, 1323-1331.

Milne, D.B., Davis, C.D., and Nielsen, F.H. (2001). Low dietary zinc alters indices of copper function and status in postmenopausal women. *Nutrition* **17**, 701-708.

Moore, R. (1978). Bleeding gastric erosion after oral zinc sulphate. *Br. Med. J.* **1**, 754.

Moreno, M.A., Marin, C., Vinagre, F., and Ostapczuk, P. (1999). Trace element levels in whole blood samples from residents of the city of Badajoz, Spain. *Sci. Total Environ*. **229**, 209-215.

Mulhern, S.A., Stroube, W.B., Jr., and Jacobs, R.M. (1986). Alopecia induced in young mice by exposure to excess dietary zinc. *Experientia* **42**, 551-553.

National Academy of Sciences (NAS) (1977). Drinking water and health. V. Inorganic solutes. Vol. 1, pp. 205-488. National Academies Press, Washington, DC.

National Institute for Occupational Safety and Health (NIOSH) (1990). National Occupational Exposure Survey (1980-1983). Unpublished provisional data as of July 1, 1990. NIOSH, Cincinnati, OH.

National Research Council (NRC) 1979. *Zinc.* University Park Press, Baltimore, MD.

National Toxicology Program (NTP) (2016). <https://ntp.niehs.nih.gov/ntp/historical_controls/ntp2000_2016/aug-2016-hc-report-hsd-rats-by-route.pdf> Accessed February 14, 2017.

Nelson, L.S., Jr., Jacobs, F.A., and Brushmiller, J.G. (1985). Solubility of calcium and zinc in model solutions based on bovine and human milks. *J. Inorg. Biochem.* **24**, 255-265.

Nève, J., Hanocq, M., Peretz, A., Abi Khalil, F., Pelen, F., Famaey, J.P., and Fontaine, J. (1991). Pharmacokinetic study of orally administered zinc in humans: Evidence for an enteral recirculation. *Eur. J. Drug Metab. Pharmacokinet.* **16**, 315-323.

Newberne, P.M., Schrager, T.F., and Broitman, S. (1997). Esophageal carcinogenesis in the rat: Zinc deficiency and alcohol effects on tumor induction. *Pathobiology* **65**, 39-45.

Nishioka, H. (1975). Mutagenic activities of metal compounds in bacteria. *Mutat. Res*. **31**, 185-189.

Nyquist, R.A., and Kagel, R.O. (1971). Handbook of Infrared and Raman Spectra of Inorganic Compounds and Organic Salts, Vol. 4, *Infrared Spectra of Inorganic Compounds* (*3800-45 cm^{-1}*), pp. 84-85. Academic Press, Inc., San Diego.

Oestreicher, P., and Cousins, R.J. (1985). Copper and zinc absorption in the rat: Mechanism of mutual antagonism. *J. Nutr*. **115**, 159-166.

Ogiso, T., Ogawa, N., and Miura, T. (1979). Inhibitory effect of high dietary zinc on copper absorption in rats. II. Binding of copper and zinc to cytosol proteins in the intestinal mucosa. *Chem. Pharm. Bull*. (Tokyo) **27**, 515-521.

Oishi, S., and Hiraga, K. (1983). Testicular atrophy induced by di(2-ethylhexyl) phthalate: Effect of zinc supplement. *Toxicol. Appl. Pharmacol*. **70**, 43-48.

Olin, K.L., Shigenaga, M.K., Ames, B.N., Golub, M.S., Gershwin, M.E., Hendrickx, A.G., and Keen, C.L. (1993). Maternal dietary zinc influences DNA strand break and 8-hydroxy-2′-deoxyguanosine levels in infant rhesus monkey liver. *Proc. Soc. Exp. Biol. Med*. **203**, 461-466.

Ong, C.N., Chia, S.E., Foo, S.C., Ong, H.Y., Tsakok, M., and Liouw, P. (1993). Concentrations of heavy metals in maternal and umbilical cord blood. *BioMetals* **6**, 61-66.

Organisation for Economic Cooperation and Development (OECD) (2014). OECD Guideline for the Testing of Chemicals. In vivo Mammalian Alkaline Comet Assay. Testing Guideline 489. OECD Publishing, Paris.

Ota, E., Mori, R., Middleton, P., Tobe-Gai, R., Mahomed, K., Miyazaki, C., and Bhutta, Z.A. (2015). Zinc supplementation for improving pregnancy and infant outcome (review). *The Cochrane Library*, Issue 2. The Cochrane Collaboration, John Wiley & Sons, Ltd.

Özkul, Y., Dursun, N., Erenmemişoğlu, A., Süer, C., and Saatçi, Ç. (1996). Effect of zinc deficiency on chromosomal abnormalities in mice. *Tohoku J. Exp. Med*. **179**, 247-251.

Pařízek, J., Boursnell, J.C., Hay, M.F., Babicky, A., and Taylor, D.M. (1966). Zinc in maturing rat testis. *J. Reprod. Fert*. **12**, 501-507.

Patterson, W.P., Winkelman, M., and Perry, M.C. (1985). Zinc-induced copper deficiency: Megamineral sideroblastic anemia. *Ann. Intern. Med*. **103**, 385-386.

Pécoud, A., Donzel, P., and Schelling, J.L. (1975). Effects of foodstuffs on the absorption of zinc sulfate. *Clin. Pharmacol. Ther*. **17**, 469-474.

Peganova, S., and Eder, K. (2004). Zinc. In *Elements and Their Compounds in the Environment*, 2nd ed. (E. Merian, M. Anke, M. Ihnat, and M. Stoeppler, Eds.), pp. 1203-1238. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Pennington, J.A., Young, B.E., Wilson, D.B., Johnson, R.D., and Vanderveen, J.E. (1986). Mineral content of foods and total diets: The selected minerals in foods survey, 1982 to 1984. *J. Am. Diet. Assoc.* **86**, 876-891.

Pfuhler, S., and Wolf, H.U. (1996). Detection of DNA-crosslinking agents with the alkaline comet assay. *Environ. Mol. Mutagen.*, **27**, 196-201.

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

Porter, K.G., McMaster, D., Elmes, M.E., and Love, A.H.G (1977). Anaemia and low serum-copper during zinc therapy. *Lancet* **October 8**, 774.

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

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

Prasad, A.S. (1976). Zinc Deficiency in man. *Am. J. Dis. Child* **130**, 359-361.

Prasad, A.S., Schulert, A.R., Sandstead, H.H., Miale, A.M., Jr., and Farid, Z. (1963). Zinc, iron, and nitrogen content of sweat in normal and deficient subjects. *J. Lab. Clin. Med*. **62**, 84-89.

Prasad, A.S., Brewer, G.J., Schoomaker, E.B., and Rabbani, P. (1978). Hypocupremia induced by zinc therapy in adults. *JAMA* **240**, 2166-2168.

Raghunath, R., Tripathi, R.M., Sastry, V.N., and Krishnamoorthy, T.M. (2000). Heavy metals in maternal and cord blood. *Sci. Total Environ*. **250**, 135-141.

Recio, L., Hobbs, C., Caspary, W., and Witt, K.L. (2010). Dose-response assessment of four genotoxic chemicals in a combined mouse and rat micronucleus (MN) and comet assay protocol. *J. Toxicol. Sci.* **35**, 149-162.

Reeves, P.G. (1995). Adaptation responses in rats to long-term feeding of high-zinc diets: Emphasis on intestinal metallothionein. *J. Nutr. Biochem*. **6**, 48-54.

Reeves, P.G., and O'Dell, B.L. (1981). Zinc deficiency in rats and angiotensin-converting enzyme activity: Comparative effects on lung and testis. *J. Nutr*. **118**, 622-626.

Reeves, P.G., Nielsen, F.H., and Fahey, G.C., Jr. (1993). AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **123**, 1939-1951.

Reinhold, J.G., Faradji, B., Abadi, P., and Ismail-Beigi, F. (1976). Decreased absorption of calcium, magnesium, zinc, and phosphorous by humans due to increased fiber and phosphorous consumption as wheat bread. *J. Nutr.* **106**, 493-503.

Richards, M.P., and Cousins, R.J. (1975). Mammalian zinc homeostasis: Requirement for RNA and metallothionein synthesis. *Biochem. Biophys. Res. Commun*. **64**, 1215-1223.

Rivlin, R.S. (1983). Misuse of hair analysis for nutritional assessment. *Am. J. Med*. **75**, 489-493.

Rosado, J.L. (2003). Zinc and copper: Proposed fortification levels and recommended zinc compounds. *J. Nutr*. **133**, 2985S-2989S.

Rossowska, M.J., and Nakamoto, T. (1992). Caffeine decreases zinc and metallothionein levels in heart of newborn and adult rats. *Pediatr. Res*. **32**, 330-332.

Saltzman, B.E., Gross, S.B., Yeager, D.W., Meiners, B.G., and Gartside, P.S. (1990). Total body burdens and tissue concentrations of lead, cadmium, copper, zinc, and ash in 55 human cadavers. *Environ. Res*. **52**, 126-145.

Salzman, M.B., Smith, E.M., and Koo, C. (2002). Excessive oral zinc supplementation. *J. Pediatr. Hematol. Oncol*. **24**, 582-584.

Samman, S., and Roberts, D.C.K. (1987). The effect of zinc supplements on plasma zinc and copper levels and the reported symptoms in healthy volunteers. *Med. J. Aust.* **146**, 246-249.

Samman, S., and Roberts, D.C.K. (1988). The effect of zinc supplements on lipoproteins and copper status. *Atherosclerosis* **70**, 247-252.

Sandstead, H.H. (1973). Zinc nutrition in the United States. *Am. J. Clin. Nutr*. **26**, 1251-1260.

Sandstead, H.H. (1981). Zinc in human nutrition. In *Disorders of Mineral Metabolism. Vol. 1 Trace Minerals* (F. Bronner and J.W. Coburn, Eds.), pp. 93-157. Academic Press, Inc., New York.

Sandström, B., and Abrahamson, H. (1989). Zinc absorption and achlorhydria. *Eur. J. Clin. Nutr*. **43**, 877-879.

Sandström, B., and Cederblad, A. (1980). Zinc absorption from composite meals. II. Influence of the main protein source. *Am. J. Clin. Nutr.* **33**, 1778-1783.

Sandström, B., and Sandberg, A.S. (1992). Inhibitory effects of isolated inositol phosphates on zinc absorption in humans. *J. Trace Elem. Electrolytes Health Dis.* **6**, 99-103.

Sankako, M.K., Garcia, P.C., Piffer, R.C., Dallaqua, B., Damasceno, D.C., and Pereira, O.C.M. (2012). Possible mechanism by which zinc protects the testicular function of rats exposed to cigarette smoke. *Pharmacol. Rep*. **64**, 1537-1546.

Sasaki, Y.F., Sekihashi, K., Izumiyama, F., Nishidate, E., Saga, A., Ishida, K., and Tsuda, S. (2000). The comet assay with multiple mouse organs: Comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database. *Crit. Rev. Toxicol*. **30**, 629-799.

Sato, F., Watanabe, T., Hoshi, E., and Endo, A. (1985). Teratogenic effect of maternal zinc deficiency and its co-teratogenic effect with cadmium. *Teratology* **31**, 13-18.

Schiffer, R.B., Sunderman, F.W., Jr., Baggs, R.B., and Moynihan, J.A. (1991). The effects of exposure to dietary nickel and zinc upon humoral and cellular immunity in SJL mice. *J. Neuroimmunol*. **34**, 229-239.

Schlicker, S.A., and Cox, D.H. (1968). Maternal dietary zinc, and development and zinc, iron, and copper content of the rat fetus. *J. Nutr.* **95**, 287-294.

Schmid, W. (1975). The micronucleus test. *Mutat. Res.* **31**, 9-15.

Schrager, T.F., Busby, W.F. Jr., Goldman, M.E., and Newberne, P.M. (1986). Enhancement of methylbenzylnitrosamine-induced esophageal carcin-ogenesis in zinc-deficient rats: Effects on incorporation of [^{3}H]thymidine into DNA of esophageal epithelium and liver. *Carcinogenesis* **7**, 1121-1126.

Schroeder, H.A., Nason, A.P., Tipton, I.H., and Balassa, J.J. (1967). Essential trace metals in man: Zinc. Relation to environmental cadmium. *J. Chronic Dis*. **20**, 179-210.

Science, M., Johnstone, J., Roth, D.E., Guyatt, G., and Loeb, M. (2012). Zinc for treatment of the common cold: A systematic review and meta-analysis of randomized controlled trials. *CMAJ* **184**, E551-E561.

Seal, C.J., and Heaton, F.W. (1983). Chemical factors affecting the intestinal absorption of zinc *in vitro* and *in vivo*. *Br. J. Nutr.* **50**, 317-324.

Shacklette, H.T., and Boerngen, J.G. (1984). Element Concentrations in Soils and Other Surficial Materials of the Conterminous United States. U.S. Geological Survey Professional Paper 1270, United States Department of the Interior. United States Government Printing Office, Washington, DC.

Sharif, R., Thomas, P., Zalewski, P., Graham, R.D., and Fenech, M. (2011). The effect of zinc sulphate and zinc carnosine on genome stability and cytotoxicity in the WIL2-NS human lymphoblastoid cell line. *Mutat. Res.* **720**, 22-33.

Sharif, R., Thomas, P., Zalewski, P., and Fenech, M. (2012). Zinc deficiency or excess within the physiological range increases genome instability and cytotoxicity, respectively, in human oral keratinocyte cells. *Genes Nutr*. **7**, 139-154.

Shirley, E. (1977). A non-parametric equivalent of Williams' test for contrasting increasing dose levels of a treatment. *Biometrics* **33**, 386-389.

Simmer, K., and Thompson, R.P.H. (1985). Zinc in the fetus and newborn. *Acta. Paediatr. Scand. Suppl*. **319**, 158-163.

Simpson, J.L., Bailey, L.B., Pietrzik, K., Shane, B., and Holzgreve, W. (2011). Micronutrients and women of reproductive potential: Required dietary intake and consequences of dietary deficiency or excess. Part II – vitamin D, vitamin A, iron, zinc, iodine, essential fatty acids. *J. Matern. Fetal Neonatal. Med*. **24**, 1-24.

Smith, P.A., Sunter, J.P., and Case, R.M. (1982). Progressive atrophy of pancreatic acinar tissue in rats fed a copper-deficient diet supplemented with *D*-penicillamine or triethylene tetramine: Morphological and physiological studies. *Digestion* **23**, 16-30.

Soltan, M.H., and Jenkins, D.M. (1982). Maternal and fetal plasma zinc concentration and fetal abnormality. *Br. J. Obstetr. Gynaecol.* **89**, 56-58.

Song, Y., Leonard, S.W., Traber, M.G., and Ho, E. (2009a). Zinc deficiency affects DNA damage, oxidative stress, antioxidant defenses, and DNA repair in rats. *J. Nutr.* **139**, 1626-1631.

Song, Y., Chung, C.S., Bruno, R.S., Traber, M.G., Brown, K.H., King, J.C., and Ho, E. (2009b). Dietary zinc restriction and repletion affects DNA integrity in healthy men. *Am. J. Clin. Nutr*. **90**, 321-328.

Spencer, H., Osis, D., Kramer, L., and Norris, C. (1976). Intake, excretion, and retention of zinc in man. In *Trace Elements in Human Health and Disease. Vol. 1. Zinc and Copper* (A.S. Prasad and D. Oberleas, Eds.), pp. 345-361. Academic Press, New York.

Spencer, H., Kramer, L., and Osis, D. (1985). Zinc metabolism in man. *J. Environ. Pathol. Toxicol. Oncol*. **5**, 265-278.

Srivastava, A., and Setty, B.S. (1985). The distribution of zinc in the subcellular fractions of the Rhesus Monkey testis. *Biol. Trace Elem. Res*. **7**, 83-87.

Straube, E.F., Schuster, N.H., and Sinclair, A.J. (1980). Zinc toxicity in the ferret. *J. Comp. Pathol.* **90**, 355-361.

Straus, D.S. (1981). Somatic mutation, cellular differentiation, and cancer causation. *JNCI* **67**, 233-241.

Stroud, S. (1991). Too much zinc has a domino effect. *Am. J. Nurs*. **91**, 61.

Sturniolo, G.C., Montino, M.C., Rossetto, L., Martin, A., D'Inca, R., D'Odorico, A., and Naccarato, R. (1991). Inhibition of gastric acid secretion reduces zinc absorption in man. *J. Am. Coll. Nutr.* **10**, 372-375.

Summerfield, A.L., Steinberg, F.U., and Gonzalez, J.G. (1992). Morphological findings in bone marrow precursor cells in zinc induced copper deficiency anemia. *Am. J. Clin. Pathol*. **97**, 665-668.

Sutomo, F.X., Woutersen, R.A., and Van den Hamer, C.J.A. (1992). Effects of elevated zinc intake on the copper metabolism and the pancreas of the mouse. *J. Trace Elem. Electrolytes Health Dis.* **6**, 75-80.

Swenerton, H., and Hurley, L.S. (1968). Severe zinc deficiency in male and female rats. *J. Nutr*. **95**, 8-18.

Tacnet, F., Watkins, D.W., and Ripoche, P. (1990). Studies of zinc transport into brush-border membrane vesicles isolated from pig small intestine. *Biochim. Biophys. Acta* **1024**, 323-330.

Takagi, Y., Matsuda, S., Imai, S., Ohmori, Y., Masuda, T., Vinson, J.A., Mehra, M.C., Puri, B.K., and Kaniewski, A. (1988). Survey of trace elements in human nails: An international comparison. *Bull. Environ. Contam. Toxicol*. **41**, 690-695.

Tamura, T., Goldenberg, R.L., Johnston, K.E., and DuBard, M. (2000). Maternal plasma zinc concentrations and pregnancy outcome. *Am. J. Clin. Nutr.* **71**, 109-113.

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

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C., and Sasaki, Y.F. (2000). Single cell gel/Comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.* **35**, 206-221.

Torous, D.K., Hall, N.E., Illi-Love, A.H., Diehl, M.S., Cederbrant, K., Sandelin, K., Pontén, I., Bolcsfoldi, G., Ferguson, L.R., Pearson, A., Majeska, J.B., Tarca, J.P., Hynes, G.M., Lynch, A.M., McNamee, J.P., Bellier, P.V., Parenteau, M., Blakey, D., Bayley, J., van der Leede, B.J., Vanparys, P., Harbach, P.R., Zhao, S., Filipunas, A.L., Johnson, C.W., Tometsko, C.R., and Dertinger, S.D. (2005). Interlaboratory validation of a CD71-based flow cytometric method (Microflow) for the scoring of micronucleated reticulocytes in mouse peripheral blood. *Environ. Mol. Mutagen.* **45**, 44-55.

Underwood, E.J. (1977). Zinc. In *Trace Elements in Human and Animal Nutrition*, 4th ed., pp. 196-242. Academic Press, Inc., Cambridge, MA.

Underwood, E.J., and Somers, M. (1969). Studies of zinc nutrition in sheep. I. The relation of zinc to growth, testicular development, and spermatogenesis in young rams. *Aust. J. Agric. Res*. **20**, 889-897.

United States Environmental Protection Agency (USEPA) (1980). Exposure and Risk Assessment for Zinc. EPA440481016. PB85212009. USEPA, Office of Water Regulations and Standards (WH-553), Washington, DC.

United States Environmental Protection Agency (USEPA) (1987). Ambient Water Quality Criteria for Zinc─1987. EPA440587003. PB87153581. USEPA, Office of Water Regulations and Standards, Washington, DC.

United States Geological Survey (USGS) (2002). Trace Elements and Organic Compounds in Streambed Sediment and Fish Tissue of Coastal New England Streams, 1998-99. Water-Resources Investigations Report 02-4179. USGS, Denver, CO.

United States Geological Survey (USGS) (2016). Zinc. Mineral Commodity Summaries. <http: //minerals.usgs.gov/minerals/pubs/commodity/zinc/mcs-2016-zinc.pdf> Accessed September 26, 2016.

Van Campen, D.R., and Scaife, P.U. (1967). Zinc interference with copper absorption in rats. *J. Nutr*. **91**, 473-476.

Van Rensburg, S.J. (1981). Epidemiologic and dietary evidence for a specific nutritional predisposition to esophageal cancer. *JNCI* **67**, 243-251.

Verburg, D.J., Burd, L.I., Hoxtell E.O., and Merill, L.K. (1974). Acrodermatitis enteropathica and pregnancy. *Obstet. Gynecol*. **44**, 233-237.

Waalkes, M.P., and Rehm, S. (1992). Carcinogenicity of oral cadmium in the male Wistar (WF/NCr) Rat: Effect of chronic dietary zinc deficiency. *Fundam. Appl. Toxicol*. **19**, 512-520.

Walsh, C.T., Sandstead, H.H., Prasad, A.S., Newberne, P.M., and Fraker, P.J. (1994). Zinc: Health effects and research priorities for the 1990s. *Environ. Health Perspect.* **102**, 5-46.

Walters, M., and Roe, F.J.C. (1965). A study of the effects of zinc and tin administered orally to mice over a prolonged period. *Food Cosmet. Toxicol.* **3**, 271-276.

Wapnir, R.A., and Balkman, C. (1991). Inhibition of copper absorption by zinc. *Biol. Trace Elem. Res.* **29**, 193-202.

Wapnir, R.A., and Stiel, L. (1986). Zinc intestinal absorption in rats: Specificity of amino acids as ligands. *J. Nutr*. **116**, 2171-2179.

Warkany, J., and Petering, H.G. (1972). Congenital malformation of the central nervous system in rats produced by maternal zinc deficiency. *Teratology* **5**, 319-334.

Wastney, M.E., Aamodt, R.L., Rumble, W.F., and Henkin, R.I. (1986). Kinetic analysis of zinc metabolism and its regulation in normal humans. *Am. J. Physiol*. **251**, R398-R408.

Watanabe, T., and Endo, A. (1997). Cytogenetic effects of cadmium on unfertilized oocytes in short-term zinc deficiency in hamsters. *Mutat. Res*. **395**, 113-118.

Wilhelm, M., Hafner, D., Lombeck, I., and Ohnesorge, F.K. (1991). Monitoring of cadmium, copper, lead and zinc status in young children using toenails: Comparison with scalp hair. *Sci. Total Environ*. **103**, 199-207.

Williams, D.A. (1971). A test for differences between treatment means when several dose levels are compared with a zero dose control. *Biometrics* **27**, 103-117.

Williams, D.A. (1972). The comparison of several dose levels with a zero dose control. *Biometrics* **28**, 519-531.

Williams, D.A. (1986). A note on Shirley's nonparametric test for comparing several dose levels with a zero-dose control. *Biometrics* **42**, 183-186.

Willis, M.S., Monaghan, S.A., Miller, M.L., McKenna, R.W., Perkins, W.D., Levinson, B.S., Bhushan, V., and Kroft, S.H. (2005). Zinc-induced copper deficiency: A report of three cases initially recognized on bone marrow examination. *Am. J. Clin. Pathol*. **123**, 125-131.

Witt, K.L., Knapton, A., Wehr, C.M., Hook, G.J., Mirsalis, J., Shelby, M.D., and MacGregor, J.T. (2000). Micronucleated erythrocyte frequency in peripheral blood of B6C3F1 mice from short-term, prechronic, and chronic studies of the NTP Carcinogenesis Bioassay Program. *Environ. Mol. Mutagen*. **36**, 163-194.

Witt, K.L., Livanos, E., Kissling, G.E., Torous, D.K., Caspary, W., Tice, R.R., and Recio, L. (2008). Comparison of flow cytometry- and microscopy-based methods for measuring micronucleated reticulocyte frequencies in rodents treated with nongenotoxic and genotoxic chemicals. *Mutat. Res.* **649**, 101-113.

World Health Organization (WHO) (2001). Zinc. Environmental Health Criteria 221. WHO, Geneva. <http://www.inchem.org/documents/ehc/ehc/ehc221.htm> Accessed February 26, 2016.

Xu, B., Chia, S.-E., and Ong, C.-N. (1994). Concentrations of cadmium, lead, selenium, and zinc in human blood and seminal plasma. *Biol. Trace Elem. Res.* **40**, 49-57.

Yadrick, M.K., Kenney, M.A., and Winterfeldt, E.A. (1989). Iron, copper, and zinc status: Response to supplementation with zinc or zinc and iron in adult females. *Am. J. Clin. Nutr*. **49**, 145-150.

Yan, M., Song, Y., Wong, C.P., Hardin, K., and Ho, E. (2008). Zinc deficiency alters DNA damage response genes in normal human prostate epithelial cells. *J. Nutr.* **138**, 667-673.

Yang, C.S. (1980). Research on esophageal cancer in China: A review. *Cancer Res*. **40**, 2633-2644.

