NATIONAL TOXICOLOGY PROGRAM
Technical Report Series
No. 435

TOXICOLOGY AND CARCINOGENESIS

STUDIES OF 4,4′-THIOBIS(6-*t*-BUTYL-*m*-CRESOL)

(CAS NO. 96-69-5)

IN F344/N RATS AND B6C3F$_1$ MICE

(FEED STUDIES)

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

FOREWORD

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

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

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

These studies are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected chemicals in laboratory animals (usually two species, rats and mice). Chemicals selected for NTP toxicology and carcinogenesis studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. Selection *per se* is not an indicator of a chemical's carcinogenic potential.

These NTP Technical Reports are available for sale from the National Technical Information Service, U.S. Department of Commerce, 5285 Port Royal Road, Springfield, VA 22161 (703-487-4650). Single copies of this Technical Report are available without charge while supplies last from NTP Central Data Management, NIEHS, P.O. Box 12233, MD A0-01, Research Triangle Park, NC 27709 (919-541-1371).

NTP TECHNICAL REPORT

ON THE

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P.O. Box 12233
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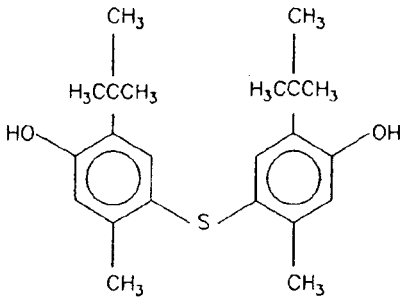
ABSTRACT



4,4′-THIOBIS(6-*t*-BUTYL-*m*-CRESOL)

CAS No. 96-69-5

Chemical Formula: $C_{22}H_{30}SO_2$ Molecular Weight: 358.52

Synonyms: 4,4′-Thiobis(6-*t*-butyl-3-cresol); bis(3-*t*-butyl-4-hydroxy-6-methylphenyl)sulfide
Trade names: Santonox; Santowhite Crystals; Sumilizer; Thioalkofen; Yoshinox

4,4′-Thiobis(6-*t*-butyl-*m*-cresol) (TBBC) is used in the rubber and plastics industries as an antioxidant. TBBC is also used as a stabilizer in polyethylene and polyolefin packaging materials for foodstuffs. Toxicology and carcinogenesis studies were conducted by administering TBBC (99% pure) in feed to groups of male and female F344/N rats and B6C3F$_1$ mice for 15 days, 13 weeks, and 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium* and cultured Chinese hamster ovary cells.

15-DAY STUDY IN RATS

Groups of 10 male and 10 female F344/N rats were fed diets containing 0, 1,000, 2,500, 5,000, 10,000 or 25,000 ppm TBBC for 15 days. Rats given to 1,000, 2,500, 5,000, or 10,000 ppm received approximate doses of 95, 235, 335, or 365 mg TBBC per kilogram body weight per day (males) or 85, 220, 325, or 270 mg/kg per day (females). Approximate doses for rats receiving 25,000 ppm could not be calculated due to early deaths. All 25,000 ppm rats and three male and four female 10,000 ppm rats died. Surviving rats in the 10,000 ppm groups had a significant weight loss and the final mean body weights of 5,000 and 10,000 ppm male and female rats were significantly lower than those of the controls. Male and female rats exposed to 5,000, 10,000, or 25,000 ppm TBBC consumed markedly less feed than the controls.

Diarrhea occurred in 5,000, 10,000, and 25,000 ppm males and females. The principal lesions attributed to the administration of TBBC were renal papillary and tubule necroses which occurred in 10,000 ppm rats. Focal necrosis or erosions of the glandular stomach also occurred in some 10,000 ppm rats. Changes observed in the thymus and spleen were attributed to debilitation or stress; bone marrow depletion was attributed to nutrient deficiency accompanying weight loss.

15-DAY STUDY IN MICE

Groups of 10 male and 10 female B6C3F$_1$ mice were fed diets containing 0, 1,000, 2,500, 5,000, 10,000, or 25,000 ppm TBBC for 15 days. Mice given 1,000, 2,500, or 5,000 ppm received approximate doses of 285, 585, or 475 mg TBBC per kilogram body weight per day (males) or 360, 950, or 1,030 mg/kg per day (females). Approximate doses for mice given 10,000 or 25,000 ppm could not be calculated due to early deaths. All 10,000 and 25,000 ppm mice died, as did eight males and eight females given 5,000 ppm. A

significant weight loss occurred in surviving 5,000 ppm males and females and the final mean body weights of 2,500 ppm females and 5,000 ppm males and females were significantly lower than those of the controls. Feed consumption by mice given 5,000, 10,000, or 25,000 ppm was markedly reduced. Diarrhea occurred in all 25,000 ppm mice and in most male and female mice given 5,000 or 10,000 ppm. Renal tubule necrosis occurred in eight males and three females in the 5,000 ppm groups. Lymphocytic depletion of lymphoid tissues in many 5,000 ppm males and females was attributed to debilitation and stress or to nutrient deficiency accompanying weight loss.