APPENDIX A
SUMMARY OF LESIONS IN MALE RATS
IN THE 2-YEAR FEED STUDY
OF DIETARY ZINC

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of Dietary Zinc[a]

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Disposition Summary					
Animals initially in study	60	60	60	60	60
Early deaths					
Special study animals	10	10	9	10	10
Moribund	11	12	10	15	7
Natural deaths	19	7	13	14	22
Survivors					
Died last week of study	2		1		1
Terminal euthanasia	18	31	27	21	20
Animals examined microscopically	50	50	50	50	50
Alimentary System					
Esophagus	(50)	(50)	(50)	(50)	(50)
Intestine large, cecum	(50)	(50)	(50)	(50)	(47)
Intestine, large, colon	(49)	(50)	(49)	(50)	(48)
Intestine large, rectum	(50)	(50)	(50)	(50)	(48)
Adenocarcinoma			1 (2%)		
Intestine small, duodenum	(50)	(49)	(50)	(49)	(48)
Adenocarcinoma	1 (2%)				
Intestine small, ileum	(50)	(49)	(48)	(50)	(45)
Intestine small, jejunum	(50)	(49)	(50)	(50)	(47)
Adenocarcinoma	1 (2%)		1 (2%)	1 (2%)	3 (6%)
Schwannoma malignant, metastatic, skin			1 (2%)		
Liver	(50)	(50)	(50)	(50)	(46)
Pheochromocytoma malignant, metastatic, adrenal medulla				1 (2%)	
Mesentery	(0)	(1)	(0)	(0)	(0)
Paraganglioma		1 (100%)			
Oral mucosa	(1)	(0)	(2)	(1)	(1)
Squamous cell carcinoma				1 (100%)	
Pancreas	(49)	(50)	(48)	(48)	(48)
Schwannoma malignant, metastatic, skin			1 (2%)		
Acinus, adenoma	6 (12%)	8 (16%)	9 (19%)	5 (10%)	6 (13%)
Acinus, adenoma, multiple	5 (10%)	13 (26%)	10 (21%)	8 (17%)	4 (8%)
Acinus, carcinoma	1 (2%)				
Salivary glands	(49)	(50)	(50)	(50)	(49)
Stomach, forestomach	(50)	(50)	(50)	(50)	(49)
Squamous cell carcinoma		2 (4%)			
Stomach, glandular	(50)	(50)	(50)	(50)	(49)
Tongue	(0)	(0)	(0)	(0)	(1)
Tooth	(3)	(0)	(1)	(0)	(2)
Cardiovascular System					
Blood vessel	(50)	(50)	(50)	(50)	(50)
Schwannoma malignant, metastatic, skin			1 (2%)		
Heart	(50)	(50)	(50)	(50)	(50)
Schwannoma malignant, metastatic, skin			1 (2%)		
Endocardium, schwannoma malignant		1 (2%)	2 (4%)	1 (2%)	
Epicardium, paraganglioma		1 (2%)			

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Endocrine System					
Adrenal cortex	(50)	(50)	(50)	(50)	(50)
Adrenal medulla	(50)	(50)	(50)	(50)	(50)
Pheochromocytoma benign	5 (10%)	3 (6%)	8 (16%)	10 (20%)	3 (6%)
Pheochromocytoma complex		1 (2%)			
Pheochromocytoma malignant	1 (2%)			1 (2%)	3 (6%)
Bilateral, pheochromocytoma benign			2 (4%)		
Bilateral, pheochromocytoma malignant		1 (2%)	1 (2%)		
Islets, pancreatic	(50)	(50)	(50)	(49)	(49)
Adenoma	2 (4%)	6 (12%)	2 (4%)	2 (4%)	2 (4%)
Carcinoma	1 (2%)		1 (2%)	1 (2%)	1 (2%)
Parathyroid gland	(47)	(41)	(44)	(40)	(42)
Pituitary gland	(50)	(50)	(50)	(50)	(49)
Schwannoma malignant, metastatic, peripheral nerve	1 (2%)				
Pars distalis, adenoma	2 (4%)	8 (16%)	4 (8%)	7 (14%)	6 (12%)
Pars distalis, carcinoma	1 (2%)				
Pars intermedia, adenoma			2 (4%)		
Pars intermedia, carcinoma	1 (2%)				
Thyroid gland	(50)	(50)	(50)	(50)	(49)
Bilateral, C-cell, adenoma			1 (2%)		
Bilateral, C-cell, carcinoma	1 (2%)				
C-cell, adenoma	2 (4%)	5 (10%)		2 (4%)	2 (4%)
C-cell, carcinoma		2 (4%)	3 (6%)	2 (4%)	1 (2%)
General Body System					
None					
Genital System					
Epididymis	(50)	(50)	(50)	(50)	(50)
Penis	(0)	(0)	(1)	(0)	(0)
Preputial gland	(50)	(50)	(50)	(50)	(50)
Carcinoma				1 (2%)	
Prostate	(50)	(50)	(50)	(50)	(50)
Seminal vesicle	(50)	(50)	(50)	(50)	(50)
Testes	(50)	(50)	(50)	(50)	(50)
Interstitial cell, adenoma		2 (4%)			
Hematopoietic System					
Bone marrow	(50)	(50)	(50)	(50)	(50)
Lymph node	(6)	(1)	(4)	(3)	(2)
Lymph node, mandibular	(49)	(50)	(50)	(50)	(48)
Lymph node, mesenteric	(50)	(50)	(49)	(50)	(49)
Schwannoma malignant, metastatic, skin			1 (2%)		
Spleen	(50)	(50)	(49)	(48)	(45)
Thymus	(47)	(49)	(50)	(47)	(58)
Schwannoma malignant, metastatic, skin			1 (2%)		
Integumentary System					
Mammary gland	(50)	(49)	(50)	(48)	(49)
Fibroadenoma	3 (6%)	3 (6%)		1 (2%)	1 (2%)
Fibroma			2 (4%)	1 (2%)	1 (2%)
Schwannoma malignant		1 (2%)	1 (2%)		

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Integumentary System (continued)					
Skin	(50)	(50)	(50)	(50)	(50)
Basal cell adenoma				1 (2%)	
Basal cell carcinoma	1 (2%)		1 (2%)		1 (2%)
Keratoacanthoma	3 (6%)		2 (4%)		1 (2%)
Schwannoma malignant			1 (2%)		
Schwannoma malignant, metastatic, peripheral nerve	1 (2%)				
Squamous cell carcinoma					1 (2%)
Trichoepithelioma	1 (2%)				
Head, neural crest tumor	1 (2%)				
Sebaceous gland, adenoma			1 (2%)		
Subcutaneous tissue, carcinoma, metastatic, thyroid gland			1 (2%)		
Subcutaneous tissue, fibroma	2 (4%)	4 (8%)	3 (6%)	1 (2%)	
Subcutaneous tissue, fibrosarcoma	1 (2%)	1 (2%)			
Subcutaneous tissue, fibrosarcoma, multiple		1 (2%)			
Subcutaneous tissue, hemangioma	1 (2%)				
Subcutaneous tissue, hemangiosarcoma	1 (2%)				
Subcutaneous tissue, lipoma		2 (4%)		1 (2%)	1 (2%)
Subcutaneous tissue, schwannoma malignant				1 (2%)	
Tail, papilloma			1 (2%)		
Musculoskeletal System					
Bone	(50)	(50)	(50)	(50)	(50)
Cranium, schwannoma malignant, metastatic, peripheral nerve	1 (2%)				
Femur, osteosarcoma					1 (2%)
Humerus, osteosarcoma				1 (2%)	
Skeletal muscle	(0)	(0)	(1)	(2)	(0)
Rhabdomyosarcoma				1 (50%)	
Schwannoma malignant			1 (100%)	1 (50%)	
Nervous System					
Brain	(48)	(50)	(50)	(50)	(50)
Granular cell tumor malignant				1 (2%)	
Schwannoma malignant, metastatic, peripheral nerve	1 (2%)				
Cerebrum, astrocytoma malignant		1 (2%)			
Cerebrum, meningioma malignant				1 (2%)	
Cerebrum, oligodendroglioma malignant		1 (2%)		1 (2%)	
Peripheral nerve	(1)	(1)	(1)	(1)	(0)
Schwannoma malignant	1 (100%)				
Spinal cord	(0)	(1)	(1)	(1)	(0)

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Respiratory System					
Lung	(50)	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma		1 (2%)			
Carcinoma, metastatic, thyroid gland			1 (2%)		
Cystic keratinizing epithelioma			1 (2%)		
Neuroblastoma, metastatic, nose			1 (2%)		
Osteosarcoma, metastatic, bone					1 (2%)
Schwannoma malignant, metastatic, skin			1 (2%)		
Nose	(50)	(50)	(50)	(50)	(49)
Schwannoma malignant, metastatic, peripheral nerve	1 (2%)				
Olfactory epithelium, neuroblastoma			1 (2%)		
Trachea	(50)	(50)	(50)	(50)	(50)
Special Senses System					
Eye	(50)	(49)	(50)	(50)	(49)
Optic nerve, schwannoma malignant				1 (2%)	
Harderian gland	(50)	(49)	(50)	(50)	(49)
Schwannoma malignant, metastatic, peripheral nerve	1 (2%)				
Zymbal's gland	(1)	(0)	(0)	(0)	(0)
Carcinoma	1 (100%)				
Urinary System					
Kidney	(50)	(50)	(50)	(50)	(50)
Schwannoma malignant, metastatic, skin			1 (2%)		
Renal tubule, adenoma				1 (2%)	1 (2%)
Urinary bladder	(50)	(50)	(50)	(50)	(49)
Systemic Lesions					
Multiple organs[b]	(50)	(50)	(50)	(50)	(50)
Histiocytic sarcoma	1 (2%)				
Leukemia mononuclear	1 (2%)	2 (4%)		1 (2%)	1 (2%)
Lymphoma malignant	1 (2%)	1 (2%)	1 (2%)	3 (6%)	
Mesothelioma malignant	1 (2%)				
Neoplasm Summary					
Total animals with primary neoplasms[c]	32	38	39	35	28
Total primary neoplasms	50	72	63	60	40
Total animals with benign neoplasms	22	36	36	29	22
Total benign neoplasms	32	57	48	40	28
Total animals with malignant neoplasms	16	15	14	17	12
Total malignant neoplasms	17	15	15	20	12
Total animals with metastatic neoplasms	1		4	1	1
Total metastatic neoplasms	6		11	1	1
Total animals with uncertain neoplasms-benign or malignant	1				
Total uncertain neoplasms	1				

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

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Adrenal Medulla: Benign Pheochromocytoma					
Overall rate[a]	5/50 (10%)	3/50 (6%)	10/50 (20%)	10/50 (20%)	3/50 (6%)
Adjusted rate[b]	12.8%	6.9%	23.7%	25.0%	7.6%
Terminal rate[c]	2/18 (11%)	3/31 (10%)	6/27 (22%)	6/21 (29%)	2/20 (10%)
First incidence (days)	652	734 (T)	659	363	715
Poly-3 test[d]	P=0.230N	P=0.298N	P=0.160		
Poly-3 test[e]	P=0.311N			P=0.133	P=0.351N
Adrenal Medulla: Malignant Pheochromocytoma					
Overall rate	1/50 (2%)	1/50 (2%)	1/50 (2%)	1/50 (2%)	3/50 (6%)
Adjusted rate	2.6%	2.3%	2.4%	2.6%	7.6%
Terminal rate	1/18 (6%)	1/31 (3%)	1/27 (4%)	0/21 (0%)	1/20 (5%)
First incidence (days)	734 (T)	734 (T)	734 (T)	713	669
Poly-3 test	—[f]	—	—		
Poly-3 test	P=0.209			P=0.760N	P=0.315
Adrenal Medulla: Benign, Complex, or Malignant Pheochromocytoma					
Overall rate	6/50 (12%)	5/50 (10%)	11/50 (22%)	11/50 (22%)	5/50 (10%)
Adjusted rate	15.4%	11.5%	26.1%	27.5%	12.6%
Terminal rate	3/18 (17%)	5/31 (16%)	7/27 (26%)	6/21 (29%)	2/20 (10%)
First incidence (days)	652	734 (T)	659	363	669
Poly-3 test	P=0.336N	P=0.424N	P=0.177		
Poly-3 test	P=0.427N			P=0.146	P=0.490N
Small Intestine (Jejunum): Carcinoma					
Overall rate	1/50 (2%)	0/50 (0%)	1/50 (2%)	1/50 (2%)	3/50 (6%)
Adjusted rate	2.6%	0.0%	2.4%	2.6%	7.6%
Terminal rate	0/18 (0%)	0/31 (0%)	0/27 (0%)	0/21 (0%)	0/20 (0%)
First incidence (days)	586	—[g]	670	680	715
Poly-3 test	—	—	—		
Poly-3 test	P=0.204			P=0.760	P=0.309
Small Intestine (Duodenum or Jejunum): Carcinoma					
Overall rate	2/50 (4%)	0/50 (0%)	1/50 (2%)	1/50 (2%)	3/50 (6%)
Adjusted rate	5.1%	0.0%	2.4%	2.6%	7.6%
Terminal rate	1/18 (6%)	0/31 (0%)	0/27 (0%)	0/21 (0%)	0/20 (0%)
First incidence (days)	586	—	670	680	715
Poly-3 test	P=0.129N	P=0.213N	P=0.475N		
Poly-3 test	P=0.404			P=0.502N	P=0.506
Mammary Gland: Fibroadenoma					
Overall rate	3/50 (6%)	3/50 (6%)	0/50 (0%)	1/50 (2%)	1/50 (2%)
Adjusted rate	7.7%	6.9%	0%	2.6%	2.5%
Terminal rate	0/18 (0%)	1/31 (3%)	0/27 (0%)	1/21 (5%)	0/20 (0%)
First incidence (days)	638	698	—	734 (T)	541
Poly-3 test	P=0.585N	P=0.611N	P=0.108N		
Poly-3 test	P=198N			P=0.310N	P=0.298N
Mammary Gland: Fibroma or Fibroadenoma					
Overall rate	3/50 (6%)	3/50 (6%)	2/50 (4%)	2/50 (4%)	2/50 (4%)
Adjusted rate	7.7%	6.9%	4.8%	5.2%	5.0%
Terminal rate	0/18 (0%)	1/31 (3%)	1/27 (4%)	2/21 (10%)	1/20 (5%)
First incidence (days)	638	698	660	734 (T)	541
Poly-3 test	P=0.548N	P=0.611N	P=0.471N		
Poly-3 test	P=0.400N			P=0.508N	P=0.492N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Pancreas: Adenoma					
Overall rate	11/49 (22%)[h]	21/50 (42%)	19/48 (40%)	13/48 (27%)	10/48 (21%)
Adjusted rate	28.4%	46.5%	46.5%	34.7%	26.1%
Terminal rate	8/18 (44%)	14/31 (45%)	16/27 (59%)	11/21 (52%)	9/20 (45%)
First incidence (days)	652	467	701	648	729
Poly-3 test	P=0.064	P=0.065	P=0.069		
Poly-3 test	P=0.462N			P=0.363	P=0.512N
Pancreatic Islets: Adenoma					
Overall rate	2/50 (4%)	6/50 (12%)	2/50 (4%)	2/49 (4%)	2/49 (4%)
Adjusted rate	5.2%	13.7%	4.8%	5.2%	5.1%
Terminal rate	2/18 (11%)	5/31 (16%)	1/27 (4%)	1/21 (5%)	1/20 (5%)
First incidence (days)	734 (T)	663	641	551	717
Poly-3 test	P=0.103	P=0.178	P=0.663N		
Poly-3 test	P=0.594N			P=0.694	P=0.688N
Pancreatic Islets: Adenoma or Carcinoma					
Overall rate	3/50 (6%)	6/50 (12%)	3/50 (6%)	3/49 (6%)	3/49 (6%)
Adjusted rate	7.8%	13.7%	7.2%	7.8%	7.7%
Terminal rate	2/18 (11%)	5/31 (16%)	2/27 (7%)	1/21 (5%)	2/20 (10%)
First incidence (days)	656	663	641	551	717
Poly-3 test	P=0.224	P=0.306	P=0.627N		
Poly-3 test	P=0.578N			P=0.660	P=0.658N
Pituitary Gland (Pars Distalis): Adenoma					
Overall rate	2/50 (4%)	8/50 (16%)	4/50 (8%)	7/50 (14%)	6/49 (12%)
Adjusted rate	5.2%	18.3%	9.2%	17.7%	15.2%
Terminal rate	1/18 (6%)	7/31 (23%)	1/27 (4%)	2/21 (10%)	2/20 (10%)
First incidence (days)	673	687	406	461	645
Poly-3 test	P=0.042	P=0.067	P=0.391		
Poly-3 test	P=0.128			P=0.082	P=0.138
Pituitary Gland (Pars Distalis): Adenoma or Carcinoma					
Overall rate	3/50 (6%)	8/50 (16%)	4/50 (8%)	7/50 (14%)	6/49 (12%)
Adjusted rate	7.6%	18.3%	9.2%	17.7%	15.2%
Terminal rate	1/18 (6%)	7/31 (23%)	1/27 (4%)	2/21 (10%)	2/20 (10%)
First incidence (days)	488	687	406	461	645
Poly-3 test	P=0.087	P=0.133	P=0.552		
Poly-3 test	P=0.209			P=0.156	P=0.242
Skin: Keratoacanthoma					
Overall rate	3/50 (6%)	0/50 (0%)	2/50 (4%)	0/50 (0%)	1/50 (2%)
Adjusted rate	7.8%	0.0%	4.8%	0.0%	2.5%
Terminal rate	2/18 (11%)	0/31 (0%)	2/27 (7%)	0/21 (0%)	0/20 (0%)
First incidence (days)	701	—	734 (T)	—	707
Poly-3 test	P=0.066N	P=0.098N	P=0.466N		
Poly-3 test	P=0.172N			P=0.118N	P=0.296N
Skin: Keratoacanthoma or Squamous Cell Carcinoma					
Overall rate	3/50 (6%)	0/50 (0%)	2/50 (4%)	0/50 (0%)	2/50 (4%)
Adjusted rate	7.8%	0.0%	4.8%	0.0%	5.0%
Terminal rate	2/18 (11%)	0/31 (0%)	2/27 (7%)	0/21 (0%)	0/20 (0%)
First incidence (days)	701	—	734 (T)	—	649
Poly-3 test	P=0.066N	P=0.098N	P=0.466N		
Poly-3 test	P=0.386N			P=0.118N	P=0.487N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Skin: Keratoacanthoma, Trichoepithelioma, Basal Cell Adenoma, Basal Cell Carcinoma, or Squamous Cell Carcinoma					
Overall rate	5/50 (10%)	0/50 (0%)	3/50 (6%)	1/50 (2%)	3/50 (6%)
Adjusted rate	12.8%	0.0%	7.2%	2.6%	7.5%
Terminal rate	3/18 (17%)	0/31 (0%)	3/27 (11%)	0/21 (0%)	0/20 (0%)
First incidence (days)	586	—	734 (T)	713	649
Poly-3 test	P=0.015N	P=0.022N	P=0.321N		
Poly-3 test	P=0.255N			P=0.102N	P=0.342N
Skin (Subcutaneous Tissue): Fibroma					
Overall rate	2/50 (4%)	4/50 (8%)	3/50 (6%)	1/50 (2%)	0/50 (0%)
Adjusted rate	5.2%	9.0%	7.2%	2.6%	0.0%
Terminal rate	1/18 (6%)	1/31 (3%)	1/27 (4%)	1/21 (5%)	0/20 (0%)
First incidence (days)	701	504	660	734 (T)	—
Poly-3 test	P=0.0.334	P=0.407	P=0.539		
Poly-3 test	P=0.139N			P=0.500N	P=0.232N
Skin (Subcutaneous Tissue): Fibroma or Fibrosarcoma					
Overall rate	3/50 (6%)	6/50 (12%)	3/50 (6%)	1/50 (2%)	0/50 (0%)
Adjusted rate	7.7%	13.2%	7.2%	2.6%	0.0%
Terminal rate	1/18 (6%)	1/31 (3%)	1/27 (4%)	1/21 (5%)	0/20 (0%)
First incidence (days)	538	504	660	734 (T)	—
Poly-3 test	P=0.238	P=0.322	P=0.631N		
Poly-3 test	P=0.059N			P=0.310N	P=0.117N
Thyroid Gland (C-Cell): Adenoma					
Overall rate	2/50 (4%)	5/50 (10%)	1/50 (2%)	2/50 (4%)	2/49 (4%)
Adjusted rate	5.2%	11.1%	2.4%	5.2%	5.2%
Terminal rate	1/18 (6%)	1/31 (3%)	0/27 (0%)	2/21 (10%)	1/20 (5%)
First incidence (days)	611	505	728	734 (T)	597
Poly-3 test	P=0.164	P=0.281	P=0.477N		
Poly-3 test	P=0.602N			P=0.692	P=0.695N
Thyroid Gland (C-Cell): Carcinoma					
Overall rate	1/50 (2%)	2/50 (4%)	3/50 (6%)	2/50 (4%)	1/49 (2%)
Adjusted rate	2.6%	4.6%	7.2%	5.2%	2.6%
Terminal rate	0/18 (0%)	1/31 (3%)	3/27 (11%)	2/21 (10%)	1/20 (5%)
First incidence (days)	733	686	734 (T)	734 (T)	734 (T)
Poly-3 test	P=0.471	P=0.545	P=0.333		
Poly-3 test	P=0.622N			P=0.501	P=0.761
Thyroid Gland (C-Cell): Adenoma or Carcinoma					
Overall rate	3/50 (6%)	7/50 (14%)	4/50 (8%)	3/50 (6%)	3/49 (6%)
Adjusted rate	7.7%	15.4%	9.6%	7.8%	7.7%
Terminal rate	1/18 (6%)	2/31 (7%)	3/27 (11%)	3/21 (14%)	2/20 (10%)
First incidence (days)	611	505	728	734 (T)	597
Poly-3 test	P=0.167	P=0.227	P=0.537		
Poly-3 test	P=0.585N			P=0.660	P=0.664N
All Organs: Malignant Lymphoma					
Overall rate	1/50 (2%)	1/50 (2%)	1/50 (2%)	3/50 (6%)	0/50 (0%)
Adjusted rate	2.6%	2.3%	2.4%	7.8%	0.0%
Terminal rate	0/18 (0%)	1/31 (3%)	0/27 (0%)	2/21 (10%)	0/20 (0%)
First incidence (days)	693	734 (T)	701	677	—
Poly-3 test	—	—	—		
Poly-3 test	P=0.369N			P=0.305	P=0.496N

TABLE A2
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
All Organs: Benign Neoplasms					
Overall rate	22/50 (44%)	36/50 (72%)	36/50 (72%)	29/50 (58%)	22/50 (44%)
Adjusted rate	53.7%	74.6%	78.5%	68.5%	53.0%
Terminal rate	12/18 (67%)	21/31 (68%)	22/27 (82%)	17/21 (81%)	12/20 (60%)
First incidence (days)	586	467	406	363	541
Poly-3 test	P=0.024	P=0.026	P=0.007		
Poly-3 test	P=0.512N			P=0.105	P=0.563N
All Organs: Malignant Neoplasms					
Overall rate	16/50 (32%)	15/50 (30%)	14/50 (28%)	17/50 (34%)	12/50 (24%)
Adjusted rate	37.7%	32.5%	31.8%	40.6%	29.4%
Terminal rate	4/18 (22%)	7/31 (23%)	7/27 (26%)	7/21 (33%)	4/20 (20%)
First incidence (days)	488	285	490	330	597
Poly-3 test	P=0.350N	P=0.385N	P=0.361N		
Poly-3 test	P=0.254N			P=0.480	P=0.283N
All Organs: Benign or Malignant Neoplasms					
Overall rate	32/50 (64%)	38/50 (76%)	39/50 (78%)	35/50 (70%)	28/50 (56%)
Adjusted rate	73.0%	77.3%	83.1%	77.7%	65.9%
Terminal rate	14/18 (78%)	22/31 (71%)	23/27 (85%)	18/21 (86%)	13/20 (56%)
First incidence (days)	488	285	406	330	541
Poly-3 test	P=0.380	P=0.404	P=0.161		
Poly-3 test	P=0.260N			P=0.387	P=0.302N

(T) Terminal euthanasia

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

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

[c] Observed incidence at terminal euthanasia

[d] Beneath the control incidence is the P value associated with the trend test between the control group and the deficient exposure groups. Beneath the deficient exposure group incidence are the P values corresponding to pairwise comparisons between the controls and that deficient exposure group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal euthanasia. A negative trend or a lower incidence in an exposure group is indicated by **N**.

[e] Beneath the control incidence is the P value associated with the trend test between the control group and the excess exposure groups. Beneath the excess exposure group incidence are the P values corresponding to pairwise comparisons between the controls and that excess exposure group. A negative trend or a lower incidence in an exposure group is indicated by **N**.

[f] Poly-3 test was not run

[g] Not applicable; no neoplasms in animal group

[h] A single incidence of carcinoma occurred in an animal that also had multiple adenoma.

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of Dietary Zinc[a]

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Disposition Summary					
Animals initially in study	60	60	60	60	60
Early deaths					
Special study animals	10	10	9	10	10
Moribund	11	12	10	15	7
Natural deaths	19	7	13	14	22
Survivors					
Died last week of study	2		1		1
Terminal euthanasia	18	31	27	21	20
Animals examined microscopically	50	50	50	50	50
Alimentary System					
Esophagus	(50)	(50)	(50)	(50)	(50)
Intestine large, cecum	(50)	(50)	(50)	(50)	(47)
Erosion	1 (2%)				
Epithelium, necrosis					1 (2%)
Intestine, large, colon	(49)	(50)	(49)	(50)	(48)
Parasite, metazoan					1 (2%)
Ulcer	1 (2%)				
Intestine, large, rectum	(50)	(50)	(50)	(50)	(48)
Intestine small, duodenum	(50)	(49)	(50)	(49)	(48)
Epithelium, hyperplasia	1 (2%)				
Intestine small, ileum	(50)	(49)	(48)	(50)	(45)
Intestine small, jejunum	(50)	(49)	(50)	(50)	(47)
Peyer's patch, hyperplasia					1 (2%)
Liver	(50)	(50)	(50)	(50)	(46)
Atrophy					1 (2%)
Basophilic focus		1 (2%)		1 (2%)	
Clear cell focus	18 (36%)	26 (52%)	19 (38%)	21 (42%)	23 (50%)
Eosinophilic focus	4 (8%)		1 (2%)	1 (2%)	
Fatty change	5 (10%)	4 (8%)	5 (10%)	2 (4%)	4 (9%)
Hematopoietic cell proliferation					1 (2%)
Hepatodiaphragmatic nodule		1 (2%)		1 (2%)	
Inflammation				1 (2%)	1 (2%)
Mixed cell focus			1 (2%)	1 (2%)	
Bile duct, hyperplasia				3 (6%)	
Hepatocyte, atrophy	1 (2%)				
Hepatocyte, necrosis	4 (8%)		3 (6%)	1 (2%)	
Hepatocyte, vacuolization cytoplasmic				1 (2%)	1 (2%)
Serosa, inflammation, acute			1 (2%)		
Mesentery	(0)	(1)	(0)	(0)	(0)
Oral mucosa	(1)	(0)	(2)	(1)	(1)
Hyperplasia			1 (50%)		1 (100%)
Inflammation			1 (50%)		
Ulcer	1 (100%)				
Pancreas	(49)	(50)	(48)	(48)	(48)
Inflammation, acute					1 (2%)
Inflammation, chronic active					1 (2%)
Mineralization	1 (2%)				
Acinus, atrophy	3 (6%)	3 (6%)	4 (8%)	3 (6%)	13 (27%)
Acinus, basophilic focus	1 (2%)		1 (2%)		2 (4%)
Acinus, hyperplasia	23 (47%)	32 (64%)	23 (48%)	21 (44%)	28 (58%)
Duct, hyperplasia, cystic		1 (2%)			

[a] Number of core study 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 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Alimentary System (continued)					
Salivary glands	(49)	(50)	(50)	(50)	(49)
Cyst	1 (2%)	1 (2%)			
Stomach, forestomach	(50)	(50)	(50)	(50)	(49)
Mineralization	2 (4%)				
Ulcer	3 (6%)		1 (2%)	2 (4%)	2 (4%)
Epithelium, hyperplasia	11 (22%)	16 (32%)	11 (22%)	14 (28%)	7 (14%)
Stomach, glandular	(50)	(50)	(50)	(50)	(49)
Metaplasia, squamous			1 (2%)		
Mineralization	2 (4%)		1 (2%)	1 (2%)	1 (2%)
Tongue	(0)	(0)	(0)	(0)	(1)
Hemorrhage					1 (100%)
Inflammation, chronic					1 (100%)
Ulcer					1 (100%)
Tooth	(3)	(0)	(1)	(0)	(2)
Inflammation					1 (50%)
Malformation			1 (100%)		
Necrosis	3 (100%)				2 (100%)
Cardiovascular System					
Blood vessel	(50)	(50)	(50)	(50)	(50)
Inflammation	24 (48%)	29 (58%)	29 (58%)	27 (54%)	16 (32%)
Mineralization	3 (6%)				
Necrosis			1 (2%)		
Aorta, mineralization		1 (2%)			
Heart	(50)	(50)	(50)	(50)	(50)
Cardiomyopathy	42 (84%)	38 (76%)	39 (78%)	35 (70%)	32 (64%)
Mineralization	2 (4%)			1 (2%)	
Atrium, thrombosis	3 (6%)		4 (8%)	3 (6%)	
Valve, inflammation			1 (2%)		
Endocrine System					
Adrenal cortex	(50)	(50)	(50)	(50)	(50)
Degeneration, cystic		1 (2%)			
Hyperplasia	7 (14%)	3 (6%)	7 (14%)	5 (10%)	5 (10%)
Hypertrophy		1 (2%)			
Necrosis	1 (2%)		1 (2%)		
Thrombosis				1 (2%)	
Vacuolization, cytoplasmic	1 (2%)	1 (2%)			
Adrenal medulla	(50)	(50)	(50)	(50)	(50)
Hyperplasia	16 (32%)	21 (42%)	14 (28%)	15 (30%)	11 (22%)
Bilateral, hyperplasia	6 (12%)	3 (6%)	8 (16%)	4 (8%)	5 (10%)
Islets, pancreatic	(50)	(50)	(50)	(49)	(49)
Atrophy		1 (2%)			1 (2%)
Hyperplasia	1 (2%)		3 (6%)		4 (8%)
Parathyroid gland	(47)	(41)	(44)	(40)	(42)
Hyperplasia	6 (13%)	2 (5%)	2 (5%)	6 (15%)	5 (12%)
Pituitary gland	(50)	(50)	(50)	(50)	(49)
Inflammation					1 (2%)
Pars distalis, hyperplasia	12 (24%)	17 (34%)	17 (34%)	13 (26%)	15 (31%)
Pars intermedia, hyperplasia	1 (2%)	1 (2%)		1 (2%)	
Thyroid gland	(50)	(50)	(50)	(50)	(49)
Mineralization				1 (2%)	
Thrombosis, chronic	1 (2%)				
C-cell, hyperplasia	16 (32%)	16 (32%)	13 (26%)	10 (20%)	12 (24%)
Follicular cell, hyperplasia	1 (2%)				1 (2%)

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
General Body System					
None					
Genital System					
Epididymis	(50)	(50)	(50)	(50)	(50)
Degeneration				2 (4%)	
Hyperplasia				1 (2%)	
Penis	(0)	(0)	(1)	(0)	(0)
Developmental malformation			1 (100%)		
Preputial gland	(50)	(50)	(50)	(50)	(50)
Inflammation			2 (4%)		
Prostate	(50)	(50)	(50)	(50)	(50)
Inflammation	1 (2%)			1 (2%)	
Epithelium, hyperplasia	2 (4%)		1 (2%)		
Seminal vesicle	(50)	(50)	(50)	(50)	(50)
Inflammation	1 (2%)				
Testes	(50)	(50)	(50)	(50)	(50)
Edema		2 (4%)			
Mineralization		1 (2%)			
Bilateral, germ cell, degeneration		1 (2%)		1 (2%)	1 (2%)
Bilateral, germinal epithelium, atrophy		7 (14%)	1 (2%)		1 (2%)
Germinal epithelium, atrophy	5 (10%)	3 (6%)		3 (6%)	4 (8%)
Interstitial cell, hyperplasia	1 (2%)				
Seminiferous tubule, dilation	1 (2%)	1 (2%)		1 (2%)	
Hematopoietic System					
Bone marrow	(50)	(50)	(50)	(50)	(50)
Atrophy	1 (2%)	1 (2%)	1 (2%)	1 (2%)	2 (4%)
Hyperplasia	4 (8%)	4 (8%)		1 (2%)	1 (2%)
Myelofibrosis				1 (2%)	
Lymph node	(6)	(1)	(4)	(3)	(2)
Deep cervical, inflammation	1 (17%)				
Inguinal, ectasia				1 (33%)	
Mediastinal, atrophy	1 (17%)		2 (50%)		
Mediastinal, ectasia					1 (50%)
Mediastinal, hemorrhage	2 (33%)	1 (100%)	4 (100%)	1 (33%)	
Mediastinal, hemorrhage, chronic	1 (17%)				
Mediastinal, pigmentation, hemosiderin	1 (17%)				
Pancreatic, hemorrhage		1 (100%)			1 (50%)
Pancreatic, hyperplasia					1 (50%)
Renal, hemorrhage	1 (17%)			1 (33%)	
Lymph node, mandibular	(49)	(50)	(50)	(50)	(48)
Atrophy	2 (4%)	1 (2%)	2 (4%)	2 (4%)	2 (4%)
Ectasia		1 (2%)	1 (2%)	1 (2%)	
Hyperplasia	15 (31%)	14 (28%)	11 (22%)	10 (20%)	11 (23%)
Infiltration, cellular, histiocyte				1 (2%)	
Inflammation, plasma cell	12 (24%)	9 (18%)	13 (26%)	12 (24%)	10 (21%)
Lymph node, mesenteric	(50)	(50)	(49)	(50)	(49)
Atrophy	2 (4%)			1 (2%)	
Hyperplasia		2 (4%)		2 (4%)	4 (8%)
Infiltration, cellular, plasma cell		1 (2%)			
Inflammation, granulomatous	1 (2%)		1 (2%)	1 (2%)	
Inflammation, chronic active	1 (2%)				

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Hematopoietic System (continued)					
Spleen	(50)	(50)	(49)	(48)	(45)
Atrophy			1 (2%)		
Hematopoietic cell proliferation	27 (54%)	37 (74%)	24 (49%)	26 (54%)	24 (53%)
Pigmentation, hemosiderin	44 (88%)	43 (86%)	49 (100%)	40 (83%)	34 (76%)
Capsule, inflammation				1 (2%)	
Lymphoid follicle, atrophy	6 (12%)	1 (2%)	3 (6%)	3 (6%)	4 (9%)
Lymphoid follicle, hyperplasia	7 (14%)	8 (16%)	10 (20%)	8 (17%)	3 (7%)
Thymus	(47)	(49)	(50)	(47)	(48)
Atrophy	37 (79%)	40 (82%)	35 (70%)	36 (77%)	37 (77%)
Hyperplasia		1 (2%)	3 (6%)	1 (2%)	1 (2%)
Integumentary System					
Mammary gland	(50)	(49)	(50)	(48)	(49)
Skin	(50)	(50)	(50)	(50)	(50)
Cyst epithelial inclusion	1 (2%)	1 (2%)	1 (2%)		2 (4%)
Dysplasia				1 (2%)	
Fibrosis	1 (2%)			1 (2%)	
Inflammation	4 (8%)		2 (4%)	1 (2%)	
Ulcer	3 (6%)			1 (2%)	2 (4%)
Epidermis, hyperplasia	2 (4%)	2 (4%)	2 (4%)	1 (2%)	
Hair follicle, cyst	2 (4%)	1 (2%)		5 (10%)	2 (4%)
Hair follicle, cyst, multiple	1 (2%)				
Hair follicle, hyperplasia		1 (2%)			
Pinna, hyperplasia, squamous					1 (2%)
Musculoskeletal System					
Bone	(50)	(50)	(50)	(50)	(50)
Fibrous osteodystrophy	3 (6%)		1 (2%)	1 (2%)	
Inflammation	1 (2%)				
Skeletal muscle	(0)	(0)	(1)	(2)	(0)
Nervous System					
Brain	(48)	(50)	(50)	(50)	(50)
Edema				1 (2%)	1 (2%)
Inflammation			1 (2%)		
Mineralization				1 (2%)	
Cerebrum, gliosis		1 (2%)		1 (2%)	1 (2%)
Cerebrum, neuron, necrosis				1 (2%)	
Ventricle, developmental malformation	1 (2%)				
Venule, mineralization				1 (2%)	
Peripheral nerve	(1)	(1)	(1)	(1)	(0)
Sciatic, degeneration				1 (100%)	
Spinal cord	(0)	(1)	(1)	(1)	(0)
Axon, degeneration		1 (100%)	1 (100%)	1 (100%)	
Respiratory System					
Lung	(50)	(50)	(50)	(50)	(50)
Edema	1 (2%)				
Hemorrhage					1 (2%)
Infiltration cellular, histiocyte	18 (36%)	21 (42%)	21 (42%)	14 (28%)	14 (28%)
Inflammation	3 (6%)	4 (8%)	7 (14%)	10 (20%)	4 (8%)
Necrosis	1 (2%)				
Alveolar epithelium, hyperplasia				1 (2%)	
Interstitium, thrombosis	1 (2%)				

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Respiratory System (continued)					
Nose	(50)	(50)	(50)	(50)	(49)
Accumulation, hyaline droplet		1 (2%)			
Inflammation	8 (16%)		4 (8%)	2 (4%)	4 (8%)
Olfactory epithelium. atrophy			1 (2%)		
Respiratory epithelium, hyperplasia			3 (6%)	2 (4%)	1 (2%)
Respiratory epithelium, metaplasia, squamous	1 (2%)				
Trachea	(50)	(50)	(50)	(50)	(50)
Special Senses System					
Eye	(50)	(49)	(50)	(50)	(49)
Atrophy		1 (2%)	1 (2%)	1 (2%)	
Cataract		1 (2%)			
Anterior chamber, inflammation, acute	1 (2%)		1 (2%)		
Anterior chamber, bilateral, inflammation, acute	1 (2%)				
Bilateral, cornea, inflammation, acute	2 (4%)	1 (2%)	2 (4%)		
Bilateral, cornea inflammation, chronic active	1 (2%)	1 (2%)	2 (4%)	3 (6%)	1 (2%)
Bilateral, cornea, necrosis	1 (2%)		1 (2%)	1 (2%)	
Cornea, inflammation, acute		1 (2%)		2 (4%)	1 (2%)
Cornea, inflammation, chronic active			2 (4%)		1 (2%)
Cornea, necrosis				3 (6%)	
Cornea, ulcer		1 (2%)			
Lens, cataract	1 (2%)				
Harderian gland	(50)	(49)	(50)	(50)	(49)
Inflammation			1 (2%)		
Zymbal's gland	(1)	(0)	(0)	(0)	(0)
Urinary System					
Kidney	(50)	(50)	(50)	(50)	(50)
Cyst		1 (2%)	4 (8%)	4 (8%)	
Infarct, chronic		3 (6%)	1 (2%)		3 (6%)
Nephropathy	49 (98%)	50 (100%)	48 (96%)	49 (98%)	49 (98%)
Pelvis, inflammation, chronic active				1 (2%)	
Renal tubule, hyperplasia, atypical			1 (2%)		
Urinary bladder	(50)	(50)	(50)	(50)	(49)
Inflammation	2 (4%)			1 (2%)	
Ulcer				1 (2%)	

APPENDIX B
SUMMARY OF LESIONS IN FEMALE RATS IN THE 2-YEAR FEED STUDY OF DIETARY ZINC

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of Dietary Zinc[a]

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Disposition Summary					
Animals initially in study	60	60	60	60	60
Special study animals	9	10	9	9	9
Early deaths					
Moribund	21	11	12	19	14
Natural deaths	5	7	5	5	6
Survivors					
Terminal euthanasia	25	32	34	27	31
Animals examined microscopically	50	50	50	50	50
Alimentary System					
Esophagus	(50)	(49)	(50)	(50)	(49)
Intestine large, cecum	(50)	(49)	(50)	(49)	(48)
Intestine, large, colon	(50)	(49)	(50)	(50)	(49)
Intestine large, rectum	(50)	(50)	(50)	(50)	(49)
Intestine small, duodenum	(50)	(49)	(50)	(49)	(49)
Leiomyoma			1 (2%)	1 (2%)	
Intestine small, ileum	(50)	(49)	(50)	(49)	(49)
Intestine small, jejunum	(50)	(49)	(50)	(49)	(49)
Liver	(50)	(50)	(50)	(50)	(50)
Cholangioma	1 (2%)		1 (2%)		
Pancreas	(50)	(48)	(49)	(49)	(49)
Schwannoma malignant					1 (2%)
Acinus, adenoma	1 (2%)				
Salivary glands	(50)	(49)	(50)	(48)	(49)
Stomach, forestomach	(50)	(50)	(50)	(50)	(49)
Stomach, glandular	(50)	(50)	(50)	(50)	(49)
Tooth	(1)	(1)	(0)	(6)	(0)
Cardiovascular System					
Blood vessel	(50)	(50)	(50)	(50)	(50)
Heart	(50)	(50)	(50)	(50)	(50)
Endocardium, schwannoma malignant			1 (2%)		
Myocardium, schwannoma malignant		1 (2%)			
Endocrine System					
Adrenal cortex	(50)	(50)	(50)	(50)	(50)
Carcinoma				1 (2%)	
Adrenal medulla	(50)	(50)	(50)	(50)	(50)
Pheochromocytoma benign			1 (2%)	2 (4%)	
Pheochromocytoma malignant	1 (2%)				
Islets, pancreatic	(50)	(50)	(50)	(49)	(49)
Adenoma				1 (2%)	
Parathyroid gland	(42)	(43)	(41)	(42)	(43)
Pituitary gland	(50)	(50)	(50)	(50)	(50)
Pars distalis, adenoma	11 (22%)	13 (26%)	9 (18%)	3 (6%)	8 (16%)
Pars distalis, carcinoma	1 (2%)		1 (2%)		
Thyroid gland	(50)	(49)	(50)	(50)	(48)
Bilateral, C-cell, adenoma				1 (2%)	
C-cell, adenoma	1 (2%)	2 (4%)	1 (2%)		3 (6%)
C-cell, carcinoma	1 (2%)			2 (4%)	

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
General Body System					
None					
Genital System					
Clitoral gland	(49)	(50)	(50)	(49)	(50)
Ovary	(50)	(50)	(50)	(50)	(49)
Granulosa cell tumor malignant					1 (2%)
Uterus	(50)	(50)	(49)	(50)	(50)
Adenocarcinoma	1 (2%)		1 (2%)		1 (2%)
Hemangioma				1 (2%)	
Leiomyoma	1 (2%)	1 (2%)			
Polyp stromal	3 (6%)	2 (4%)	1 (2%)		2 (4%)
Schwannoma malignant		1 (2%)			
Squamous cell carcinoma			1 (2%)		
Cervix, schwannoma malignant					1 (2%)
Hematopoietic System					
Bone marrow	(50)	(49)	(50)	(50)	(50)
Lymph node	(1)	(0)	(0)	(0)	(1)
Pancreatic, schwannoma malignant					1 (100%)
Lymph node, mandibular	(50)	(49)	(49)	(48)	(48)
Lymph node, mesenteric	(50)	(49)	(50)	(50)	(49)
Hemangiosarcoma					1 (2%)
Spleen	(50)	(50)	(50)	(50)	(49)
Thymus	(48)	(50)	(48)	(49)	(49)
Thymoma malignant					1 (2%)
Integumentary System					
Mammary gland	(50)	(50)	(50)	(50)	(50)
Adenocarcinoma	4 (8%)	4 (8%)	4 (8%)	2 (4%)	4 (8%)
Adenocarcinoma, multiple				2 (4%)	
Adenoma	3 (6%)	2 (4%)		3 (6%)	1 (2%)
Fibroadenoma	23 (46%)	20 (40%)	21 (42%)	22 (44%)	13 (26%)
Fibroadenoma, multiple	6 (12%)	11 (22%)	12 (24%)	9 (18%)	14 (28%)
Fibrosarcoma				1 (2%)	
Schwannoma malignant				1 (2%)	1 (2%)
Skin	(50)	(50)	(50)	(50)	(50)
Sebaceous gland, adenoma	1 (2%)				
Subcutaneous tissue, fibroma			1 (2%)		
Subcutaneous tissue, schwannoma malignant		1 (2%)			
Musculoskeletal System					
Bone	(50)	(50)	(50)	(50)	(50)
Carcinoma, metastatic, Zymbal's gland				1 (2%)	
Nervous System					
Brain	(50)	(50)	(50)	(50)	(50)
Cerebrum, oligodendroglioma malignant	1 (2%)				
Peripheral nerve	(0)	(0)	(0)	(1)	(1)
Spinal cord	(0)	(0)	(0)	(1)	(1)

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Respiratory System					
Lung	(50)	(50)	(50)	(50)	(50)
Adenocarcinoma, metastatic, mammary gland			1 (2%)		
Adenocarcinoma, metastatic, uterus			1 (2%)		
Carcinoma, metastatic, adrenal cortex				1 (2%)	
Carcinoma, metastatic, thyroid gland	1 (2%)				
Cystic keratinizing epithelioma		1 (2%)			
Squamous cell carcinoma, metastatic, uterus			1 (2%)		
Nose	(50)	(50)	(50)	(50)	(50)
Carcinoma, metastatic, Zymbal's gland				1 (2%)	
Trachea	(50)	(50)	(50)	(50)	(50)
Special Senses System					
Eye	(50)	(50)	(50)	(49)	(50)
Harderian gland	(50)	(50)	(50)	(49)	(50)
Zymbal's gland	(0)	(0)	(0)	(1)	(0)
Carcinoma				1 (100%)	
Urinary System					
Kidney	(50)	(50)	(50)	(50)	(49)
Urinary bladder	(50)	(50)	(50)	(50)	(49)
Systemic Lesions					
Multiple organs[b]	(50)	(50)	(50)	(50)	(50)
Histiocytic sarcoma	1 (2%)				1 (2%)
Leukemia mononuclear	1 (2%)	1 (2%)			
Lymphoma malignant		1 (2%)		1 (2%)	
Neoplasm Summary					
Total animals with primary neoplasms[c]	40	41	42	40	39
Total primary neoplasms	62	61	56	54	54
Total animals with benign neoplasms	35	39	39	34	32
Total benign neoplasms	51	52	48	43	41
Total animals with malignant neoplasms	11	8	8	11	11
Total malignant neoplasms	11	9	8	11	13
Total animals with metastatic neoplasms	1		3	2	
Total metastatic neoplasms	1		3	3	

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

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Mammary Gland: Fibroadenoma					
Overall rate[a]	29/50 (58%)	31/50 (62%)	33/50 (66%)	31/50 (62%)	27/50 (54%)
Adjusted rate[b]	65.1%	64.5%	68.7%	68.0%	59.8%
Terminal rate[c]	14/25 (56%)	16/32 (50%)	22/34 (65%)	15/27 (56%)	15/31 (48%)
First incidence (days)	435	323	447	179	396
Poly-3 test[d]	P=0.513N	P=0.563N	P=0.441		
Poly-3 test[e]	P=0.335N			P=0.473	P=0.379N
Mammary Gland: Adenoma					
Overall rate	3/50 (6%)	2/50 (4%)	0/50 (0%)	3/50 (6%)	1/50 (2%)
Adjusted rate	7.6%	4.7%	0.0%	7.7%	2.5%
Terminal rate	3/25 (12%)	1/32 (3%)	0/34 (0%)	3/27 (11%)	1/31 3%)
First incidence (days)	725 (T)	659	—[f]	725 (T)	725 (T)
Poly-3 test	P=0.384N	P=0.468N	P=0.099N		
Poly-3 test	P=0.229N			P=0.659	P=0.296N
Mammary Gland: Fibroadenoma or Adenoma					
Overall rate	29/50 (58%)	31/50 (62%)	33/50 (66%)	31/50 (62%)	28/50 (56%)
Adjusted rate	65.1%	64.5%	68.7%	68.0%	62.0%
Terminal rate	14/25 (56%)	16/32 (50%)	22/34 (65%)	15/27 (56%)	16/31 (52%)
First incidence (days)	435	323	447	179	396
Poly-3 test	P=0.513N	P=0.563N	P=0.441		
Poly-3 test	P=0.419N			P=0.473	P=0.465N
Mammary Gland: Carcinoma					
Overall rate	4/50 (8%)	4/50 (8%)	4/50 (8%)	4/50 (8%)	4/50 (8%)
Adjusted rate	9.8%	9.4%	8.9%	10%	9.9%
Terminal rate	1/25 (4%)	3/32 (3%)	2/34 (6%)	2/27 (7%)	4/31 (13%)
First incidence (days)	540	601	483	449	725 (T)
Poly-3 test	P=0.553N	P=0.623N	P=0.589N		
Poly-3 test	P=0.566			P=0.632	P=0.637
Mammary Gland: Adenoma or Carcinoma					
Overall rate	7/50 (14%)	6/50 (12%)	4/50 (8%)	7/50 (14%)	5/50 (10%)
Adjusted rate	17.1%	14.0%	8.9%	17.5%	12.4%
Terminal rate	4/25 (16%)	4/32 (13%)	2/34 (6%)	5/27 (19%)	5/31 (16%)
First incidence (days)	540	601	483	449	725 (T)
Poly-3 test	P=0.413N	P=0.465N	P=0.206N		
Poly-3 test	P=0.333N			P=0.598	P=0.387N
Mammary Gland: Fibroadenoma, Adenoma, or Carcinoma					
Overall rate	33/50 (66%)	33/50 (66%)	35/50 (70%)	34/50 (68%)	32/50 (64%)
Adjusted rate	71.7%	68.6%	71.6%	73.3%	70.8%
Terminal rate	15/25 (60%)	18/32 (56%)	22/34 (65%)	17/27 (63%)	20/31 (65%)
First incidence (days)	435	323	447	179	396
Poly-3 test	P=0.414N	P=0.459N	P=0.588N		
Poly-3 test	P=0.511N			P=0.524	P=0.557N
Pituitary Gland (Pars Distalis): Adenoma					
Overall rate	11/50 (22%)	13/50 (26%)	9/50 (18%)	3/50 (6%)	8/50 (16%)
Adjusted rate	27.1%	30.2%	19.8%	7.6%	19.1%
Terminal rate	6/25 (24%)	9/32 (28%)	4/34 (12%)	2/27 (7%)	4/31 (13%)
First incidence (days)	609	601	615	687	562
Poly-3 test	P=0.408	P=0.472	P=0.293N		
Poly-3 test	P=0.217N			P=0.020N	P=0.273N