13-Week Study in Rats

Groups of 10 male and 10 female F344/N rats were fed diets containing 0, 250, 500, 1,000, 2,500, or 5,000 ppm TBBC for 13 weeks. These exposure levels delivered approximate doses of 15, 30, 60, 165, or 315 mg TBBC per kilogram body weight per day (males) or 15, 35, 70, 170, or 325 mg/kg per day (females). All rats survived to the end of the study. The final mean body weight of 5,000 ppm males was 40% lower than that of the controls; the final mean body weight of 5,000 ppm females was 27% lower than that of the controls. Feed consumption by male and female rats exposed to 5,000 ppm TBBC was markedly lower than that by the controls throughout the study. The absolute and relative liver weights of 5,000 ppm females were significantly greater than those of the controls.

Serum alkaline phosphatase (ALP) levels were significantly higher in 2,500 and 5,000 ppm males and slightly higher in 5,000 ppm females. Serum alanine aminotransferase levels were significantly higher in 2,500 and 5,000 ppm males and females. Hematocrit and hemoglobin concentrations and mean erythrocyte volume (MCV) values were significantly lower in 1,000, 2,500, and 5,000 ppm males than in controls; MCV values were also significantly lower in 5,000 ppm females. A dose-related significant increase in forelimb and hindlimb grip strength was observed in exposed male and female rats.

Histopathologic findings in the liver of 2,500 and 5,000 ppm males and females included hypertrophy of Kupffer cells, bile duct hyperplasia, and individual cell necrosis of hepatocytes; centrilobular hepatocyte hypertrophy also occurred in males and females exposed to 5,000 ppm TBBC. Macrophages were increased in size and number in the mesenteric lymph nodes of males and females exposed to 5,000 ppm, and to a lesser extent in 2,500 ppm male and female rats. Pigmentation and degeneration of the renal cortical tubule epithelial cells was also present in males and females in the 2,500 and 5,000 ppm groups; cortical tubule necrosis occurred in 5,000 ppm males and females.

13-Week Study in Mice

Groups of up to 10 male and 10 female B6C3F$_1$ mice were fed diets containing 0, 100, 250, 500, 1,000, or 2,500 ppm TBBC for 13 weeks. These exposure levels delivered approximate doses of 15, 30, 65, 145, or 345 mg TBBC per kilogram body weight per day (males) or 10, 35, 60, 165, or 340 mg/kg per day (females). All mice survived to the end of the study. The final mean body weights of 2,500 ppm males and of 500, 1,000, or 2,500 ppm females were significantly lower than those of the controls. Feed consumption by 2,500 ppm males averaged 24% lower than that by controls through week 3 and was similar to that by controls for the remainder of the study. Feed consumption by females receiving 2,500 ppm averaged 27% less than that by the controls during most of the study. The absolute and relative liver weights of males and females exposed to 2,500 ppm TBBC were slightly but significantly greater than those of the controls. Males exposed to 500, 1,000, or 2,500 ppm and females exposed to 2,500 ppm had significantly increased absolute and relative spleen weights. No clinical findings in mice were considered chemical related.

Hematocrit concentrations and erythrocyte counts of males receiving 1,000 or 2,500 ppm were significantly less than those of the controls; hemoglobin concentration in males receiving 2,500 ppm was significantly less and mean erythrocyte volume was significantly less in males receiving 2,500 ppm. Females in the 1,000 and 2,500 ppm groups had significantly decreased hematocrit concentrations and erythrocyte counts; 2,500 ppm females also had significantly decreased hemoglobin concentrations and mean erythrocyte volumes.

Kupffer cell hypertrophy, bile duct hyperplasia, and an increase in size and number of macrophages in mesenteric lymph nodes were present in 2,500 ppm male and female mice.

2-YEAR STUDY IN RATS

Doses selected for the 2-year study of TBBC were based on the lower body weights and liver and kidney toxicity observed at 5,000 ppm in the 13-week study.

Groups of 115 male and 75 female F344/N rats were fed diets containing 0, 500, 1,000, or 2,500 ppm TBBC for 2 years. Based on average daily feed consumption, these exposure levels resulted in a daily ingestion of TBBC of approximately 20, 40, or 100 mg/kg body weight for males and 20, 45, or 120 mg/kg body weight for females. Hematology, clinical chemistry, and urinalysis evaluations were performed on 15 male and 15 female rats from each group at 3, 9, and 15 months. Also at 15 months, an additional 10 male and 10 female rats from each group were evaluated for histopathology, hematology, and clinical chemistry. Forty male rats per group were evaluated for neurotoxic effects.

Survival, Body Weights, Feed Consumption, and Clinical Findings

Two-year survival rates and mean body weights of exposed male and female rats were generally similar to those of the controls. The mean body weights of 2,500 ppm male rats were slightly lower than those of the controls throughout the study. At week 65, the mean body weight of 2,500 ppm females was 14% lower than that of the controls, but the final mean body weight of this group was 6% lower than that of the control group. Feed consumption, behavior, and general health and appearance of exposed male and female rats were similar to those of the controls.

Hematology and Clinical Chemistry

Results of the hematology evaluation were not uniformly consistent at 3, 9, and 15 months in one set of rats, nor were they consistent between the two sets of rats evaluated at 15 months. Slight but significant decreases in hematocrit levels, hemoglobin concentrations, and erythrocyte counts were observed in the 1,000 and 2,500 ppm groups in one set of males at 15 months. Similar significant decreases in hematocrit level and hemoglobin concentration occurred in 2,500 ppm females at 9 months. Mean erythrocyte hemoglobin and mean erythrocyte hemoglobin concentration of 2,500 ppm females were also significantly lower than those of controls at 9 months and in both sets of female rats evaluated at 15 months. Platelet counts of 2,500 ppm male and female rats were slightly but significantly higher than those of controls at 3 and 9 months. Platelet counts were also slightly but significantly increased in 2,500 ppm males of one set evaluated at 15 months, and in 2,500 ppm females of the second set evaluated at 15 months.

Serum activities of alkaline phosphatase, alanine aminotransferase, and sorbitol dehydrogenase in 2,500 ppm males were significantly greater than those in the controls at 3, 9, and 15 months. Alkaline phosphatase activities in both sets of 1,000 ppm males evaluated at 15 months were also significantly greater than those of controls. Serum activities of alanine aminotransferase and sorbitol dehydrogenase in 2,500 ppm females were also significantly greater than those in controls at 3, 9, and 15 months.

Neurotoxicity Findings

There were no significant inhibitory effects of TBBC on motor nerve excitability or conduction, neuromuscular transmission, or muscle contractility. There were no microscopic lesions in the sciatic nerve, quadriceps muscle, or teased nerve preparations of sciatic nerve that could be attributed to TBBC administration.

Pathology Findings

At the 15-month interim evaluation, the absolute and relative liver weights of 2,500 ppm female rats were significantly greater than those of controls; at 15 months and at the end of the study, the incidences of Kupffer cell hypertrophy, hepatocyte cytoplasmic vacuolization, and mixed cell foci were also significantly increased. At the end of the study, the incidence of hepatocellular fatty change was significantly increased in 2,500 ppm females. The incidence of Kupffer cell hypertrophy was significantly increased in 2,500 ppm males at 15 months and at 2 years; the incidence of cytoplasmic vacuolization was significantly increased in all exposed males at 15 months but only moderately increased in 1,000 and 2,500 ppm males at 2 years; the incidence of basophilic foci was significantly increased in 2,500 ppm males at 15 months and the incidence of mixed cell foci was significantly increased in 1,000 and 2,500 ppm male rats at 2 years. The incidences of hepatocellular adenoma or carcinoma (combined)

in exposed male rats were not significantly greater than that in the controls (0 ppm, 1/50; 500 ppm, 3/50; 1,000 ppm, 3/50; 2,500 ppm, 5/49), were within the historical control range, and were not considered chemical related. The severity of nephropathy was significantly increased in 2,500 ppm female rats.

There was a significant negative trend in the incidence of mammary gland fibroadenoma, adenoma, or carcinoma (combined) in female rats (32/50, 24/50, 11/50, 16/50), and the incidences of fibroadenoma in 1,000 and 2,500 ppm females were significantly less than that of the controls.