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Pituitary Gland (Pars Distalis): Adenoma or Carcinoma					
Overall rate	12/50 (24%)	13/50 (26%)	10/50 (20%)	3/50 (6%)	8/50 (16%)
Adjusted rate	29.6%	30.2%	21.8%	7.6%	19.1%
Terminal rate	7/25 (28%)	9/32 (28%)	4/34 (12%)	2/27 (7%)	4/31 (13%)
First incidence (days)	609	601	572	687	562
Poly-3 test	P=0.510	P=0.571	P=0.280N		
Poly-3 test	P=0.144N			P=0.011N	P=0.195N
Thyroid Gland (C-Cell): Adenoma					
Overall rate	1/50 (2%)	2/49 (4%)	1/50 (2%)	1/50 (2%)	3/48 (6%)
Adjusted rate	2.5%	4.8%	2.3%	2.6%	7.5%
Terminal rate	1/25 (4%)	2/32 (6%)	0/34 (0%)	0/27 (0%)	1/31 (3%)
First incidence (days)	725 (T)	725 (T)	676	687	610
Poly-3 test	P=0.394	P=0.517	P=0.733N		
Poly-3 test	P=0.205			P=0.760	P=0.312
Thyroid Gland (C-Cell): Adenoma or Carcinoma					
Overall rate	2/50 (4%)	2/49 (4%)	1/50 (2%)	3/50 (6%)	3/48 (6%)
Adjusted rate	5.1%	4.8%	2.3%	7.5%	7.5%
Terminal rate	1/25 (4%)	2/32 (6%)	0/34 (0%)	1/27 (4%)	1/31 (3%)
First incidence (days)	700	725 (T)	676	568	610
Poly-3 test	P=0.603N	P=0.678N	P=0.459N		
Poly-3 test	P=0.424			P=0.502	P=0.508
Uterus: Stromal Polyp					
Overall rate	3/50 (6%)	2/50 (4%)	1/50 (2%)	0/50 (0%)	2/50 (4%)
Adjusted rate	7.5%	4.8%	2.2%	0.0%	4.9%
Terminal rate	1/25 (4%)	2/32 (6%)	0/34 (0%)	0/27 (0%)	1/31 (3%)
First incidence (days)	677	725 (T)	663	—	635
Poly-3 test	P=0.388N	P=0.474N	P=0.266N		
Poly-3 test	P=0.386N			P=0.121N	P=0.490N
All Organs: Benign Neoplasms					
Overall rate	35/50 (70%)	39/50 (78%)	39/50 (78%)	34/50 (68%)	32/50 (64%)
Adjusted rate	77.1%	81.1%	79.9%	73.7%	69.6%
Terminal rate	17/25 (68%)	24/32 (75%)	25/34 (74%)	17/27 (63%)	18/31 (58%)
First incidence (days)	435	323	447	179	396
Poly-3 test	P=0.362	P=0.408	P=0.464		
Poly-3 test	P=0.239N			P=0.446N	P=0.278N
All Organs: Malignant Neoplasms					
Overall rate	11/50 (22%)	8/50 (16%)	8/50 (16%)	11/50 (22%)	11/50 (22%)
Adjusted rate	26.1%	18.1%	17.3%	25.8%	26.2%
Terminal rate	4/25 (16%)	5/32 (16%)	4/34 (12%)	3/27 (11%)	8/31 (26%)
First incidence (days)	540	350	483	449	362
Poly-3 test	P=0.226N	P=0.264N	P=0.231N		
Poly-3 test	P=0.541			P=0.589N	P=0.590

TABLE B2
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
All Organs: Benign or Malignant Neoplasms					
Overall rate	40/50 (80%)	41/50 (82%)	42/50 (84%)	40/50 (80%)	39/50 (78%)
Adjusted rate	84.3%	82.5%	84.0%	82.7%	82.5%
Terminal rate	18/25 (72%)	24/32 (75%)	26/34 (77%)	19/27 (70%)	23/31 (74%)
First incidence (days)	435	323	447	179	362
Poly-3 test	P=0.457N	P=0.511N	P=0.592N		
Poly-3 test	P=0.462N			P=0.526N	P=0.517N

(T) Terminal euthanasia

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

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

[c] Observed incidence at terminal euthanasia

[d] Beneath the control incidence is the P value associated with the trend test between the control group and the deficient exposure groups. Beneath the deficient exposure group incidence are the P values corresponding to pairwise comparisons between the controls and that deficient exposure group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal euthanasia. A negative trend or a lower incidence in an exposure group is indicated by **N**.

[e] Beneath the control incidence is the P value associated with the trend test between the control group and the excess exposure groups. Beneath the excess exposure group incidence are the P values corresponding to pairwise comparisons between the controls and that excess exposure group. A negative trend or a lower incidence in an exposure group is indicated by **N**.

[f] Not applicable; no neoplasms in animal group

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of Dietary Zinc[a]

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Disposition Summary					
Animals initially in study	60	60	60	60	60
Special study animals	9	10	9	9	9
Early deaths					
Moribund	21	11	12	19	14
Natural deaths	5	7	5	5	6
Survivors					
Terminal euthanasia	25	32	34	27	31
Animals examined microscopically	50	50	50	50	50
Alimentary System					
Esophagus	(50)	(49)	(50)	(50)	(49)
Intestine large, cecum	(50)	(49)	(50)	(49)	(48)
Intestine, large, colon	(50)	(49)	(50)	(50)	(49)
Parasite, metazoan				1 (2%)	1 (2%)
Intestine large, rectum	(50)	(50)	(50)	(50)	(49)
Intestine small, duodenum	(50)	(49)	(50)	(49)	(49)
Intestine small, ileum	(50)	(49)	(50)	(49)	(49)
Intestine small, jejunum	(50)	(49)	(50)	(49)	(49)
Liver	(50)	(50)	(50)	(50)	(50)
Angiectasis		2 (4%)	3 (6%)	1 (2%)	1 (2%)
Basophilic focus	1 (2%)				1 (2%)
Clear cell focus	2 (4%)	2 (4%)	7 (14%)	5 (10%)	5 (10%)
Developmental malformation		1 (2%)			
Eosinophilic focus		1 (2%)	3 (6%)	1 (2%)	2 (4%)
Fatty change	6 (12%)	4 (8%)	11 (22%)	4 (8%)	3 (6%)
Hematopoietic cell proliferation	1 (2%)	2 (4%)			
Hepatodiaphragmatic nodule	1 (2%)				
Inflammation	1 (2%)				
Mixed cell focus		1 (2%)	2 (4%)	4 (8%)	
Bile duct, cyst		1 (2%)			
Bile duct, hyperplasia		2 (4%)			
Hepatocyte, hypertrophy				1 (2%)	1 (2%)
Hepatocyte, inclusion body intracytoplasmic		1 (2%)			
Hepatocyte, necrosis		1 (2%)	2 (4%)		1 (2%)
Hepatocyte, vacuolization cytoplasmic	1 (2%)	1 (2%)		2 (4%)	
Pancreas	(50)	(48)	(49)	(49)	(49)
Thrombosis		1 (2%)			
Acinus, atrophy	2 (4%)	4 (8%)	2 (4%)	5 (10%)	10 (20%)
Acinus, basophilic focus			2 (4%)		
Acinus, depletion secretory					1 (2%)
Acinus, hyperplasia	2 (4%)	1 (2%)	5 (10%)		1 (2%)
Duct, hyperplasia, cystic		1 (2%)			1 (2%)
Salivary glands	(50)	(49)	(50)	(48)	(49)
Stomach, forestomach	(50)	(50)	(50)	(50)	(49)
Edema	1 (2%)				
Inflammation				1 (2%)	
Ulcer	1 (2%)	1 (2%)		1 (2%)	1 (2%)
Epithelium, hyperplasia	5 (10%)	8 (16%)	4 (8%)	2 (4%)	4 (8%)
Stomach, glandular	(50)	(50)	(50)	(50)	(49)
Erosion	1 (2%)				

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

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Alimentary System (continued)					
Tooth	(1)	(1)	(0)	(6)	(0)
Inflammation				3 (50%)	
Malformation		1 (100%)			
Necrosis	1 (100%)			3 (50%)	
Cardiovascular System					
Blood vessel	(50)	(50)	(50)	(50)	(50)
Inflammation	1 (2%)	1 (2%)		1 (2%)	1 (2%)
Heart	(50)	(50)	(50)	(50)	(50)
Cardiomyopathy	7 (14%)	10 (20%)	4 (8%)	8 (16%)	6 (12%)
Endocardium, fibrosis	1 (2%)				
Endocardium, hyperplasia			1 (2%)	1 (2%)	
Myocardium, inflammation, chronic active	1 (2%)				
Endocrine System					
Adrenal cortex	(50)	(50)	(50)	(50)	(50)
Degeneration, cystic	3 (6%)	4 (8%)	1 (2%)	1 (2%)	2 (4%)
Hyperplasia	6 (12%)	5 (10%)	5 (10%)	4 (8%)	5 (10%)
Necrosis	1 (2%)				1 (2%)
Thrombosis			1 (2%)	2 (4%)	
Adrenal medulla	(50)	(50)	(50)	(50)	(50)
Hyperplasia	6 (12%)	4 (8%)	3 (6%)	3 (6%)	2 (4%)
Islets, pancreatic	(50)	(50)	(50)	(49)	(49)
Hyperplasia			1 (2%)		
Parathyroid gland	(42)	(43)	(41)	(42)	(43)
Hyperplasia		1 (2%)			
Pituitary gland	(50)	(50)	(50)	(50)	(50)
Angiectasis	1 (2%)				
Hemorrhage				1 (2%)	
Pars distalis, angiectasis		1 (2%)		1 (2%)	
Pars distalis, hyperplasia	15 (30%)	15 (30%)	22 (44%)	13 (26%)	17 (34%)
Thyroid gland	(50)	(49)	(50)	(50)	(48)
C-cell, hyperplasia	18 (36%)	14 (29%)	9 (18%)	14 (28%)	5 (10%)
General Body System					
None					
Genital System					
Clitoral gland	(49)	(50)	(50)	(49)	(50)
Cyst			1 (2%)		
Hyperplasia				1 (2%)	
Ovary	(50)	(50)	(50)	(50)	(49)
Atrophy	31 (62%)	29 (58%)	30 (60%)	29 (58%)	26 (53%)
Cyst	12 (24%)	8 (16%)	5 (10%)	11 (22%)	4 (8%)
Inflammation	1 (2%)				
Bilateral, cyst			2 (4%)		
Granulosa cell, hyperplasia	1 (2%)		1 (2%)		
Interstitial cell, hyperplasia		2 (4%)			1 (2%)

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Genital System (continued)					
Uterus	(50)	(50)	(49)	(50)	(50)
Cyst		1 (2%)			
Hemorrhage					1 (2%)
Inflammation	1 (2%)	1 (2%)		1 (2%)	
Metaplasia, squamous	21 (42%)	19 (38%)	18 (37%)	15 (30%)	20 (40%)
Pigmentation, hemosiderin	2 (4%)				
Polyp, inflammatory			1 (2%)		
Thrombosis	1 (2%)				
Cervix, adenomyosis				1 (2%)	
Cervix, hypertrophy	1 (2%)				
Endometrium, hyperplasia, cystic	9 (18%)	13 (26%)	5 (10%)	7 (14%)	7 (14%)
Hematopoietic System					
Bone marrow	(50)	(49)	(50)	(50)	(50)
Atrophy	1 (2%)		1 (2%)	2 (4%)	
Hyperplasia	14 (28%)	10 (20%)	9 (18%)	14 (28%)	18 (36%)
Lymph node	(1)	(0)	(0)	(0)	(1)
Mediastinal, hemorrhage	1 (100%)				
Lymph node, mandibular	(50)	(49)	(49)	(48)	(48)
Atrophy		2 (4%)	1 (2%)	1 (2%)	
Hyperplasia	8 (16%)	12 (24%)	8 (16%)	8 (17%)	6 (13%)
Infiltration, cellular, plasma cell	15 (30%)	18 (37%)	8 (16%)	14 (29%)	10 (21%)
Lymph node, mesenteric	(50)	(49)	(50)	(50)	(49)
Atrophy		1 (2%)	1 (2%)		
Hyperplasia	1 (2%)	1 (2%)			1 (2%)
Infiltration, cellular, plasma cell	1 (2%)	3 (6%)	2 (4%)		3 (6%)
Spleen	(50)	(50)	(50)	(50)	(49)
Hematopoietic cell proliferation	25 (50%)	28 (56%)	31 (62%)	39 (78%)	34 (69%)
Hemorrhage	1 (2%)				
Inflammation		1 (2%)			
Pigmentation, hemosiderin	39 (78%)	38 (76%)	43 (86%)	42 (84%)	33 (67%)
Lymphoid follicle, atrophy	1 (2%)	2 (4%)	1 (2%)	3 (6%)	
Lymphoid follicle, hyperplasia	11 (22%)	9 (18%)	7 (14%)	7 (14%)	8 (16%)
Thymus	(48)	(50)	(48)	(49)	(49)
Atrophy	35 (73%)	34 (68%)	31 (65%)	32 (65%)	33 (67%)
Hyperplasia			1 (2%)		
Epithelial cell, hyperplasia		1 (2%)			
Integumentary System					
Mammary gland	(50)	(50)	(50)	(50)	(50)
Cyst			1 (2%)		
Hyperplasia	2 (4%)	5 (10%)	2 (4%)	2 (4%)	2 (4%)
Skin	(50)	(50)	(50)	(50)	(50)
Inflammation		1 (2%)			
Ulcer					1 (2%)
Musculoskeletal System					
Bone	(50)	(50)	(50)	(50)	(50)
Osteopetrosis				1 (2%)	
Joint, degeneration				1 (2%)	
Maxilla, fibrosis				2 (4%)	

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Nervous System					
Brain	(50)	(50)	(50)	(50)	(50)
Hemorrhage	1 (2%)	1 (2%)			
Hydrocephalus					1 (2%)
Cerebrum, gliosis	1 (2%)				
Glial cell, hyperplasia	1 (2%)				
Peripheral nerve	(0)	(0)	(0)	(1)	(1)
Spinal cord	(0)	(0)	(0)	(1)	(1)
Axon, degeneration				1 (100%)	1 (100%)
Respiratory System					
Lung	(50)	(50)	(50)	(50)	(50)
Cyst, squamous				1 (2%)	
Infiltration cellular, histiocyte	37 (74%)	38 (76%)	40 (80%)	38 (76%)	35 (70%)
Inflammation		1 (2%)			1 (2%)
Metaplasia, squamous					1 (2%)
Alveolar epithelium, hyperplasia	1 (2%)			1 (2%)	1 (2%)
Nose	(50)	(50)	(50)	(50)	(50)
Inflammation	1 (2%)	2 (4%)		2 (4%)	4 (8%)
Metaplasia, squamous					1 (2%)
Respiratory epithelium, hyperplasia			1 (2%)		
Trachea	(50)	(50)	(50)	(50)	(50)
Special Senses System					
Eye	(50)	(50)	(50)	(49)	(50)
Cataract		1 (2%)			
Harderian gland	(50)	(50)	(50)	(49)	(50)
Hyperplasia				1 (2%)	
Zymbal's gland	(0)	(0)	(0)	(1)	(0)
Urinary System					
Kidney	(50)	(50)	(50)	(50)	(49)
Cyst	2 (4%)				
Hydronephrosis	1 (2%)				
Infarct, chronic		1 (2%)			
Mineralization		1 (2%)			2 (4%)
Nephropathy	27 (54%)	28 (56%)	21 (42%)	31 (62%)	29 (59%)
Bilateral, papilla, inflammation, acute				1 (2%)	
Cortex, inflammation, chronic active	1 (2%)				
Pelvis, inflammation				1 (2%)	
Pelvis, inflammation, acute				1 (2%)	
Urinary bladder	(50)	(50)	(50)	(50)	(49)
Inflammation				1 (2%)	

APPENDIX C
GENETIC TOXICOLOGY

GENETIC TOXICOLOGY

RAT PERIPHERAL BLOOD MICRONUCLEUS TEST PROTOCOL

A detailed discussion of this assay is presented by Witt *et al.* (2008) and Torous *et al*. (2005). At day 19, and at months 3, 6, 9, and 12 in the 2-year study of dietary zinc, small blood samples (~120 µL) were obtained from male and female Hsd:Sprague Dawley SD special study rats in EDTA tubes. Samples were immediately refrigerated, and then shipped with cold packs by overnight courier to the analytical laboratory where they were immediately fixed in ultracold methanol (MicroFlow® Basic Kits, Litron Laboratories, Rochester NY; Dertinger *et al*., 2004) and stored in a −80° C freezer until analysis. Flow cytometric analysis was conducted using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Immature erythrocytes or reticulocytes (polychromatic erythrocytes, PCEs) were identified by the presence of an active transferrin receptor (CD71+) on the cell surface; mature erythrocytes were identified as CD71 negative. For these rat blood samples, the analysis was restricted to the youngest reticulocytes (i.e., the subpopulation of immature erythrocytes with the highest CD71 expression) to focus on the population of reticulocytes that were least altered by the efficient action of the rat spleen in sequestering and destroying micronucleated red blood cells (MacGregor *et al*., 2006). Using flow cytometry, micronucleated cells are detected using the DNA staining dye propidium iodide (PI) in conjunction with RNase treatment. Therefore, micronucleated reticulocytes express high levels of CD71 (CD71+) and PI-associated fluorescence. CD71+ reticulocytes without micronuclei show no PI-associated fluorescence. Twenty thousand CD71+ reticulocytes were scored per animal for presence of micronuclei, and approximately 1×10^6 mature erythrocytes were counted for the presence of micronuclei and to determine of the percentage of reticulocytes (% PCEs) as a measure of chemical-induced bone marrow toxicity.

In this assay, the animal is the experimental unit and approximately 20,000 reticulocytes and/or 1×10^6 erythrocytes are evaluated per animal for presence of micronuclei. In addition, the % PCEs was determined in approximately 1×10^6 erythrocytes. The optimum number of cells to score for micronuclei using flow cytometric approaches was determined in earlier studies (Kissling *et al*., 2007). Data from each treatment group are summarized as the mean frequency of micronucleated reticulocytes per 1,000 reticulocytes, plus or minus the standard error of the mean. With the large number of cells counted by flow cytometry, it is assumed that the number of micronucleated cells is normally distributed. Levene's test is used to determine if variances among treatment groups were equal. When they are, linear regression analysis is used to test for linear trend and pairwise differences with the control group are evaluated using Williams' test, after linearizing the data by averaging data points that violate a linear trend. When variances are unequal, nonparametric methods are used to analyze the data: Jonckheere's test is used to evaluate linear trend and Dunn's test is used to assess the significance of pairwise differences with the control group. To maintain the overall significance level at $P\leq0.05$, the trend as well as the pairwise differences from the control group are declared statistically significant if $P\leq0.025$. Ultimately, the scientific staff determines the final call after considering the results of statistical analyses, reproducibility of any effects observed where applicable, and the magnitudes of those effects.

COMET ASSAY PROTOCOL FOR DNA DAMAGE ASSESSMENT

The same rats sampled for the peripheral blood micronucleus assay were sampled for assessment of DNA damage in cells from the blood and colon. The general tissue sample preparation procedures have been described in detail previously (Recio *et al*., 2010). In brief, blood samples (~50 µL) were obtained at the same five time points that were used to assess micronucleus frequencies (Tice *et al*., 2000; Ghanayem *et al*., 2005; Burlinson *et al*., 2007). Blood samples were placed into tubes containing 1 mL of mincing solution (Mg^{+2} and Ca^{+2} free Hank's Balanced Salt Solution with 20 mM EDTA pH 7.4 to 7.7 and 10% v/v fresh DMSO), flash frozen in liquid nitrogen and stored at −80° C prior to shipping. At 12 months, the colon was removed, opened, rinsed thoroughly with cold mincing solution to remove food and debris, and gently scraped to release epithelial cells into 1 mL of mincing solution. Colon samples were flash frozen in liquid nitrogen and stored at −80° C along with the blood samples prior to shipping on dry ice to the genetic toxicology laboratory. Upon arrival at the genetic toxicology laboratory, frozen samples were stored in a −80° C freezer until thawing and processing for DNA damage analysis.

Thawed cell samples were diluted with phosphate buffered saline (PBS), mixed with 0.5% low melting point agarose at 37° C, layered onto slides, and placed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, with freshly added 10% DMSO and 1% Triton X-100) overnight. After rinsing in 0.4 M Trizma base, pH 7.5, slides were treated with cold alkali (300 mM NaOH, 1 mM Na_2EDTA, pH>13) for 20 minutes to allow DNA unwinding, then electrophoresed at 4° to 10° C for 20 minutes at 1.0 V/cm, 300 mA. Slides were then neutralized with 0.4 M Trizma base (pH 7.5) for 5 minutes, incubated for 5 minutes in ice-cold 100% ethanol and allowed to air dry. Slides were stained with SYBR® Gold and 100 cells were scored per leukocyte or colon sample per animal using Comet Assay IV Imaging Software, Version 4.11 (Perceptive Instruments, Ltd., Suffolk, UK). For each cell, the extent of DNA migration was characterized using the percent tail DNA endpoint measurement (intensity of all tail pixels divided by the total intensity of all pixels in the comet, expressed as a percentage).

Five animals per sex per treatment group were analyzed, except no females were analyzed on day 19. Levene's test was used to determine if variances among treatment groups were equal. When they were, linear regression analysis was used to test for linear trend and pairwise differences with the control group were evaluated using Williams' test, after linearizing the data by averaging data points that violated a linear trend. When variances were unequal, nonparametric methods were used to analyze the data: Jonckheere's test was used to evaluate linear trend and Dunn's test was used to assess the significance of pairwise differences with the control group. To maintain the overall significance level at 0.05, the trend as well as the pairwise differences from the control group were declared statistically significant if $P \leq 0.025$. One-tailed tests were used to generate P values for percent tail DNA for the blood samples, and two-tailed tests for both trend and pairwise tests were used to generate P values for percent tail DNA for the colon samples. Ultimately, the scientific staff determined the final call after considering the results of statistical analyses, reproducibility of any effects observed where applicable, and the magnitudes of those effects.

EVALUATION PROTOCOL

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

RESULTS

The percentage of micronucleated immature erythrocytes (reticulocytes) was measured in peripheral blood at five sequential time points during the first year of the 2-year rat study (Tables C1 through C4). Although data on micronucleus frequencies were also collected for the mature erythrocyte population automatically, this cell population is not appropriate for evaluating micronucleus induction in rats due to the rat spleen's ability to efficiently sequester and destroy damaged reticulocytes soon after they emerge from the bone marrow. Therefore, evaluation of the effects of the deficient and excess zinc diets on chromosome integrity was limited to the immature erythrocyte population.

At the first sample time, 19 days after the study began, female rats maintained on the zinc-deficient diet showed a statistically significant ($P \leq 0.025$) increase in micronucleated reticulocytes at both dose levels (3.5 and 7 ppm), although the trend test was not significant ($P > 0.025$). However, the mean micronucleated reticulocyte values observed for these two treatment groups were well within the laboratory historical control range, and in fact, were within the range of values seen in female rats maintained on either deficient or excess zinc diets at all time points. In addition, no increases were seen at any subsequent sampling time, and for all these reasons, the increased frequencies of micronucleated reticulocytes seen on day 19 in female rats were not judged to be biologically

significant. No increases in micronucleated red blood cells were observed at any other sampling time for up to 12 months in either sex.

The percentage of PCEs among total erythrocytes was calculated at each sample time for each sex, and minor, statistically significant, sporadic alterations showing no pattern over time or association with specific diet were observed: zinc-deficient male rats at 19 days; both zinc-deficient and zinc-excess groups of female rats at 6 months. These were considered normal fluctuations and all values were within historical control ranges.

In blood leukocytes (Table C5) and colonic epithelium (Table C6), indications of effects on DNA integrity were observed. In blood leukocyte samples obtained from male rats at 12 months, significant increases in percent tail DNA were observed in both the zinc-deficient and the zinc-excess groups. No significant changes in percent tail DNA were observed in peripheral blood samples at any of the earlier sampling times in either dietary group of male rats. Increased levels of DNA damage were also observed in blood leukocytes of female rats fed the zinc-deficient diet at both the 9- and 12-month sampling times. No significant changes in percent tail DNA were observed in female rat blood samples at any other sampling times in either dietary group.

In colon cell samples obtained after 12 months of exposure, a significant increase in percent tail DNA was observed in male rats (trend, P=0.019) fed a diet with excess zinc, and a small, but not significant, decrease in percent tail DNA was observed in males maintained on the zinc-deficient diet. A significant increase in percent tail DNA was observed in female rats fed a diet supplemented with excess zinc, and a significant decrease in percent tail DNA was observed in females maintained on the zinc-deficient diet. This same pattern of DNA damage was seen in the male rat colon cell samples, although the decreases observed in males fed a zinc-deficient diet were not statistically significant.

Overall, indications of increased levels of DNA damage related to excess dietary levels of zinc were seen in blood leukocytes and colonic epithelial cell samples of male and female rats. In addition, colon cell samples for rats maintained on a zinc-deficient diet showed a significant decrease in DNA migration at 12 months, an observation that is consistent with DNA cross-linking.

TABLE C1
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Male Rats Following Exposure to a Zinc-Deficient Diet for Up to 12 Months[a]

	Dose (ppm)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/ 1,000 NCEs[b]	P Value	PCEs (%)[b]	P Value
Day 19								
	38[d]	5	0.860 ± 0.215		0.349 ± 0.066		2.6 ± 0.1	
	7	5	0.890 ± 0.070	0.457	0.327 ± 0.037	0.695	2.3 ± 0.2	0.426
	3.5	5	0.742 ± 0.077	1.000	0.241 ± 0.065	0.780	1.4 ± 0.1	< 0.001
			P=0.584[e]		P=0.902		P<0.001	
Month 3								
	38	5	0.846 ± 0.054		0.069 ± 0.011		1.3 ± 0.3	
	7	5	0.570 ± 0.089	0.927	0.060 ± 0.006	0.600	1.1 ± 0.1	0.494
	3.5	5	0.690 ± 0.043	0.965	0.067 ± 0.017	0.633	1.0 ± 0.1	0.388
			P=0.912		P=0.548		P=0.305	
Month 6								
	38	5	0.800 ± 0.132		0.056 ± 0.013		0.8 ± 0.0	
	7	5	0.830 ± 0.145	0.446	0.037 ± 0.006	1.000	1.0 ± 0.1	0.139
	3.5	5	0.840 ± 0.176	0.505	0.045 ± 0.003	1.000	1.0 ± 0.1	0.166
			P=0.425		P=0.479		P=0.317	
Month 9								
	38	5	0.994 ± 0.136		0.073 ± 0.008		0.9 ± 0.1	
	7	5	1.030 ± 0.101	0. 663	0.122 ± 0.015	0.053	1.1 ± 0.1	0.236
	3.5	5	0.760 ± 0.081	0.748	0.106 ± 0.024	0.065	0.9 ± 0.0	0.282
			P=0.923		P=0.106		P=0.712	
Month 12	38	5	1.140 ± 0.062		0.086 ± 0.033		1.2 ± 0.1	
	7	5	0.950 ± 0.101	0.972	0.044 ± 0.006	1.000	1.0 ± 0.1	0.223
	3.5	5	0.700 ± 0.022	0.989	0.044 ± 0.003	1.000	0.9 ± 0.1	0.064
			P=1.000		P=0.968		P=0.046	

[a] Study was performed at ILS, Inc. The detailed protocol is presented by Dertinger *et al.* (2004), MacGregor *et al.* (2006), and Witt *et al.* (2008). NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte

[b] Mean ± standard error

[c] Pairwise comparison with the control group; values are significant at P≤0.025 by Williams' or Dunn's test.

[d] 38 ppm is the control group.

[e] Dose-related trend; significant at P≤0.025 by linear regression or Jonckheere's test.

TABLE C2
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Male Rats Following Exposure to a Zinc-Excess Diet for Up to 12 Months[a]

	Dose (ppm)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/ 1,000 NCEs[b]	P Value	PCEs (%)[b]	P Value
Day 19								
	38[d]	5	0.860 ± 0.215		0.349 ± 0.066		2.6 ± 0.1	
	250	5	0.660 ± 0.070	1.000	0.243 ± 0.083	0.871	3.3 ± 0.1	0.166
	500	5	0.780 ± 0.152	1.000	0.134 ± 0.024	0.928	2.4 ± 0.1	0.196
			P=0.521[e]		P=0.987		P=0.557	
Month 3								
	38	5	0.846 ± 0.054		0.069 ± 0.011		1.3 ± 0.3	
	250	5	0.850 ± 0.122	0.640	0.057 ± 0.017	0.671	1.0 ± 0.1	0.402
	500	5	0.710 ± 0.058	0.727	0.055 ± 0.010	0.757	1.0 ± 0.1	0.224
			P=0.867		P=0.768		P=0.172	
Month 6								
	38	5	0.800 ± 0.132		0.056 ± 0.013		0.8 ± 0.0	
	250	5	0.840 ± 0.083	0.614	0.029 ± 0.003	1.000	0.9 ± 0.1	0.403
	500	5	0.640 ± 0.048	0.702	0.031 ± 0.004	1.000	0.9 ± 0.0	0.337
			P=0.873		P=0.866		P=0.264	
Month 9								
	38	5	0.994 ± 0.136		0.073 ± 0.008		0.9 ± 0.1	
	250	5	1.470 ± 0.200	0.068	0.177 ± 0.043	0.104	1.0 ± 0.1	0.467
	500	5	1.210 ± 0.111	0.082	0.074 ± 0.008	0.777	1.0 ± 0.0	0.562
			P=0.192		P=0.396		P=0.603	
Month 12								
	38	5	1.140 ± 0.062		0.086 ± 0.033		1.2 ± 0.1	
	250	5	0.910 ± 0.068	0.846	0.055 ± 0.007	1.000	1.2 ± 0.1	0.788
	500	5	0.990 ± 0.113	0.908	0.036 ± 0.004	1.000	1.1 ± 0.1	0.537
			P=0.875		P=0.988		P=0.429	

[a] Study was performed at ILS, Inc. The detailed protocol is presented by Dertinger *et al.* (2004), MacGregor *et al.* (2006), and Witt *et al.* (2008). NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte

[b] Mean ± standard error

[c] Pairwise comparison with the control group; values are significant at P≤0.025 by Williams' or Dunn's test.

[d] 38 ppm is the control group.

[e] Dose-related trend; significant at P≤0.025 by linear regression or Jonckheere's test.

TABLE C3
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Female Rats Following Exposure to a Zinc-Deficient Diet for Up to 12 Months[a]

	Dose (ppm)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/ 1,000 NCEs[b]	P Value	PCEs (%)[b]	P Value
Day 19								
	38[d]	5	0.623 ± 0.085		0.126 ± 0.041		0.9 ± 0.2	
	7	5	0.970 ± 0.086	0.011	0.232 ± 0.026	0.100	1.3 ± 0.2	0.117
	3.5	5	0.860 ± 0.064	0.013	0.180 ± 0.054	0.120	1.3 ± 0.1	0.092
			P=0.045[e]		P=0.201		P=0.074	
Month 3								
	38	5	0.840 ± 0.095		0.073 ± 0.009		0.7 ± 0.1	
	7	5	0.750 ± 0.061	0.637	0.052 ± 0.016	0.717	1.2 ± 0.1	0.022
	3.5	5	0.840 ± 0.103	0.584	0.064 ± 0.010	0.775	0.9 ± 0.1	0.578
			P=0.500		P=0.691		P=0.267	
Month 6								
	38	5	0.830 ± 0.108		0.043 ± 0.008		0.6 ± 0.1	
	7	5	1.110 ± 0.154	0.076	0.049 ± 0.015	0.427	0.7 ± 0.0	0.120
	3.5	5	1.120 ± 0.123	0.084	0.042 ± 0.006	0.504	0.9 ± 0.1	0.012
			P=0.067		P=0.520		P=0.008	
Month 9								
	38	5	1.020 ± 0.064		0.024 ± 0.003		0.8 ± 0.1	
	7	5	0.960 ± 0.171	0.564	0.040 ± 0.009	0.106	0.9 ± 0.1	0.555
	3.5	5	1.040 ± 0.126	0.538	0.036 ± 0.009	0.126	0.9 ± 0.1	0.390
			P=0.456		P=0.135		P=0.306	
Month 12								
	38	5	1.220 ± 0.046		0.054 ± 0.010		1.0 ± 0.3	
	7	5	1.290 ± 0.155	0.740	0.043 ± 0.007	0.650	1.0 ± 0.1	0.729
	3.5	5	0.788 ± 0.154	0.820	0.046 ± 0.015	0.736	2.7 ± 0.2	0.211
			P=0.976		P=0.687		P=0.164	

[a] Study was performed at ILS, Inc. The detailed protocol is presented by Dertinger *et al.* (2004), MacGregor *et al.* (2006), and Witt *et al.* (2008). NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte

[b] Mean ± standard error

[c] Pairwise comparison with the control group; values are significant at P≤0.025 by Williams' or Dunn's test.

[d] 38 ppm is the control group.

[e] Dose-related trend; significant at P≤0.025 by linear regression or Jonckheere's test.

TABLE C4
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Female Rats Following Exposure to a Zinc-Excess Diet for Up to 12 Months[a]

Dose (ppm)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/ 1,000 NCEs[b]	P Value	PCEs (%)[b]	P Value
Day 19							
38[d]	5	0.623 ± 0.085		0.126 ± 0.041		0.9 ± 0.2	
250	5	0.700 ± 0.076	0.368	0.124 ± 0.014	0.506	1.3 ± 0.3	0.185
500	5	0.620 ±0.068	0.438	0.149 ±0.025	0.349	1.6 ± 0.3	0.097
		P=0.511[e]		P=0.285		P=0.073	
Month 3							
38	5	0.840 ± 0.095		0.073 ± 0.009		0.7 ± 0.1	
250	5	0.820 ± 0.064	0.572	0.090 ± 0.016	0.576	1.2 ± 0.2	0.041
500	5	0.780 ± 0.130	0.660	0.044 ± 0.015	0.633	1.1 ± 0.2	0.048
		P=0.667		P=0.902		P=0.096	
Month 6							
38	5	0.830 ± 0.108		0.043 ± 0.008		0.6 ± 0.1	
250	5	1.030 ± 0.128	0.279	0.071 ± 0.018	0.289	1.0 ± 0.1	0.019
500	5	0.860 ± 0.164	0.334	0.043 ± 0.009	1.000	1.1 ± 0.1	0.007
		P=0.439		P=0.521		P=0.006	
Month 9							
38	5	1.020 ± 0.064		0.024 ± 0.003		0.8 ± 0.1	
250	5	0.869 ± 0.145	1.000	0.030 ± 0.007	0.224	0.9 ± 0.2	0.741
500	5	0.820 ± 0.108	1.000	0.028 ± 0.001	0.271	0.8 ± 0.1	0.861
		P=0.831		P=0.265		P=0.836	
Month 12							
38	5	1.220 ± 0.046		0.054 ± 0.010		1.0 ± 0.3	
250	5	0.975 ± 0.156	0.842	0.030 ± 0.005	0.890	1.3 ± 0.3	0.443
500	5	0.925 ± 0.131	0.911	0.030 ± 0.006	0.943	0.9 ± 0.1	0.532
		P=0.963		P=0.972		P=0.809	

[a] Study was performed at ILS, Inc. The detailed protocol is presented by Dertinger *et al.* (2004), MacGregor *et al.* (2006), and Witt *et al.* (2008). NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte

[b] Mean ± standard error

[c] Pairwise comparison with the control group; values are significant at P≤0.025 by Williams' or Dunn's test.

[d] 38 ppm is the control group.

[e] Dose-related trend; significant at P≤0.025 by linear regression or Jonckheere's test.

TABLE C5
DNA Damage in the Blood of Rats Administered Varying Levels of Zinc in the Diet for up to 12 Months[a]

Exposure Concentration (ppm)	Percent Tail DNA	P Value
Male		
Day 19		
38 (Control)	2.24 ± 0.30	
3.5	2.89 ± 0.26	0.101
7	1.91 ± 0.37	0.644
	P=0.098[d]	
250	1.92 ± 0.31	0.723
500	1.77 ± 0.31	0.805
	P=0.861	
Month 3		
38 (Control)	3.25 ± 0.37	
3.5	2.35 ± 0.52	0.910
7	2.28 ± 0.32	0.848
	P=0.928	
250	2.50 ± 0.41	0.771
500	2.35 ± 0.68	0.849
	P=0.892	
Month 6		
38 (Control)	1.46 ± 0.19	
3.5	1.94 ± 0.29	0.120
7	1.44 ± 0.27	0.510
	P=0.098	
250	2.37 ± 0.24	0.028
500	3.08 ± 1.05	0.104
	P=0.041	
Month 9		
38 (Control)	6.67 ± 1.05	
3.5	5.80 ± 0.78	0.521
7	8.00 ± 1.29	0.442
	P=0.710	
250	5.61 ± 0.65	0.660
500	6.12 ± 0.94	0.746
	P=0.667	

TABLE C5
DNA Damage in the Blood of Rats Administered Varying Levels of Zinc in the Diet for up to 12 Months

Exposure Concentration (ppm)	Percent Tail DNA	P Value
Male (continued)		
Month 12		
38 (Control)	5.25 ± 0.59	
3.5	8.90 ± 0.39	0.011
7	7.78 ± 1.07	0.056
	P=0.007	
250	8.15 ± 0.41	0.045
500	8.86 ± 1.80	0.025
	P=0.019	
Female		
Month 3		
38 (Control)	4.04 ± 0.75	
3.5	6.79 ± 1.46	0.049
7	3.80 ± 0.67	0.532
	P=0.043	
250	4.05 ± 0.58	0.649
500	3.04 ± 0.41	0.736
	P=0.877	
Month 6		
38 (Control)	2.86 ± 0.26	
3.5	2.84 ± 0.21	1.000
7	4.00 ± 0.93	0.596
	P=0.500	
250	3.71 ± 0.58	0.202
500	3.00 ± 0.31	0.245
	P=0.409	
Month 9		
38 (Control)	3.16 ± 0.43	
3.5	8.82 ± 1.19	0.001
7	4.80 ± 1.15	0.013
	P=0.001	
250	3.13 ± 0.36	0.551
500	3.00 ± 0.21	0.638
	P=0.663	

TABLE C5
DNA Damage in the Blood of Rats Administered Varying Levels of Zinc in the Diet for up to 12 Months

Exposure Concentration (ppm)	Percent Tail DNA	P Value
Female (continued)		
Month 12		
38 (Control)	7.97 ± 1.36	
3.5	17.32 ± 1.33	0.001
7	6.22 ± 2.15	0.642
	P=0.005	
250	7.32 ± 1.70	0.589
500	7.24 ± 1.07	0.677
	P=0.647	

[a] Study was performed at ILS, Inc. The detailed protocol is presented by Recio *et al.* (2010).
[b] Mean ± standard error. n=5
[c] Pairwise comparison with the control group; exposed group values are significant at P≤0.025 by Williams' or Dunn's test.
[d] Exposure concentration-related trend; significant at P≤0.025 by linear regression or Jonckheere's test.

TABLE C6
DNA Damage in the Colon of Rats Administered Varying Levels of Zinc in the Diet for 12 Months[a]

Exposure Concentration (ppm)	Percent Tail DNA	P Value
Male		
38 (Control)	12.54 ± 2.52	
3.5	8.20 ± 0.70	0.066
7	7.03 ± 1.18	0.056
	P=0.104[d]	
250	14.84 ± 1.60	0.395
500	19.36 ± 1.17	0.026
	P=0.019	
Female		
38 (Control)	14.13 ± 0.99	
3.5	4.47 ± 1.74	0.004
7	9.16 ± 1.01	0.154
	P=0.001	
250	13.45 ± 1.40	1.000
500	19.04 ± 0.81	0.009
	P=0.017	

[a] Study was performed at ILS, Inc. The detailed protocol is presented by Recio *et al.* (2010).
[b] Mean ± standard error. n=5
[c] Pairwise comparison with the control group; exposed group values are significant at P≤0.025 by Williams or Dunn's test.
[d] Exposure concentration-related trend; significant at P≤0.025 by linear regression or Jonckheere's test.

APPENDIX D
HEMATOLOGY RESULTS
AND TRACE METAL METHODS AND RESULTS

HEMATOLOGY RESULTS AND TRACE METAL METHODS AND RESULTS

MATERIALS AND METHODS FOR TRACE METAL CONCENTRATIONS

Study Design

Ten male and 10 female special study rats were exposed to 3.5, 7, 38 (control), 250, or 500 ppm zinc in the diet. Blood was collected on day 19 and at 3, 6, 9, and 12 months following exposure for determination of trace metal concentrations. All samples were frozen at −70° C and shipped to the analytical chemistry laboratory (Research Triangle Institute (RTI), Research Triangle Park, NC).

Analysis of Blood for Zinc, Copper, and Iron

Samples were analyzed for zinc concentrations in blood with a validated analytical method using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). The validation was accomplished using calibration standards prepared in an acid matrix that matched the digested blood samples and the performance of the assay in blood was demonstrated using matrix standards run on male and female rat blood. All standards used were National Institute of Standards and Technology–traceable. To mitigate the potential for contamination, high-purity, Ultrex grade acids and oxidants and approximately 18 MΩ quality deionized (DI) water were used. In addition, all new labware (pipette tips and centrifuge tubes) and reusable labware were cleaned prior to use with DI water and 20% (v/v) nitric acid, respectively, and dried under HEPA-filtered air. All sample preparation activities took place in a HEPA-filtered environment, and a high percentage of method blanks approximating 20% of the study sample numbers were processed with each sample batch to continuously monitor the analyte background contribution from the reagents and procedure to ensure the background levels were controlled (see below).

The analytical method was validated over the zinc concentration range of 0.030 to 1.00 µg/mL in the acid digestion matrix (10% v/v nitric acid), which translates to 2.40 to 80.0 µg/mL of blood using 125 µL of blood in the assay. The following criteria were met for linearity (correlation coefficient, $r \geq 0.99$), precision [relative standard deviation (RSD) ≤ 15% except at the experimental limit of quantitation (ELOQ) where RSD ≤ 20%], and accuracy relative error (RE) ≤ ± 10% (except at the ELOQ RE ≤ ± 20%). The ELOQ and estimated limit of detection (LOD) for zinc were 2.40 and 0.6 µg/mL of blood, respectively. During validation, this ELOQ was conservatively established to exceed the observed analyte background levels. Dilution verification was conducted to demonstrate that concentrations outside the validated range could be accurately quantitated after dilution. Stability of zinc in blood digests prepared for analysis stored under ambient and refrigerated conditions were demonstrated for up to 2 hours and 32 days, respectively. Stability of zinc in blood that had undergone three freeze-thaw cycles and stored at −70° C for up to 61 days was also established. The assay was qualified to quantitate copper and iron, respectively, over the concentration range of 0.010 to 0.100 and 1.00 to 20.0 µg/mL of acid digestion matrix (10% v/v nitric acid), which translates to 0.800 to 8.00 and 80.0 to 1,600 µg/mL in blood for copper and iron, respectively. The ELOQ and LOD in blood for copper were 0.800 and 0.2 µg/mL respectively, and the ELOQ for iron was 80 µg/mL.

All study samples were stored frozen at −70° C until analysis. Prior to preparation, samples were allowed to thaw at room temperature in a plastic hood. A 125 µL-aliquot of blood was transferred into a digestion tube along with 1 mL each of nitric acid and DI water. All tubes were tightly capped with digestion caps and digested in two sequences using a microwave (maximum power 800 watts) at 50% power as given below. During sequence 1, temperature was ramped to 60º C in 5 minutes and held for 20 minutes followed by ramping to 75º C in 5 minutes and held at 10 minutes. Samples were allowed to cool to room temperature, 1 mL of hydrogen peroxide was added to each tube, and subjected to a second sequence of digestion as follows; temperature was ramped to 75º C in 5 minutes, held for 10 minutes, ramped to 90º C in 5 minutes, held for 20 minutes, and ramped to 100º C in 5 minutes and held for 10 minutes. After the digestion was complete, samples were allowed to come to room temperature and brought to a final volume of 10 mL with DI water. An aliquot of the supernatant was transferred to a vial for analysis by ICP-OES.

All samples were analyzed using ICP-OES on a Perkin-Elmer Optima 4300DV (Waltham, MA). Analyses wave lengths were set for zinc at 213.857 nm, copper at 327.393 nm, and iron at 259.939 nm using a cyclonic spray chamber with three scans/analysis at radio frequency power of 1,450 watts. The concentration of each analyte was calculated using its individual response, the regression equation, and dilution when applicable. Samples with responses greater than the highest calibration standard were

diluted with the diluent to get a response within the range. The concentrations of zinc, copper, and iron were expressed as µg/mL of blood. Concentrations less than the LOD were reported as not detected.

A large number of control samples (approximately 50% of the number of study samples) were prepared and analyzed with each batch of study samples, including method blanks, method controls, quality control (QC) samples and certified reference material (CRM) (International Atomic Energy Agency CRM IAEA-A-13, trace elements in freeze dried animal blood). Calibration standards of zinc (0 to 1.00 µg/mL), copper (0 to 0.100 µg/mL) and iron (0 to 20 µg/mL) were prepared with a minimum of six concentrations in acid digestion matrix (10% v/v nitric acid). The performance of the calibration curve was evaluated prior to the analysis of each sample set. A successful calibration was indicated by an acceptable correlation coefficient ($r > 0.99$) and error within ± 20% of the nominal concentration for the lowest standard and within ± 10% of the nominal concentration for all other standards.

With each batch of 25 study samples, five method blanks, two method controls, and three CRMs were bracketed by two QC sets. Method blanks were prepared in a manner identical to the study samples but excluding blood to monitor for assay background levels. Method controls were included to determine analyte recovery in the absence of the blood matrix. Method controls were prepared in a manner identical to the study samples excluding blood but adding analytes to give final representative blood concentrations of 16, 4, and 400 µg/mL, for zinc, copper, and iron, respectively. CRM samples were prepared with each sample batch in a manner identical to the study samples where final concentrations were 13.0, 4.3, and 2,400 mg analyte/kg blood for zinc, copper and iron, respectively.

The mean percent recoveries for CRM were ≥ 93.1 for zinc, ≥ 73.5 for copper, and ≥ 86.6 for iron, of the recommended concentration in CRM. The mean percent recoveries for method controls were ≥ 91.3 for zinc, ≥ 85.8 for copper and ≥ 92.5 for iron of the nominal value. Analyte levels in method blanks were below the ELOQ for both copper and iron. For zinc, levels were below the ELOQ for all (n=97) except three samples; the average values (± standard deviation) with and without the three samples above ELOQ were, 0.662 (0.650) and 0.570 (0.369) µg/mL blood, respectively.

Data from study samples were considered valid if they were bracketed by valid QC sets. A QC set passed when the measured concentration for the QC standard was within 10% of its nominal value. If the QC standard failed, it was necessary to reanalyze the bracketed samples. Any samples with a response greater than the calibration range required dilution into range and reanalysis. All analyte values above the LOD were reported.

Statistical Analyses

Data were analyzed using the nonparametric multiple comparison methods of Shirley (1977) (as modified by Williams, 1986) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1957) were examined by NTP personnel, and implausible values were eliminated from the analysis. All values above the LOD and below the ELOQ were used as reported. In groups with at least 20% of the values above the LOD, the samples that were below the LOD were replaced with half of the LOD value in order to provide non-zero values for statistical analyses.

TABLE D1
Hematology Data for Rats in the 2-Year Feed Study of Dietary Zinc[a]

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Male					
n	10	10	9	10	10
Hematocrit (auto) (%)					
Day 19	44.7 ± 0.6[b]	44.1 ± 0.5[c]	42.0 ± 0.4**[d]	42.4 ± 0.7*[b]	41.7 ± 0.8**
Month 3	50.0 ± 0.6	49.1 ± 0.5	49.8 ± 0.7	50.2 ± 0.4[b]	49.0 ± 0.7
Month 6	49.0 ± 0.5	48.9 ± 0.7	48.3 ± 0.6	47.9 ± 0.4	47.2 ± 0.3*
Month 9	47.9 ± 0.5	47.9 ± 0.4[b]	47.0 ± 0.5	47.9 ± 0.6	48.1 ± 0.3[b]
Month 12	49.8 ± 0.4	48.6 ± 0.6[c]	48.3 ± 0.7	50.3 ± 0.5	49.6 ± 0.2
Hematocrit (manual) (%)					
Day 19	43.2 ± 0.7[b]	43.3 ± 0.7[c]	41.6 ± 0.5[d]	41.9 ± 0.7[b]	40.7 ± 0.7
Month 3	54.0 ± 0.9	51.9 ± 0.8	58.0 ± 3.2	54.2 ± 1.0[b]	52.9 ± 1.3[b]
Month 6	49.2 ± 0.5	48.8 ± 0.8	48.2 ± 0.6	48.3 ± 0.5	48.0 ± 0.4
Month 9	48.7 ± 0.7	49.2 ± 0.6[b]	48.3 ± 0.5[c]	48.2 ± 0.3	49.2 ± 0.5[b]
Month 12	48.8 ± 0.4	48.9 ± 0.6[c]	49.0 ± 0.7	49.3 ± 0.4	48.7 ± 0.4
Hemoglobin (g/dL)					
Day 19	13.6 ± 0.2[b]	13.9 ± 0.1[c]	13.0 ± 0.1*[d]	13.1 ± 0.2[b]	12.7 ± 0.2**
Month 3	15.3 ± 0.2	15.1 ± 0.1	15.2 ± 0.2	15.3 ± 0.1[b]	15.0 ± 0.2
Month 6	15.6 ± 0.2	15.2 ± 0.2	15.2 ± 0.1	15.1 ± 0.2	14.9 ± 0.1*
Month 9	15.3 ± 0.1	15.5 ± 0.2[b]	15.3 ± 0.2	15.2 ± 0.2	15.2 ± 0.1[b]
Month 12	15.6 ± 0.1	15.4 ± 0.2[c]	15.5 ± 0.2	15.6 ± 0.1	15.3 ± 0.1
Erythrocytes (10^6/µL)					
Day 19	7.21 ± 0.09[b]	7.23 ± 0.10[c]	6.78 ± 0.07**[d]	6.91 ± 0.12[b]	7.14 ± 0.14
Month 3	8.82 ± 0.08	8.53 ± 0.10	8.73 ± 0.09	8.87 ± 0.06[b]	8.93 ± 0.12
Month 6	8.69 ± 0.09	8.50 ± 0.12	8.55 ± 0.09	8.42 ± 0.08	8.56 ± 0.07
Month 9	8.52 ± 0.09	8.49 ± 0.08[b]	8.35 ± 0.05	8.50 ± 0.09	8.70 ± 0.12[b]
Month 12	8.77 ± 0.09	8.65 ± 0.08[c]	8.64 ± 0.09	8.80 ± 0.09	8.83 ± 0.11
Reticulocytes (10^3/µL)					
Day 19	356.5 ± 11.2[b]	230.0 ± 16.3**[c]	312.4 ± 11.6*[d]	358.9 ± 11.4[b]	325.4 ± 15.0
Month 3	199.0 ± 15.8	177.3 ± 4.6	180.3 ± 6.2	193.5 ± 7.3[b]	182.7 ± 8.3
Month 6	188.1 ± 9.9	185.3 ± 7.6	190.7 ± 10.7	175.3 ± 6.3	161.1 ± 6.4
Month 9	190.2 ± 8.3	177.4 ± 5.7[b]	186.7 ± 9.0	186.8 ± 12.2	171.9 ± 8.8[b]
Month 12	216.6 ± 10.0	178.2 ± 9.5*[c]	205.9 ± 9.4	206.1 ± 13..1	179.7 ± 9.7*
Mean cell volume (fL)					
Day 19	62.0 ± 0.4[b]	60.9 ± 0.4[c]	62.0 ± 0.4[d]	61.4 ± 0.2[b]	58.4 ± 0.6**
Month 3	56.7 ± 0.4	57.5 ± 0.2	57.1 ± 0.5	56.6 ± 0.3[b]	54.9 ± 0.5**
Month 6	56.5 ± 0.4	57.5 ± 0.3	56.5 ± 0.4	56.9 ± 0.3	55.2 ± 0.5
Month 9	56.2 ± 0.4	56.5 ± 0.4[b]	56.3 ± 0.5	56.4 ± 0.4	55.3 ± 0.6[b]
Month 12	56.8 ± 0.4	56.2 ± 0.4[c]	55.9 ± 0.6	57.2 ± 0.4	56.2 ± 0.6
Mean cell hemoglobin (pg)					
Day 19	18.9 ± 0.2[b]	19.2 ± 0.1[c]	19.2 ± 0.1[d]	19.0 ± 0.1[b]	17.8 ± 0.2**
Month 3	17.4 ± 0.1	17.7 ± 0.1	17.4 ± 0.1	17.3 ± 0.1[b]	16.8 ± 0.2**
Month 6	17.9 ± 0.1	17.9 ± 0.1	17.8 ± 0.1	18.0 ± 0.1	17.4 ± 0.2*
Month 9	18.0 ± 0.1	18.3 ± 0.2[b]	18.3 ± 0.2	17.9 ± 0.1	17.5 ± 0.2[b]
Month 12	17.8 ± 0.2	17.9 ± 0.1[c]	17.9 ± 0.2	17.8 ± 0.2	17.3 ± 0.2
Mean cell hemoglobin concentration (g/dL)					
Day 19	30.5 ± 0.2[b]	31.5 ± 0.1**[c]	30.9 ± 0.1[d]	30.9 ± 0.1[b]	30.5 ± 0.2
Month 3	30.7 ± 0.2	30.8 ± 0.2	30.5 ± 0.2	30.5 ± 0.1[b]	30.5 ± 0.1
Month 6	31.7 ± 0.3	31.1 ± 0.1	31.4 ± 0.2	31.6 ± 0.1	31.6 ± 0.1
Month 9	32.0 ± 0.2	32.4 ± 0.2[b]	32.5 ± 0.2	31.8 ± 0.1	31.6 ± 0.2[b]
Month 12	31.3 ± 0.2	31.8 ± 0.1[c]	32.0 ± 0.1**	31.0 ± 0.1	30.8 ± 0.1*

TABLE D1
Hematology Data for Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Male (continued)					
n	10	10	9	10	10
Platelets ($10^3/\mu L$)					
Day 19	1,074 ± 40[b]	930 ± 42[c]	1,032 ± 57[d]	974 ± 73[b]	984 ± 38
Month 3	765 ± 46	752 ± 50	830 ± 44	769 ± 34[b]	752 ± 29
Month 6	763 ± 52	777 ± 29	786 ± 20	806 ± 50	811 ± 23
Month 9	713 ± 36	799 ± 38[b]	653 ± 49	709 ± 67	702 ± 61[b]
Month 12	491 ± 51	546 ± 24[c]	521 ± 64	486 ± 21	546 ± 36
Leukocytes ($10^3/\mu L$)					
Day 19	14.56 ± 0.62[b]	13.20 ± 1.08[c]	12.93 ± 0.48[d]	12.52 ± 0.63[b]	12.38 ± 0.91
Month 3	10.95 ± 0.57	11.17 ± 0.53	11.63 ± 0.60	11.09 ± 0.49[b]	9.95 ± 0.52
Month 6	10.96 ± 0.56	11.53 ± 0.94	11.84 ± 0.83	10.54 ± 0.33	9.81 ± 0.43
Month 9	10.54 ± 0.60	11.48 ± 0.44[b]	11.94 ± 0.58	12.07 ± 0.87	10.33 ± 0.65[b]
Month 12	11.93 ± 0.51	8.84 ± 0.42**[c]	9.77 ± 0.44*	12.03 ± 0.71	11.16 ± 0.63
Segmented neutrophils ($10^3/\mu L$)					
Day 19	1.43 ± 0.19[b]	1.72 ± 0.17[c]	1.60 ± 0.07[d]	1.24 ± 0.12[b]	1.21 ± 0.12
Month 3	1.23 ± 0.15	1.42 ± 0.16	0.98 ± 0.07	1.07 ± 0.07[b]	0.96 ± 0.07
Month 6	1.44 ± 0.15	1.85 ± 0.50	1.63 ± 0.31	1.38 ± 0.12	1.33 ± 0.08
Month 9	1.60 ± 0.12	1.44 ± 0.11[b]	1.82 ± 0.16	2.36 ± 0.55	1.59 ± 0.20[b]
Month 12	2.24 ± 0.22	2.18 ± 0.36[c]	1.83 ± 0.13	1.74 ± 0.16	1.75 ± 0.17
Lymphocytes ($10^3/\mu L$)					
Day 19	12.48 ± 0.48[b]	10.91 ± 0.88[c]	10.76 ± 0.43*[d]	10.73 ± 0.52[b]	10.62 ± 0.78
Month 3	9.24 ± 0.46	9.31 ± 0.38	10.20 ± 0.53	9.64 ± 0.43[b]	8.65 ± 0.45
Month 6	9.10 ± 0.49	9.15 ± 0.60	9.75 ± 0.66	8.70 ± 0.30	7.99 ± 0.35
Month 9	8.33 ± 0.53	9.39 ± 0.36[b]	9.40 ± 0.51	8.92 ± 0.59	8.17 ± 0.40[b]
Month 12	8.83 ± 0.39	6.04 ± 0.32**[c]	7.17 ± 0.35**	9.39 ± 0.51	8.67 ± 0.45
Monocytes ($10^3/\mu L$)					
Day 19	0.55 ± 0.03[b]	0.40 ± 0.03**[c]	0.44 ± 0.03*[d]	0.44 ± 0.05[b]	0.42 ± 0.05
Month 3	0.32 ± 0.03	0.27 ± 0.03	0.28 ± 0.04	0.25 ± 0.02[b]	0.20 ± 0.02**
Month 6	0.30 ± 0.02	0.32 ± 0.04	0.33 ± 0.06	0.30 ± 0.03	0.32 ± 0.03
Month 9	0.38 ± 0.04	0.39 ± 0.04[b]	0.45 ± 0.04	0.55 ± 0.10	0.36 ± 0.05[b]
Month 12	0.58 ± 0.05	0.39 ± 0.04**[c]	0.53 ± 0.07	0.59 ± 0.10	0.49 ± 0.05
Basophils ($10^3/\mu L$)					
Day 19	0.05 ± 0.00[b]	0.04 ± 0.01[c]	0.04 ± 0.00[d]	0.04 ± 0.00[b]	0.04 ± 0.01
Month 3	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.00[b]	0.03 ± 0.00
Month 6	0.02 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Month 9	0.04 ± 0.00	0.05 ± 0.00[b]	0.05 ± 0.00	0.06 ± 0.01	0.04 ± 0.01[b]
Month 12	0.02 ± 0.01	0.02 ± 0.00[c]	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00
Eosinophils ($10^3/\mu L$)					
Day 19	0.05 ± 0.01[b]	0.12 ± 0.03*[c]	0.09 ± 0.02[d]	0.09 ± 0.02[b]	0.09 ± 0.02
Month 3	0.13 ± 0.01	0.14 ± 0.03	0.12 ± 0.01	0.10 ± 0.01[b]	0.10 ± 0.01
Month 6	0.11 ± 0.02	0.18 ± 0.04	0.10 ± 0.02	0.13 ± 0.01	0.15 ± 0.01
Month 9	0.20 ± 0.03	0.21 ± 0.03[b]	0.22 ± 0.05	0.19 ± 0.03	0.18 ± 0.03[b]
Month 12	0.26 ± 0.05	0.22 ± 0.03[c]	0.21 ± 0.02	0.27 ± 0.06	0.22 ± 0.04

TABLE D1
Hematology Data for Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Female					
n	10	10	9	9	10
Hematocrit (auto) (%)					
Day 19	42.4 ± 0.6	45.3 ± 0.8**[e]	43.7 ± 0.6	42.8 ± 0.5[d]	41.9 ± 0.5
Month 3	44.2 ± 0.7	45.4 ± 0.5	46.3 ± 0.3[d]	45.4 ± 0.6	44.0 ± 0.6
Month 6	42.8 ± 0.7	44.8 ± 0.5*	43.5 ± 1.1[d]	44.2 ± 0.4	42.8 ± 0.5
Month 9	42.2 ± 0.7	43.2 ± 0.6	43.8 ± 0.5	44.5 ± 0.8	42.5 ± 0.5[b]
Month 12	44.9 ± 1.1[b]	45.9 ± 0.9	46.2 ± 0.6	46.2 ± 0.6	44.6 ± 0.7[c]
Hematocrit (manual) (%)					
Day 19	42.4 ± 0.5	45.6 ± 0.7**[e]	43.9 ± 0.6*	43.7 ± 0.6[d]	42.2 ± 0.6
Month 3	44.6 ± 0.9	46.4 ± 0.7	47.0 ± 0.5[d]	46.0 ± 0.7	45.3 ± 0.5
Month 6	44.0 ± 0.7	45.0 ± 0.5	44.9 ± 1.1[d]	44.6 ± 0.4	43.6 ± 0.5
Month 9	44.6 ± 0.7 [c]	45.0 ± 0.6	45.6 ± 0.5	45.8 ± 0.7	44.2 ± 0.3[b]
Month 12	44.3 ± 1.1[b]	46.5 ± 1.0	45.8 ± 0.7	45.8 ± 0.7	43.8 ± 0.8[c]
Hemoglobin (g/dL)					
Day 19	13.5 ± 0.2	14.2 ± 0.1**[e]	13.8 ± 0.2	13.6 ± 0.2[d]	13.0 ± 0.2
Month 3	14.1 ± 0.2	14.5 ± 0.1	14.5 ± 0.1[d]	14.3 ± 0.1	13.9 ± 0.2
Month 6	13.8 ± 0.2	14.7 ± 0.2**	13.9 ± 0.4[d]	14.5 ± 0.1	13.7 ± 0.2
Month 9	14.0 ± 0.2	14.3 ± 0.2	14.3 ± 0.2	14.5 ± 0.3	13.8 ± 0.2[b]
Month 12	14.0 ± 0.3[b]	14.4 ± 0.3	14.4 ± 0.2	14.4 ± 0.2	13.9 ± 0.2[c]
Erythrocytes (10^6/μL)					
Day 19	6.97 ± 0.10	7.46 ± 0.12*[e]	7.11 ± 0.14[d]	7.08 ± 0.07[d]	7.10 ± 0.08
Month 3	7.84 ± 0.09	8.05 ± 0.08	8.14 ± 0.10 [d]	8.00 ± 0.06	7.87 ± 0.08
Month 6	7.52 ± 0.09	7.77 ± 0.09	7.39 ± 0.34 [d]	7.76 ± 0.08	7.56 ± 0.08
Month 9	7.57 ± 0.10	7.66 ± 0.08	7.71 ± 0.09	7.87 ± 0.13	7.62 ± 0.07[b]
Month 12	7.67 ± 0.18[b]	7.73 ± 0.28	7.84 ± 0.13	7.97 ± 0.13	7.73 ± 0.11[c]
Reticulocytes (10^3/μL)					
Day 19	194.3 ± 18.2	216.4 ± 13.8[e]	214.0 ± 14.1	221.3 ± 18.3[d]	213.9 ± 20.4
Month 3	163.3 ± 11.6	187.4 ± 14.7	204.5 ± 9.4*[d]	188.0 ± 15.6	171.0 ± 10.8
Month 6	166.5 ± 17.0	191.6 ± 15.3	293.4 ± 125.6[d]	164.1 ± 15.5	157.0 ± 14.3
Month 9	143.9 ± 8.0	176.2 ± 10.3	171.0 ± 5.9	169.2 ± 17.8	144.1 ± 12.3[b]
Month 12	172.2 ± 19.0[b]	258.9 ± 66.5	201.8 ± 9.2	215.4 ± 26.9	158.7 ± 13.2[c]
Mean cell volume (fL)					
Day 19	60.8 ± 0.2	60.7 ± 0.3[e]	61.5 ± 0.[b]	60.5 ± 0.5[d]	58.9 ± 0.4*
Month 3	56.3 ± 0.5	56.5 ± 0.4	56.9 ± 0.4	56.7 ± 0.5	55.9 ± 0.5
Month 6	56.9 ± 0.6	57.6 ± 0.4	59.6 ± 2.0	56.9 ± 0.3	56.6 ± 0.5
Month 9	55.7 ± 0.4	56.4 ± 0.5	56.8 ± 0.5[b]	56.5 ± 0.5	55.7 ± 0.6[b]
Month 12	58.6 ± 0.6[b]	59.8 ± 1.6	59.1 ± 0.4[b]	58.0 ± 0.3	57.8 ± 0.7[c]
Mean cell hemoglobin (pg)					
Day 19	19.3 ± 0.1	19.0 ± 0.2[e]	19.4 ± 0.2	19.2 ± 0.1[d]	18.3 ± 0.1**
Month 3	18.0 ± 0.2	18.0 ± 0.1	17.9 ± 0.2[d]	17.9 ± 0.1	17.7 ± 0.2
Month 6	18.4 ± 0.2	19.0 ± 0.2	19.0 ± 0.5[d]	18.7 ± 0.2	18.1 ± 0.2
Month 9	18.4 ± 0.2	18.6 ± 0.2	18.6 ± 0.2	18.4 ± 0.1	18.1 ± 0.2[b]
Month 12	18.2 ± 0.2[b]	18.8 ± 0.4	18.4 ± 0.2	18.1 ± 0.1	18.0 ± 0.2[c]
Mean cell hemoglobin concentration (g/dL)					
Day 19	31.8 ± 0.2	31.3 ± 0.3[e]	31.5 ± 0.2	31.8 ± 0.2[d]	31.0 ± 0.1**
Month 3	32.0 ± 0.1	31.8 ± 0.2	31.4 ± 0.1*[d]	31.5 ± 0.1*	31.6 ± 0.1
Month 6	32.3 ± 0.2	32.9 ± 0.2	32.0 ± 0.3[d]	32.8 ± 0.1*	32.0 ± 0.2
Month 9	33.0 ± 0.2	33.0 ± 0.2	32.7 ± 0.1	32.6 ± 0.2	32.6 ± 0.1[b]
Month 12	31.1 ± 0.1[b]	31.4 ± 0.2	31.1 ± 0.1	31.2 ± 0.2	31.1 ± 0.1[c]

TABLE D1
Hematology Data for Rats in the 2-Year Feed Study of Dietary Zinc

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Female (continued)					
n	10	10	9	9	10
Platelets (10^3/μL)					
Day 19	953 ± 53	932 ± 55[e]	937 ± 47	1,006 ± 53[d]	1,092 ± 82
Month 3	902 ± 38	881 ± 35	831 ± 37[d]	863 ± 63	800 ± 55
Month 6	813 ± 46	764 ± 23	816 ± 52[d]	775 ± 42	786 ± 52
Month 9	844 ± 49	761 ± 18	724 ± 43	767 ± 41	753 ± 49[b]
Month 12	749 ± 63[b]	577 ± 47	594 ± 22	617 ± 61	601 ± 43[c]
Leukocytes (10^3/μL)					
Day 19	9.18 ± 0.78	9.98 ± 0.71[e]	10.50 ± 0.44*	10.05 ± 0.73[d]	9.11 ± 0.67
Month 3	9.22 ± 0.80	9.20 ± 0.38	9.84 ± 0.63[d]	8.76 ± 0.47	7.66 ± 0.40
Month 6	7.44 ± 0.57	7.74 ± 0.38	8.70 ± 0.72[d]	8.16 ± 0.20	8.09 ± 0.49
Month 9	8.70 ± 0.52	10.03 ± 0.85	8.45 ± 0.64	8.31 ± 0.37	7.88 ± 0.35[b]
Month 12	7.17 ± 0.85[b]	6.98 ± 0.61	5.88 ± 0.73	7.25 ± 0.43	6.78 ± 0.48[c]
Segmented neutrophils (10^3/μL)					
Day 19	0.78 ± 0.10	1.02 ± 0.15[e]	1.08 ± 0.09	0.89 ± 0.08[d]	0.79 ± 0.09
Month 3	0.93 ± 0.23	0.85 ± 0.09	0.90 ± 0.11[d]	0.87 ± 0.11	0.71 ± 0.08
Month 6	1.22 ± 0.19	0.74 ± 0.05*	0.98 ± 0.18[d]	1.05 ± 0.09	1.23 ± 0.19
Month 9	1.49 ± 0.24	1.64 ± 0.34	1.20 ± 0.30	1.36 ± 0.23	1.16 ± 0.09[b]
Month 12	1.41 ± 0.21[b]	1.43 ± 0.32	1.06 ± 0.13	1.12 ± 0.20	1.13 ± 0.16[c]
Lymphocytes (10^3/μL)					
Day 19	8.01 ± 0.73	8.50 ± 0.58[e]	9.00 ± 0.36	8.70 ± 0.73[d]	7.89 ± 0.57
Month 3	7.94 ± 0.58	8.01 ± 0.39	8.59 ± 0.57[d]	7.51 ± 0.40	6.67 ± 0.35
Month 6	5.84 ± 0.47	6.68 ± 0.34	7.39 ± 0.63[d]	6.68 ± 0.19	6.46 ± 0.39
Month 9	6.65 ± 0.50	7.83 ± 0.54	6.75 ± 0.59	6.46 ± 0.21	6.25 ± 0.34[b]
Month 12	5.32 ± 0.71[b]	5.18 ± 0.39	4.47 ± 0.59	5.81 ± 0.29	5.12 ± 0.28[c]
Monocytes (10^3/μL)					
Day 19	0.27 ± 0.04	0.31 ± 0.05[e]	0.28 ± 0.03	0.31 ± 0.03[d]	0.31 ± 0.04
Month 3	0.23 ± 0.03	0.23 ± 0.02	0.23 ± 0.02[d]	0.28 ± 0.03	0.15 ± 0.01
Month 6	0.24 ± 0.03	0.21 ± 0.02	0.25 ± 0.04[d]	0.29 ± 0.03	0.26 ± 0.02
Month 9	0.33 ± 0.03	0.38 ± 0.05	0.31 ± 0.03	0.32 ± 0.02	0.32 ± 0.04[b]
Month 12	0.29 ± 0.07[b]	0.24 ± 0.04	0.20 ± 0.06	0.23 ± 0.06	0.33 ± 0.04[c]
Basophils (10^3/μL)					
Day 19	0.02 ± 0.00	0.03 ± 0.01[e]	0.03 ± 0.00	0.03 ± 0.00[d]	0.02 ± 0.00
Month 3	0.03 ± 0.01	0.02 ± 0.00	0.03 ± 0.00[d]	0.02 ± 0.00	0.02 ± 0.00
Month 6	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00[d]	0.02 ± 0.00	0.02 ± 0.00
Month 9	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.00**[b]
Month 12	0.02 ± 0.01[b]	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00[c]
Eosinophils (10^3/μL)					
Day 19	0.10 ± 0.01	0.12 ± 0.04[e]	0.11 ± 0.02	0.12 ± 0.03[d]	0.10 ± 0.03
Month 3	0.09 ± 0.01	0.08 ± 0.01	0.10 ± 0.01[d]	0.08 ± 0.01	0.10 ± 0.02
Month 6	0.13 ± 0.02	0.11 ± 0.02	0.07 ± 0.01*[d]	0.13 ± 0.02	0.13 ± 0.02
Month 9	0.19 ± 0.03	0.15 ± 0.02	0.14 ± 0.02	0.14 ± 0.02	0.13 ± 0.02 [b]
Month 12	0.14 ± 0.03[b]	0.12 ± 0.02	0.15 ± 0.03	0.08 ± 0.02	0.18 ± 0.03 [c]

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
** P≤0.01
[a] Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=9
[c] n=8
[d] n=10
[e] n=7

TABLE D2
Trace Metal Concentrations in Blood of Rats in the 2-Year Feed Study of Dietary Zinc[a]

	Control 38 ppm	3.5 ppm	7 ppm	250 ppm	500 ppm
Male					
n	10	10	9	10	10
Zinc (µg/mL)					
Day 19	6.668 ± 0.392	6.235 ± 0.557	6.541 ± 0.387[b]	8.662 ± 0.403**	9.840 ± 0.382**
Month 3	6.630 ± 0.148	6.287 ± 0.263	6.766 ± 0.342	6.887 ± 0.162	6.659 ± 0.156
Month 6	6.860 ± 0.366	6.151 ± 0.199	6.148 ± 0.220	6.536 ± 0.143	6.302 ± 0.178
Month 9	6.028 ± 0.197	5.822 ± 0.154	6.041 ± 0.250	6.078 ± 0.128	6.057 ± 0.136
Month 12	5.379 ± 0.158	5.273 ± 0.132	5.217 ± 0.112	5.604 ± 0.251	5.722 ± 0.150
Copper (µg/mL)					
Day 19	0.438 ± 0.056	0.452 ± 0.026	0.441 ± 0.021[b]	ND	ND
Month 3	0.634 ± 0.024	0.604 ± 0.030	0.628 ± 0.022	0.584 ± 0.021	0.553 ± 0.038
Month 6	0.532 ± 0.042	0.543 ± 0.040	0.527 ± 0.065	0.480 ± 0.034[c]	0.440 ± 0.035
Month 9	0.795 ± 0.041	0.831 ± 0.040	0.844 ± 0.066	0.862 ± 0.088	0.712 ± 0.040
Month 12	0.710 ± 0.025	0.745 ± 0.031	0.675 ± 0.023	0.705 ± 0.054	0.714 ± 0.028
Iron (µg/mL)					
Day 19	453.1 ± 8.6	462.0 ± 6.5	447.6 ± 4.9[b]	442.7 ± 6.6	434.9 ± 8.1
Month 3	490.7 ± 8.6	495.0 ± 7.4	497.8 ± 5.6	484.1 ± 7.7	479.6 ± 5.7
Month 6	486.1 ± 6.2	492.8 ± 6.6	475.2 ± 5.0	482.9 ± 4.4	480.0 ± 5.0
Month 9	476.2 ± 7.1	478.6 ± 6.9	477.8 ± 6.0	470.5 ± 5.4	471.7 ± 7.6
Month 12	473.1 ± 8.7	458.8 ± 5.9	462.2 ± 8.9	473.2 ± 8.6	476.0 ± 6.1
Female					
n	10	10	10	9	10
Zinc (µg/mL)					
Day 19	4.458 ± 0.188	3.928 ± 0.169	4.502 ± 0.131	6.358 ± 0.580[b]**	7.754 ± 0.202**
Month 3	5.505 ± 0.138	5.187 ± 0.176	5.301 ± 0.153	5.120 ± 0.057	5.448 ± 0.185
Month 6	4.864 ± 0.072	4.982 ± 0.124	5.052 ± 0.133	4.903 ± 0.203	4.608 ± 0.091
Month 9	5.021 ± 0.149	4.638 ± 0.213	4.998 ± 0.120[c]	5.136 ± 0.131	5.200 ± 0.173[c]
Month 12	4.668 ± 0.197[c]	4.595 ± 0.377	4.537 ± 0.146[c]	4.380 ± 0.209	4.733 ± 0.143[c]
Copper (µg/mL)					
Day 19	0.663 ± 0.029	0.590 ± 0.034	0.669 ± 0.022	0.331 ± 0.044[b]**	0.167 ± 0.027**
Month 3	0.769 ± 0.049	0.665 ± 0.030	0.733 ± 0.023	0.733 ± 0.041	0.699 ± 0.072
Month 6	0.275 ± 0.045	0.159 ± 0.025	0.236 ± 0.069	0.432 ± 0.079	0.546 ± 0.064**
Month 9	0.791 ± 0.037	0.690 ± 0.031	0.743 ± 0.041[c]	0.713 ± 0.019	0.685 ± 0.059[c]
Month 12	0.457 ± 0.042[c]	0.382 ± 0.032	0.399 ± 0.032[c]	0.417 ± 0.065	0.451 ± 0.058[c]
Iron (µg/mL)					
Day 19	411.1 ± 9.9	400.6 ± 13.6	436.0 ± 10.2	444.9 ± 9.4[b]	423.6 ± 5.4
Month 3	448.0 ± 5.0	461.3 ± 7.3	445.4 ± 9.6	430.8 ± 7.1	419.9 ± 6.7**
Month 6	420.3 ± 6.7	428.0 ± 9.2	418.9 ± 16.0	398.3 ± 17.8	378.8 ± 8.2**
Month 9	405.1 ± 5.3	378.2 ± 7.7*	409.8 ± 7.0[c]	411.9 ± 6.6	389.4 ± 9.6[c]
Month 12	402.8 ± 8.6[c]	376.8 ± 13.8	385.6 ± 14.9[c]	383.7 ± 17.5	412.1 ± 12.9[c]

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
** Significantly different (P≤0.01) from the control group by Shirley's test
[a] Data are presented as mean ± standard error. Statistical tests were performed on unrounded data. ND= All values below the limit of detection at this dose level
[b] n=10
[c] n=9

APPENDIX E
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF DIETARY ZINC

Zinc carbonate basic {$[ZnCO_3]_2 \cdot [Zn(OH)_2]_3$} was obtained from Sigma-Aldrich (St. Louis, MO) in one lot (1217764) that was used in the 2-year study to create dietary levels of zinc. Analyses to determine the identity, purity, and storage stability were conducted by the analytical chemistry laboratory at Research Triangle Institute (RTI) (Research Triangle Park, NC) and the study laboratory at Battelle Columbus (Columbus, OH) and its sister laboratory at Battelle Toxicology Northwest (BTNW) (Richland, WA). Reports on analyses performed in support of the dietary zinc study are on file at the National Institute of Environmental Health Sciences.

Zinc carbonate basic (lot 1217764) was a fine white powder. The lot was analyzed by RTI using inductively coupled plasma/optical emission spectroscopy (ICP/OES) for zinc and a panel of secondary elements (a total of 18 including zinc) using Thermo Jarrell Ash Atomscan-16 ICP-OES (Franklin, MA, USA). The instrument was calibrated with standards prepared from National Institute of Standards and Technology (NIST)-traceable, single element standards for each element. NIST-traceable yttrium and gold was used as the internal standard and to stabilize mercury during analysis. Zinc carbonate basic aliquots were dissolved in high-purity nitric acid (J.T. Baker, Phillipsburg, NJ), internal standard was added and diluted with deionized water prior to analyses. The zinc content (at the primary zinc wavelength of 213.856 nm) was calculated to be 56.6%, which is lower than the theoretical value of 59.6% based on the molecular formula of zinc carbonate basic suggesting other zinc based compounds may have been present. Of the other elements, heavy metal levels (e.g., arsenic, cadmium, chromium, mercury, lead, and thallium) were determined to be below the limit of quantitation of 0.01% and levels of calcium and magnesium were 0.0916% and 1.32%, respectively.

The moisture content of lot 1217764 was determined by RTI using weight loss on drying and by Galbraith Laboratories, Inc. (Knoxville, TN), using Karl Fischer titration. Purity of the test article was assessed using elemental analyses conducted by Quantitative Technologies, Inc. (Whitehouse, NJ), and Galbraith Laboratories, Inc. Ion chromatography (IC) was conducted by Quantitative Technologies, Inc., to measure the concentrations of a number of possible anionic and cationic impurities in the test article.

Weight loss on drying determined a water content of 0.30% and Karl Fisher titration indicated 2.52% water. Elemental analyses yielded for carbon (3.58%) and hydrogen (1.07%) were slightly different than the theoretical (carbon, 4.38%; hydrogen, 1.10%) suggesting the presence of other zinc-based compounds. IC analysis revealed the presence of minor ionic impurities, including chloride (429 µg/g), sulfate (1,871 µg/g), and sodium (698 µg/g), that were consistent with the vendor's certificate of analysis.

RTI conducted several additional analyses using x-ray diffraction (XRD) spectroscopy, Fourier-transform infrared (FTIR) spectroscopy, thermogravimetric analysis (TGA), and qualitative X-ray fluorescence (XRF) spectroscopy. XRD was performed using a Shimadzu XRD-6000 instrument (Shimadzu Scientific Instruments, Kyoto, Japan), with a step size of 0.02° and a two-theta angle range of 5° to 65°. FTIR was performed on pelletized aliquots of the test article using a ThermoFisher Nicolet 6700 instrument (ThermoFisher Scientific, Waltham, MA). TGA was performed on a Q50 TGA instrument (TA Instruments, New Castle, DE) with nitrogen as both the sample and balance gasses and ramping at 5° C/minute to 400° C. Qualitative XRF was performed using a Thermo ARL Quant'X instrument (Thermo Fisher Scientific) to determine if elements with atomic weights ranging from sodium to uranium were present. After preliminary detection of zinc, copper, magnesium, and sulfur, XRF instrumental conditions were optimized to analyze for these four elements using either no filter, a cellulose filter, or a thin or thick palladium filter.

XRD patterns collected from the zinc carbonate basic test article were searched against a database from the International Center for Diffraction Data (ICDD, Newton Square, PA) Powder Diffraction File (PDF4+) for potential matches. The diffraction patterns did not yield a conclusive match as zinc carbonate basic because a zinc carbonate basic reference spectrum does not exist in the database. The database returned 43 possible matches, but only three scored with match figures of merit less than 5.0 (a perfect match has figure of merit equal to 1.0). These

potential matches included zinc oxide (ZnO; PDF4+ reference 03-065-3411, figure of merit 3.0), zinc hydroxide [$Zn(OH)_2$; PDF4+ reference 00-048-1066, figure of merit 3.5], and zinc carbonate hydroxide hydrate [$Zn_4(CO_3)_2(OH)_6 \cdot H_2O$, PDF4+ reference 00-003-0787, figure of merit 4.0]. The ICDD PDF4+ database was also queried to produce the closest possible chemical formula match to zinc carbonate basic and returned reference spectra for both hydrozincite [$Zn_5(CO_3)_2(OH)_6$, PDF4+ reference 00-014-0256] and smithsonite ($ZnCO_3$, PDF4+ reference 00-001-1036). Peak diffraction angles and relative intensities from the zinc carbonate basic were closely compared against these five reference compounds but a match was not found. In contrast, all of the peak diffraction angles for zinc oxide corresponded with matching peak diffraction angles from the test lot, but relative peak height intensity distributions of the most abundant peaks for zinc oxide did not correlate with the zinc carbonate basic test lot. Taken collectively, these data suggest that zinc oxide is likely present in the sample, but as a possible minor component.

Because none of the database reference cards produced an unequivocal match with the observed diffraction pattern from the zinc carbonate basic lot, zinc carbonate hydroxide (CAS No. 3486-35-9) with the same nominal formula as the zinc carbonate basic lot (both are $[ZnCO_3]_2 \cdot [Zn(OH)_2]_3$) was procured and analyzed by XRD. (It was difficult to identify and procure potential reference compounds with the same nominal formula, so only this one additional compound was obtained.) Zinc carbonate hydroxide had the same nominal formula as the test article but had a different CAS number at the time of purchase. However, the zinc carbonate hydroxide vendor now offers this compound under the same CAS number as the zinc carbonate basic (5263-02-5). This change in CAS numbers suggests that zinc carbonate hydroxide and the test zinc carbonate basic article may be the same material. Zinc oxide was also procured and analyzed. Diffraction patterns from zinc carbonate hydroxide and zinc carbonate basic generally correspond with each other (Figure E1). These same diffraction patterns are overlaid with data obtained from zinc oxide. Each of the overlapping peaks for the procured zinc oxide is paralleled with an increase in the peak abundance in the zinc carbonate basic test article spectrum relative to the procured zinc carbonate hydroxide spectrum. Taken collectively, XRD data suggest that the test article is structurally similar or equivalent to the procured zinc carbonate hydroxide, but with zinc oxide as a minor component.

The FTIR spectrum from the zinc carbonate basic lot agrees with a reference spectrum (Nyquist and Kagel, 1971). For additional comparison, FTIR spectra were obtained for aliquots of the zinc carbonate hydroxide and zinc oxide compounds. Visual inspection of the FTIR spectra from the zinc carbonate basic test article and zinc carbonate hydroxide and comparison of prominent peaks further suggests that these compounds are structurally very similar to each other and to the zinc carbonate basic reference spectrum. A strong zinc oxide stretch peak (approximately 450 cm^{-1}) was also observed from the test chemical spectrum and the spectra from the procured zinc carbonate hydroxide and zinc oxide compounds, suggesting that zinc oxide is a plausible minor component in the lot.

A single peak was observed in TGA depicting simultaneous decarbonation and dehydroxylation of zinc carbonate basic as represented in the equation (Koga and Tanaka, 2005):

$$Zn_5(CO_3)_2(OH)_6 \rightarrow 5ZnO + 2CO_2 + 3H_2O$$

Because zinc carbonate basic degrades to zinc oxide upon heating, theoretical weight loss due to loss of water and carbon dioxide for the above equation is 25.9%. However, the observed weight loss was 23.3%, suggesting that nonvolatile impurities may be present in the test lot.

A preliminary XRF scan was obtained to determine whether elements with atomic weights ranging from sodium to uranium were present. From these scans, zinc, calcium, magnesium, and sulfur were observed, and the test article was reanalyzed using optimal instrumental conditions for these elements. Peaks were observed for calcium and sulfur by using the optimal cellulose filter condition for these elements and for magnesium and sulfur by using the optimal no filter condition for these elements. Sulfur is a plausible impurity in the zinc carbonate test article because zinc sulfate compounds can be used in the synthesis of zinc carbonate hydroxides (Cao *et al*. 2009, Du *et al.*, 2009) and because zinc carbonates are effective sulfide scavengers (Cameron, 2005).

Stability studies of the bulk chemical were performed by RTI using the ICP/OES method previously described. These studies indicated that zinc carbonate basic was stable as a bulk chemical for 15 days when stored in capped plastic bottles at temperatures up to 60° C. To ensure stability, the bulk chemical was stored at room temperature in capped amber glass bottles. Prior to study start, the study laboratory analyzed the bulk chemical and the frozen

reference standard using XRD (H&M Analytical Services, Inc., Allentown, NJ) and ICP/atomic emission spectroscopy (AES) (IRIS Intrepid, Thermo Electron Corporation, Waltham, MA) using the method described above for ICP/OES. Results of these analyses showed that the bulk test article and frozen reference samples of the same lot were consistent with each other. Periodic analyses of the bulk chemical and the frozen reference standard were performed by the study laboratory at least every 6 months during the 2-year study with ICP/AES, and no degradation of the bulk chemical was detected.

BACKGROUND ZINC CONTENT OF BASE DIET

Aliquots of four batches (with nine manufacture dates from June 29, 2009, to April 11, 2011) of the base diet (AIN-93M Modified Low Zinc Feed; Zeigler Brothers, Inc., Gardners, PA), were analyzed by BTNW to prescreen for possible background zinc in the blank vehicle using ICP/AES. All batches of the zinc-deficient base diet were determined to contain less than 1 mg Zn/kg diet and were considered acceptable to be used for formulation preparations.

For ICP/AES or ICP/OES, each feed sample (approximately 10 g) was weighed, 1 mL of internal standard solution Y and 100 mL of concentrated nitric acid were added, and the mixture was stirred with a magnetic stir bar until the feed material was thoroughly wetted. The samples were covered or capped loosely and allowed to sit overnight at room temperature in a fume hood.

Samples were restirred with the magnetic stir bars and 5 mL were transferred from each container to separate centrifuge tubes containing 2 mL of deionized water. The centrifuge tubes were capped with vented microwave digestion caps and the samples were subjected to three stages of microwave digestion: a 1 minute ramp to 60° C with a 20 minute hold, then a 2 minute ramp to 80° C with a 20 minute hold, and finally a 2 minute ramp to 100° C with a 10 minute hold. After cooling for 15 minutes, hydrogen peroxide was added, the tubes were recapped with vented caps, and the samples were subjected to a second three-stage digestion as described above. After the second digestion and cooling for 15 minutes, the samples were diluted with deionized water, capped with solid (unvented) caps, mixed well, and analyzed for zinc (213.856 nm) and internal standard solution Y (371.030 nm) on an IRIS Intrepid, Intrepid II, or Thermo Jarrell Ash AtomScan-16 spectrometer (Thermo Electron Corporation, Waltham, MA) or on an Optima 4300 DV spectrometer (PerkinElmer, Waltham, MA).

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared monthly by mixing zinc carbonate basic with AIN-93M feed (Table E1). The theoretical value of zinc content (59.6%) was used to calculate the amount of zinc in the dose formulations, therefore the doses used in this study are approximately 3% lower than what is stated. A premix was prepared by hand and then blended with additional feed in a Patterson-Kelly twin-shell blender for approximately 15 minutes. Formulations were stored in sealed plastic bag-lined buckets at room temperature for up to 42 days. The 38 ppm formulation was used as the control formulation for the 2-year study.

Homogeneity studies of 3.5, 7, 38, 250, 500, and 1,000 ppm formulations and of 3.5, 38, and 500 ppm dose formulations were performed by RTI and BTNW, respectively. These studies were conducted with ICP/AES or ICP/OES and measured Zn in digested samples of the formulations. ICP-OES was also used in stability studies of 3.5 and 7 ppm dose formulations that were performed by RTI. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in sealed plastic bags under freezer, refrigerated, and room temperature conditions; stability was also confirmed for at least 7 days under simulated animal room conditions. Periodic analyses of the dose formulations of zinc carbonate basic were conducted by BTNW using ICP/AES.

During the 2-year study, the dose formulations were analyzed every 2 to 3 months and animal room samples were also analyzed (Table E2). Of the dose formulations analyzed and used during the study, 102 of 110 were within 10% of the target concentrations; all 20 animal room samples were within 10% of the target concentrations.

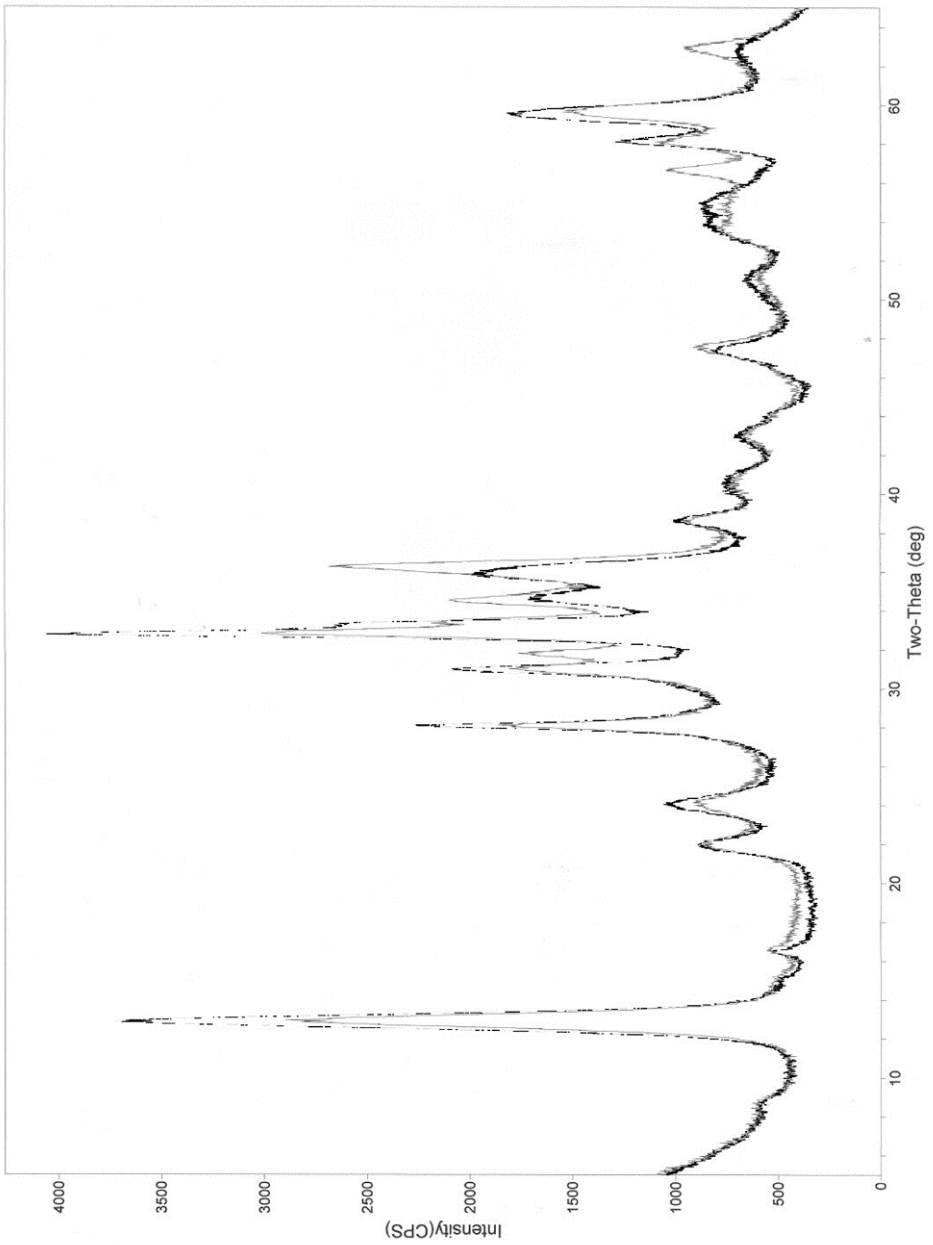


FIGURE E1
X-ray Diffraction Patterns of Zinc Carbonate Basic Test Article (gray) and Zinc Carbonate Basic (black)

TABLE E1
Preparation and Storage of Dose Formulations in the 2-Year Feed Study of Dietary Zinc

Preparation
Appropriate amounts of blank AIN-93M Modified Low Zinc Feed and zinc carbonate basic test article (adjusted for the theoretical Zn content of 59.6%) were weighed into separate weighing containers. The test article was transferred with rinses to a mortar and pestle and thoroughly ground. The ground mixtures were transferred with rinses into a stainless steel container, and the remaining blank premix feed was incrementally added to obtain a final premix size of 1 kg; the contents of the stainless steel container were thoroughly mixed at each step using a spatula. Each premix was layered into the remaining blank feed and blended in a Patterson-Kelly twin-shell blender for approximately 15 minutes. The dose formulations were prepared monthly during the study.

Chemical Lot Number
1217764

Maximum Storage Time
42 days

Storage Conditions
Stored in sealed plastic bag-lined buckets at room temperature

Study Laboratory
Battelle Columbus Operations (Columbus, OH)

TABLE E2
Results of Analyses of Dose Formulations Administered to Rats in the 2-Year Feed Study of Dietary Zinc

Date Prepared	Date Analyzed	Target Concentration[a] (ppm)	Determined Concentration[b] (ppm)	Difference from Target (%)
August 21, 2009	August 28, 2009	3.5	3.49	0
		3.5	3.53	+1
		7	6.67	–5
		7	6.75	–4
		38	35.9	–6
		38	35.9	–6
		250	234	–6
		250	234	–6
		500	472	–6
		500	468	–6
	October 22, 2009[c]	3.5	3.48	–1
		7	6.83	–2
		38	36.7	–3
		250	232	–7
		500	465	–7
October 16, 2009	October 22, 2009	3.5	3.51	0
		3.5	3.57	+2
		7	7.02	0
		7	6.86	–2
		38	37.3	–2
		38	36.6	–4
		250	242	–3
		250	244	–2
		500	483	–3
		500	482	–4
January 8, 2010	January 14-15, 2010	3.5	3.40	–3
		3.5	3.45	–1
		7	6.98	0
		7	6.96	–1
		38	36.6	–4
		38	36.4	–4
		250	242	–3
		250	244	–2
		500	481	–4
		500	481	–4
March 8, 2010	March 12, 2010	3.5	3.56	+2
		3.5	3.59	+3
		7	6.90	–1
		7	6.92	–1
		38	35.8	–6
		38	36.5	–4
		250	236	–6
		250	239	–4
		500	480	–4
		500	481	–4
	April 23, 2010[c]	3.5	3.40	–3
		7	6.86	–2
		38	36.6	–4
		250	238	–5
		500	478	–4

TABLE E2
Results of Analyses of Dose Formulations Administered to Rats in the 2-Year Feed Study of Dietary Zinc

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)
May 28, 2010	June 4, 2010	3.5	3.67	+5
		3.5	3.53	+1
		7	6.72	–4
		7	6.65	–5
		38	35.2	–7
		38	35.2	–7
		250	228	–9
		250	228	–9
		500	458	–8
		500	462	–8
July 23, 2010	August 2, 2010	3.5	3.72	+6
		3.5	3.70	+6
		7	7.22	+3
		7	6.95	–1
		38	38.6	+2
		38	37.4	–2
		250	244	–2
		250	236	–6
		500	467	–7
		500	471	–6
October 15, 2010	October 22, 2010	3.5	3.43	–2
		3.5	3.42	–2
		7	6.56	–6
		7	6.57	–6
		38	35.6	–6
		38	35.5	–7
		250	239	–4
		250	239	–4
		500	468	–6
		500	470	–6
	December 8, 2010[c]	3.5	3.84	+10
		7	7.22	+3
		38	36.5	–4
		250	240	–4
		500	481	–4
December 10, 2010	December 16, 2010	3.5	3.92	+12[d]
		3.5	3.94	+13[d]
		7	6.99	0
		7	7.33	+5
		38	37.7	–1
		38	38.2	+1
		250	245	–2
		250	243	–3
		500	479	–4
		500	485	–3
March 4, 2011	March 15, 2011	3.5	4.05	+16[d]
		3.5	4.00	+14[d]
		7	7.32	+5
		7	7.56	+8
		38	38.3	+1
		38	38.6	+2
		250	249	0
		250	247	–1
		500	485	–3
		500	488	–2

TABLE E2
Results of Analyses of Dose Formulations Administered to Rats in the 2-Year Feed Study of Dietary Zinc

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)
April 29, 2011	May 6, 2011	3.5	3.88	+11[d]
		3.5	3.92	+12[d]
		7	7.06	+1
		7	7.26	+4
		38	37.9	0
		38	38.0	0
		250	245	–2
		250	243	–3
		500	473	–5
		500	471	–6
	June 17, 2011[c]	3.5	3.83	+9
		7	7.15	+2
		38	36.8	–3
		250	241	–4
		500	475	–5
July 22, 2011	July 27, 2011	3.5	3.87	+11[d]
		3.5	3.93	+12[d]
		7	7.15	+2
		7	7.27	+4
		38	38.2	+1
		38	38.9	+2
		250	249	0
		250	247	–1
		500	477	–5
		500	478	–4

[a] The theoretical value of zinc content (59.6%) was used to calculate the amount of zinc in the dose formulations, therefore the doses used in this study are approximately 3% lower than what is stated.
[b] Results of duplicate analyses
[c] Animal room samples
[d] Formulation was outside the acceptable range of ± 10% of target concentration, but used at NTP's direction.

APPENDIX F
FEED AND COMPOUND CONSUMPTION IN THE 2-YEAR FEED STUDY OF DIETARY ZINC

TABLE F1
Feed and Compound Consumption by Male Rats in the 2-Year Feed Study of a Zinc-Deficient Diet

Week	Control 38 ppm			3.5 ppm			7 ppm		
	Feed[a] (g/day)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	17.4	115	5.8	15.6	115	0.5	17.0	115	1.0
2	16.5	162	3.9	13.1	150	0.3	16.1	159	0.7
3	17.1	207	3.1	12.9	176	0.3	16.5	203	0.6
4	18.6	250	2.8	14.2	202	0.2	18.1	245	0.5
5	18.4	283	2.5	15.0	225	0.2	17.9	278	0.5
6	18.4	311	2.2	15.7	250	0.2	17.8	304	0.4
7	18.0	332	2.1	15.7	271	0.2	17.6	324	0.4
8	18.0	349	2.0	16.0	289	0.2	17.7	340	0.4
9	17.4	363	1.8	15.5	306	0.2	17.1	354	0.3
10	17.4	376	1.8	15.3	319	0.2	16.7	366	0.3
11	17.4	386	1.7	15.0	330	0.2	16.8	377	0.3
12	17.5	395	1.7	15.4	341	0.2	16.7	386	0.3
13	16.4	402	1.6	14.9	350	0.1	16.0	392	0.3
14	16.2	410	1.5	14.2	361	0.1	15.6	401	0.3
17	17.6			15.9			17.1		
18	17.4	441	1.5	15.9	392	0.1	17.2	429	0.3
21	17.3			16.0			17.0		
22	17.2	462	1.4	15.7	417	0.1	16.7	450	0.3
25	17.4			16.0			16.8		
26	17.1	481	1.4	15.7	436	0.1	16.6	468	0.2
29	17.8			16.8			17.7		
30	17.1	497	1.3	15.9	455	0.1	17.4	483	0.3
33	17.6			16.2			17.3		
34	17.3	514	1.3	16.1	469	0.1	17.4	499	0.2
37	17.3			16.8			17.1		
38	17.6	530	1.3	16.8	487	0.1	17.5	512	0.2
41	17.8			16.9			17.5		
42	18.3	540	1.3	17.6	502	0.1	18.1	521	0.2
45	18.4			18.2			18.3		
46	17.6	552	1.2	16.6	511	0.1	17.0	530	0.2
49	18.0			17.4			18.0		
50	17.5	567	1.2	17.1	526	0.1	17.8	545	0.2
53	18.0			17.0			17.7		
54	17.8	583	1.2	17.0	541	0.1	17.3	557	0.2
57	18.4			17.2			17.3		
58	18.5	597	1.2	17.7	552	0.1	17.1	566	0.2
61	17.6			17.3			17.5		
62	18.0	608	1.1	17.8	565	0.1	17.6	577	0.2
65	18.3			17.3			17.4		
66	18.4	616	1.1	17.5	581	0.1	17.5	588	0.2
69	17.4			16.8			17.4		
70	17.3	621	1.1	17.1	587	0.1	16.9	595	0.2
73	17.5			17.3			17.4		
74	17.8	633	1.1	17.7	594	0.1	17.6	605	0.2
77	17.2			17.0			17.5		
78	17.2	636	1.0	17.7	596	0.1	17.5	609	0.2
81	17.6			17.1			17.1		
82	17.2	639	1.0	17.5	603	0.1	16.8	610	0.2
85	17.7			17.1			17.6		
86	17.7	638	1.1	17.1	605	0.1	17.7	614	0.2
89	17.1			16.8			16.8		
90	17.5	644	1.0	17.3	612	0.1	17.1	611	0.2
93	17.5			17.0			16.8		
94	17.3	643	1.0	17.1	604	0.1	16.5	610	0.2
97	16.6			16.3			16.4		
98	15.9	633	1.0	16.0	597	0.1	16.6	613	0.2
101	17.5			16.1			16.2		
102	16.8	647	1.0	16.4	602	0.1	15.8	603	0.2

TABLE F1
Feed and Compound Consumption by Male Rats in the 2-Year Feed Study of a Zinc-Deficient Diet

Week	Control 38 ppm Feed (g/day)	Body Weight (g)	Dose (mg/kg)	3.5 ppm Feed (g/day)	Body Weight (g)	Dose (mg/kg)	7 ppm Feed (g/day)	Body Weight (g)	Dose (mg/kg)
Mean for Weeks									
1-13	17.6	302	2.5	14.9	256	0.2	17.1	296	0.5
14-52	17.5	499	1.3	16.4	456	0.1	17.3	484	0.3
53-102	17.5	626	1.1	17.1	589	0.1	17.1	597	0.2

[a] Grams of feed consumed per animal per day
[b] Milligrams of dietary zinc consumed per kilogram body weight per day

TABLE F2
Feed and Compound Consumption by Male Rats in the 2-Year Feed Study of a Zinc-Excess Diet

Week	Control 38 ppm			250 ppm			500 ppm		
	Feed[a] (g/day)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	17.4	115	5.8	17.3	115	37.7	17.7	115	77.3
2	16.5	162	3.9	16.4	163	25.2	16.6	164	50.5
3	17.1	207	3.1	16.7	208	20.1	17.0	210	40.5
4	18.6	250	2.8	18.1	250	18.1	18.6	252	36.9
5	18.4	283	2.5	18.0	282	15.9	18.2	284	32.1
6	18.4	311	2.2	18.1	309	14.7	18.3	311	29.5
7	18.0	332	2.1	17.7	330	13.4	18.1	332	27.3
8	18.0	349	2.0	17.5	345	12.7	18.1	349	26.0
9	17.4	363	1.8	17.1	359	11.9	17.3	362	23.9
10	17.4	376	1.8	17.0	372	11.4	17.0	373	22.8
11	17.4	386	1.7	16.9	381	11.1	17.0	382	22.2
12	17.5	395	1.7	17.0	390	10.9	17.3	393	22.0
13	16.4	402	1.6	16.0	398	10.0	16.6	399	20.8
14	16.2	410	1.5	15.7	406	9.7	15.9	406	19.6
17	17.6			16.7			17.0		
18	17.4	441	1.5	17.0	435	9.8	16.8	435	19.3
21	17.3			16.8			16.7		
22	17.2	462	1.4	16.9	454	9.3	17.3	457	18.9
25	17.4			17.0			17.0		
26	17.1	481	1.4	17.1	474	9.0	17.0	479	17.8
29	17.8			17.4			17.6		
30	17.1	497	1.3	17.1	492	8.7	17.0	494	17.2
33	17.6			17.2			17.4		
34	17.3	514	1.3	16.9	505	8.4	17.8	510	17.5
37	17.3			17.5			17.9		
38	17.6	530	1.3	17.9	520	8.6	18.0	523	17.2
41	17.8			17.8			18.4		
42	18.3	540	1.3	18.5	532	8.7	18.4	537	17.1
45	18.4			18.2			18.7		
46	17.6	552	1.2	17.7	541	8.2	17.7	548	16.2
49	18.0			17.9			18.6		
50	17.5	567	1.2	17.6	555	7.9	18.1	562	16.1
53	18.0			17.6			17.8		
54	17.8	583	1.2	17.4	569	7.6	17.6	580	15.2
57	18.4			18.0			18.3		
58	18.5	597	1.2	17.8	583	7.6	18.4	593	15.5
61	17.6			17.7			18.2		
62	18.0	608	1.1	17.2	588	7.3	18.6	606	15.3
65	18.3			16.7			17.9		
66	18.4	616	1.1	17.4	597	7.3	18.3	619	14.8
69	17.4			17.1			18.2		
70	17.3	621	1.1	17.4	611	7.1	17.8	628	14.2
73	17.5			17.6			18.4		
74	17.8	633	1.1	17.5	614	7.1	18.4	637	14.4
77	17.2			17.9			18.0		
78	17.2	636	1.0	17.3	619	7.0	18.1	646	14.0
81	17.6			18.0			19.0		
82	17.2	639	1.0	17.3	624	6.9	18.8	648	14.5
85	17.7			17.9			18.6		
86	17.7	638	1.1	18.3	618	7.4	18.5	652	14.2
89	17.1			17.1			17.8		
90	17.5	644	1.0	17.5	620	7.1	17.7	657	13.5
93	17.5			17.4			17.3		
94	17.3	643	1.0	17.8	628	7.1	17.7	648	13.7
97	16.6			15.9			16.5		
98	15.9	633	1.0	16.0	623	6.4	16.8	644	13.1
101	17.5			16.7			16.6		
102	16.8	647	1.0	16.6	623	6.7	16.9	644	13.1

TABLE F2
Feed and Compound Consumption by Male Rats in the 2-Year Feed Study of a Zinc-Excess Diet

Week	Control 38 ppm Feed (g/day)	Body Weight (g)	Dose (mg/kg)	250 ppm Feed (g/day)	Body Weight (g)	Dose (mg/kg)	500 ppm Feed (g/day)	Body Weight (g)	Dose (mg/kg)
Mean for Weeks									
1-13	17.6	302	2.5	17.2	300	16.4	17.5	302	33.2
14-52	17.5	499	1.3	17.3	491	8.8	17.5	495	17.7
53-102	17.5	626	1.1	17.4	610	7.2	17.9	631	14.4

[a] Grams of feed consumed per animal per day
[b] Milligrams of dietary zinc consumed per kilogram body weight per day

TABLE F3
Feed and Compound Consumption by Female Rats in the 2-Year Feed Study of a Zinc-Deficient Diet

Week	Control 38 ppm			3.5 ppm			7 ppm		
	Feed[a] (g/day)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	13.6	97	5.4	12.8	97	0.5	13.9	97	1.0
2	12.1	126	3.6	10.3	121	0.3	12.2	128	0.7
3	12.1	150	3.1	10.8	137	0.3	12.0	150	0.6
4	12.6	171	2.8	11.3	153	0.3	13.0	172	0.5
5	12.6	190	2.5	11.3	168	0.2	12.4	191	0.5
6	12.1	205	2.2	11.6	182	0.2	12.4	206	0.4
7	12.3	214	2.2	11.5	192	0.2	12.2	216	0.4
8	12.3	223	2.1	11.6	200	0.2	12.1	223	0.4
9	11.6	230	1.9	11.4	208	0.2	11.7	229	0.4
10	11.7	235	1.9	11.1	213	0.2	11.8	233	0.4
11	11.5	240	1.8	11.4	219	0.2	11.9	241	0.3
12	11.5	244	1.8	11.1	224	0.2	11.7	244	0.3
13	11.5	248	1.8	11.2	228	0.2	11.3	248	0.3
14	11.3	253	1.7	10.5	232	0.2	11.3	251	0.3
17	11.5			11.1			11.6		
18	11.9	267	1.7	11.3	247	0.2	11.7	266	0.3
21	10.9			10.6			11.4		
22	11.2	273	1.6	10.7	256	0.1	11.4	276	0.3
25	11.2			10.9			11.2		
26	11.3	282	1.5	10.7	265	0.1	11.0	283	0.3
29	11.9			11.2			11.5		
30	11.4	290	1.5	10.7	272	0.1	11.4	289	0.3
33	11.6			11.5			12.1		
34	11.5	295	1.5	11.9	281	0.1	11.8	300	0.3
37	11.7			11.4			11.4		
38	11.6	300	1.5	11.8	286	0.1	11.7	304	0.3
41	11.6			11.7			11.8		
42	12.2	305	1.5	12.0	290	0.1	12.3	306	0.3
45	12.2			12.3			12.4		
46	11.6	308	1.4	11.5	296	0.1	12.1	316	0.3
49	12.1			11.6			12.2		
50	12.1	319	1.4	11.6	300	0.1	12.1	326	0.3
53	12.0			11.6			12.4		
54	11.9	327	1.4	11.4	302	0.1	12.1	336	0.3
57	12.6			12.4			12.4		
58	11.7	336	1.3	12.0	309	0.1	11.4	342	0.2
61	11.9			11.4			12.1		
62	12.1	344	1.3	11.5	316	0.1	12.0	352	0.2
65	12.2			11.6			11.8		
66	12.3	350	1.3	11.8	321	0.1	11.8	356	0.2
69	11.6			11.8			12.4		
70	11.9	354	1.3	11.2	324	0.1	11.9	363	0.2
73	12.5			11.9			12.1		
74	12.1	363	1.3	12.1	334	0.1	12.3	369	0.2
77	11.6			12.8			12.0		
78	11.7	368	1.2	11.6	336	0.1	12.4	376	0.2
81	12.3			12.1			11.8		
82	11.7	376	1.2	12.2	341	0.1	11.8	379	0.2
85	11.9			12.5			12.1		
86	12.3	378	1.2	12.2	350	0.1	12.2	384	0.2
89	12.7			12.3			12.8		
90	12.2	379	1.2	12.0	351	0.1	11.9	388	0.2
93	12.4			12.3			13.0		
94	12.6	377	1.3	12.0	357	0.1	12.3	388	0.2
97	11.6			11.9			12.6		
98	11.7	370	1.2	12.5	363	0.1	11.7	388	0.2
101	11.4			12.0			12.5		
102	12.1	370	1.2	11.6	355	0.1	11.9	383	0.2

TABLE F3
Feed and Compound Consumption by Female Rats in the 2-Year Feed Study of a Zinc-Deficient Diet

Week	Control 38 ppm			3.5 ppm			7 ppm		
	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
Mean for Weeks									
1-13	12.1	198	2.5	11.3	180	0.2	12.2	198	0.5
14-52	11.6	289	1.5	11.3	273	0.1	11.7	292	0.3
53-102	12.0	361	1.3	12.0	335	0.1	12.1	370	0.2

[a] Grams of feed consumed per animal per day
[b] Milligrams of dietary zinc consumed per kilogram body weight per day

TABLE F4
Feed and Compound Consumption by Female Rats in the 2-Year Feed Study of a Zinc-Excess Diet

Week	Control 38 ppm			250 ppm			500 ppm		
	Feed[a] (g/day)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	13.6	97	5.4	14.1	97	36.5	13.8	97	71.3
2	12.1	126	3.6	12.3	129	23.9	12.0	128	46.8
3	12.1	150	3.1	12.1	151	20.0	11.8	149	39.6
4	12.6	171	2.8	13.1	174	18.9	12.3	163	37.7
5	12.6	190	2.5	12.6	192	16.4	12.3	185	33.2
6	12.1	205	2.2	12.7	207	15.3	12.2	201	30.4
7	12.3	214	2.2	12.2	218	14.0	12.1	211	28.6
8	12.3	223	2.1	12.5	226	13.8	12.1	219	27.6
9	11.6	230	1.9	12.1	232	13.0	12.4	226	27.4
10	11.7	235	1.9	12.0	237	12.6	11.9	232	25.7
11	11.5	240	1.8	12.1	243	12.5	12.0	238	25.2
12	11.5	244	1.8	11.9	246	12.1	11.7	241	24.3
13	11.5	248	1.8	11.7	252	11.6	11.9	246	24.2
14	11.3	253	1.7	11.3	254	11.1	11.6	250	23.2
17	11.5			11.9			11.8		
18	11.9	267	1.7	12.1	270	11.2	11.9	265	22.5
21	10.9			11.2			12.0		
22	11.2	273	1.6	11.4	277	10.3	11.5	274	21.0
25	11.2			11.9			11.8		
26	11.3	282	1.5	11.9	290	10.3	11.5	281	20.5
29	11.9			12.3			12.0		
30	11.4	290	1.5	11.9	296	10.1	11.4	291	19.6
33	11.6			11.9			11.8		
34	11.5	295	1.5	12.2	304	10.0	12.0	299	20.1
37	11.7			11.8			12.0		
38	11.6	300	1.5	12.0	307	9.8	11.9	304	19.6
41	11.6			12.3			12.2		
42	12.2	305	1.5	13.0	311	10.4	12.0	308	19.5
45	12.2			12.8			12.7		
46	11.6	308	1.4	12.6	318	9.9	12.3	314	19.6
49	12.1			12.3			12.0		
50	12.1	319	1.4	12.4	325	9.5	12.2	323	18.9
53	12.0			12.5			12.3		
54	11.9	327	1.4	12.5	333	9.4	12.3	334	18.4
57	12.6			12.8			13.4		
58	11.7	336	1.3	11.9	342	8.7	12.1	341	17.8
61	11.9			12.3			12.7		
62	12.1	344	1.3	12.3	351	8.8	12.8	350	18.3
65	12.2			12.6			12.4		
66	12.3	350	1.3	12.6	363	8.7	12.0	359	16.7
69	11.6			12.0			12.6		
70	11.9	354	1.3	12.2	371	8.2	12.7	365	17.4
73	12.5			12.5			13.6		
74	12.1	363	1.3	12.7	376	8.4	12.7	372	17.1
77	11.6			12.8			13.0		
78	11.7	368	1.2	12.5	386	8.1	12.9	379	17.0
81	12.3			12.9			14.0		
82	11.7	376	1.2	13.3	386	8.6	13.4	384	17.4
85	11.9			13.3			13.1		
86	12.3	378	1.2	12.4	393	7.9	12.3	389	15.8
89	12.7			13.7			13.3		
90	12.2	379	1.2	13.6	405	8.4	12.8	395	16.2
93	12.4			13.3			13.0		
94	12.6	377	1.3	12.2	401	7.6	12.1	400	15.1
97	11.6			13.2			13.0		
98	11.7	370	1.2	13.7	404	8.5	12.7	410	15.5
101	11.4			13.7			12.7		
102	12.1	370	1.2	12.7	406	7.8	12.4	402	15.4

TABLE F4
Feed and Compound Consumption by Female Rats in the 2-Year Feed Study of a Zinc-Excess Diet

Week	Control 38 ppm Feed (g/day)	Body Weight (g)	Dose (mg/kg)	250 ppm Feed (g/day)	Body Weight (g)	Dose (mg/kg)	500 ppm Feed (g/day)	Body Weight (g)	Dose (mg/kg)
Mean for Weeks									
1-13	12.1	198	2.5	12.4	200	17.0	12.2	195	34.0
14-52	11.6	289	1.5	12.1	295	10.3	11.9	291	20.5
53-102	12.0	361	1.3	12.8	378	8.5	12.8	375	16.9

[a] Grams of feed consumed per animal per day
[b] Milligrams of dietary zinc consumed per kilogram body weight per day

APPENDIX G
INGREDIENTS AND NUTRIENT COMPOSITION OF AIN-93M MODIFIED LOW ZINC FEED

TABLE G1
Ingredients of AIN-93M Modified Low Zinc Feed

Ingredients	Percent by Weight
Corn starch	46.00
Dextrin	15.50
Egg white solids	14.40
Sugar granual	10.00
Solka Floc-40	5.00
Soy oil mixer: -No A	4.00
Salt mix AIN-93M MX Zn Deficient	3.50
Vitamin mix AIN-93M Zn Deficient - CO	1.00
L-Lysine 98.5%	0.35
Choline bitartrate	0.25

TABLE G2
Vitamins, Minerals, and Amino Acids in AIN-93M Modified Low Zinc Feed

	Amount	Source
Vitamins		
A	4.0 IU/kg	Stabilized Vitamin A palmitate
D_3	1.0 IU/kg	
E	86.44 IU/kg	
K	0.75 ppm	
Thiamine	6 ppm	Thiamine HCL
Riboflavin	6 ppm	
Niacin	31 ppm	
α-Pantothenic acid	16 ppm	α-Calcium pantothenate
Folic acid	2 ppm	
Pyridoxine	7 ppm	Pyridoxine hydrochloride
Biotin	0.2 ppm	α-Biotin
B_{12}	25 ppb	
Minerals		
Calcium	1.0 %	
Total phosphorus	0.20 %	
Potassium	0.36 %	
Magnesium	0.05 %	Magnesium oxide
Sodium	0.10 %	
Sulfur	0.03%	
Iron	48.33 ppm	Ferric citrate
Zinc	0.002 ppm	
Manganese	10.5 ppm	Manganese carbonate
Copper	6 ppm	Cupric carbonate
Iodine	0.2 ppm	Potassium iodate
Selenium	0.15 ppm	Sodium selenite
Amino Acids (% of total diet as published)		
Arginine	0.6926	
Lysine	1.0059	
Methionine	0.4608	
Cystine	0.2938	
Tryptophan	0.1829	
Histidine	0.2693	
Leucine	1.0325	
Isoleucine	1.7229	
Phenalalanine	0.7459	
Tyrosine	0.4766	
Threonine	0.5285	
Valine	0.8899	
Essential Fatty Acids (% of total diet as published)		
Linoleic	2	

TABLE G3
Nutrient Composition of AIN-93M Modified Low Zinc Feed

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	12.3 ± 0.18	12.1 – 12.6	6
Crude fat (% by weight)	3.85 ± 0.37	3.3 – 4.3	6
Crude fiber (% by weight)	3.01 ± 0.15	2.8 – 3.2	6
Ash (% by weight)	3.29 ± 0.08	3.1 – 3.3	6
Vitamins			
Vitamin A (IU/kg)	4,513 ± 95	3,360 – 5,640	6
Thiamine (ppm)	5.3 ± 0.93	4.2 – 6.5	6
Minerals			
Calcium (%)	0.523 ± 0.024	0.489 – 0.557	6
Phosphorus (%)	0.216 ± 0.0104	0.204 – 0.230	6

TABLE G4
Contaminant Levels in AIN-93M Modified Low Zinc Feed[a]

	Mean ± Standard Deviation[b]	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.03 ± 0.007	0.18 – 0.037	6
Cadmium (ppm)	0.01 ± 0.0	0.04 – 0.01	6
Lead (ppm)	0.01 ± 0.003	0.01 – 0.019	6
Mercury (ppm)	<0.02		6
Selenium (ppm)	0.46 ± 0.174	0.376 – 0.817	6

[a] All samples were irradiated.
[b] For values less than the limit of detection, the detection limit is given as the mean.

APPENDIX H
SENTINEL ANIMAL PROGRAM

SENTINEL ANIMAL PROGRAM

METHODS

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicological evaluation of test compounds. Under this program, the disease state of the rodents is monitored via sera or feces from extra (sentinel) or dosed animals in the study rooms. The sentinel animals and the study animals are subject to identical environmental conditions. Furthermore, the sentinel animals come from the same production source and weanling groups as the animals used for the studies of test compounds.

Blood samples were collected and allowed to clot, and the serum was separated. All samples were processed appropriately with serology testing performed by IDEXX BioResearch [formerly Research Animal Diagnostic Laboratory (RADIL), University of Missouri] (Columbia, MO) for determination of the presence of pathogens. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed.

Blood was collected from five animals per sex per time point except the 18-month collection included only four male rats and three female rats.

Method and Test	Time of Collection
RATS	
2-Year Study	
Multiplex Fluorescent Immunoassay (MFI)	
CAR Bacillus	Study termination
H-1 (Toolan's H-1 virus)	4 weeks, 6, 12, and 18 months, study termination
KRV (Kilham rat virus)	4 weeks, 6, 12, and 18 months, study termination
Mycoplasma pulmonis	4 weeks, 6, 12, and 18 months, study termination
Parvo NS-1	4 weeks and 6 months
PVM (pneumonia virus of mice)	4 weeks, 6, 12, and 18 months, study termination
RCV/SDA (rat coronavirus/sialodacryoadenitis virus)	4 weeks, 6, 12, and 18 months, study termination
RMV (rat minute virus)	4 weeks, 6, 12, and 18 months, study termination
RPV (rat parvovirus)	4 weeks, 6, 12, and 18 months, study termination
RTV (rat theliovirus)	4 weeks, 6, 12, and 18 months, study termination
Sendai	4 weeks, 6, 12, and 18 months, study termination
TMEV (Theiler's murine encephalomyelitis virus)	4 weeks, 6 months
Immunofluorescence Assay	
CAR Bacillus	Study termination
M. pulmonis	12 months and study termination
KRV	12 months
Pneumocystis carinii	Study termination

RESULTS

All test results were negative.