2-Year Study in Mice

Because of the reduction in body weights, the increase in liver and spleen weights, and the accompanying histopathologic changes in the liver of 2,500 ppm male and female mice in the 13-week study, the doses selected for the 2-year study were 250, 500, and 1,000 ppm.

Groups of 80 male and 80 female mice were fed diets containing 0, 250, 500, or 1,000 ppm TBBC for 2 years. Based on average daily feed consumption, these exposure levels resulted in the daily ingestion of approximately 30, 60, or 145 mg TBBC/kg body weight for males and 45, 110, or 255 mg TBBC/kg body weight for females. Nine or 10 animals from each exposure group were evaluated at 3, 9, and 15 months.

Survival, Body Weights, Feed Consumption, and Clinical Findings

Two-year survival rates of exposed male and female mice were similar to those of the controls. The final mean body weights of male and female mice exposed to 1,000 ppm were 8% and 18% lower than those of the controls, respectively. The final mean body weights of females exposed to 250 or 500 ppm were 8% to 9% lower than that of the controls. Feed consumption by exposed males was similar to that by controls, and there were no clinical findings attributed to TBBC administration.

Hematology and Clinical Chemistry

Hematocrit level, hemoglobin concentration, and erythrocyte count in 1,000 ppm male mice were significantly lower than those in controls at the 15-month interim evaluation. Serum alkaline phosphatase activities in 1,000 ppm males were slightly but significantly greater than those in controls at 3 and 9 months, as was the serum alkaline phosphatase activity in 1,000 ppm females at 9 months. Serum levels of total bilirubin in all exposed groups of males were significantly greater than those in controls at 9 and 15 months.

Pathology Findings

In the liver of male mice, negative trends in the incidences of fatty change, clear cell foci, and adenoma or carcinoma combined occurred at the end of the 2-year study. There were no compound-related increased incidences of neoplasms or non-neoplastic lesions in mice receiving TBBC for 2 years. A negative trend in the incidence of fatty change in the liver of male mice also occurred at 15 months.

Genetic Toxicology

4,4'-Thiobis(6-*t*-butyl-*m*-cresol) was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 with or without exogenous metabolic activation (S9). Sister chromatid exchanges were induced in cultured Chinese hamster ovary cells treated with TBBC, with and without S9, but no increases in chromosomal aberrations were noted in cultured Chinese hamster ovary cells after treatment with TBBC.

Conclusions

Under the conditions of these 2-year feed studies, there was *no evidence of carcinogenic activity** of 4,4'-thiobis(6-*t*-butyl-*m*-cresol) in male or female F344/N rats administered 500, 1,000, or 2,500 ppm or in male or female B6C3F$_1$ mice administered 250, 500, or 1,000 ppm.

Nonneoplastic lesions associated with exposure to TBBC included: Kupffer cell hypertrophy, cytoplasmic vacuolization, and mixed cell foci in the liver of male and female rats, fatty change in the liver of female rats, and an increase in the severity of nephropathy in the kidney of female rats. In addition, decreased incidences of fibroadenoma, adenoma, or carcinoma (combined) were observed in the mammary gland of female rats. Decreases also occurred in the incidences of fatty change, clear cell foci, and adenoma or carcinoma (combined) in the liver of male mice.

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

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

	Male F344/N Rats	Female F344/N Rats	Male $B6C3F_1$ Mice	Female $B6C3F_1$ Mice
Doses	0, 500, 1,000, or 2,500 ppm in feed (approximately 20, 40, or 100 mg/kg/day)	0, 500, 1,000, or 2,500 ppm in feed (approximately 20, 45, or 120 mg/kg/day)	0, 250, 500, or 1,000 ppm in feed (approximately 30, 60, or 145 mg/kg/day)	0, 250, 500, or 1,000 ppm in feed (approximately 45, 110, or 255 mg/kg/day)
Body weights	Exposed groups lower than controls	2,500 ppm group lower than controls	1,000 ppm group lower than controls	Exposed groups lower than controls
2-Year survival rates	18/50, 28/50, 22/50, 18/50	34/50, 31/50, 32/50, 28/50	42/50, 42/50, 49/50, 45/50	40/51, 38/50, 36/50, 35/50
Nonneoplastic effects	Liver: Kupffer cell hypertrophy: 2/50, 3/50, 2/50, 31/49; cytoplasmic vacuolization: 13/50, 11/50, 19/50, 18/49; mixed cell foci: 6/50, 14/50, 18/50, 15/49	Liver: Kupffer cell hypertrophy: 11/50, 10/50, 9/50, 42/50; cytoplasmic vacuolization: 12/50, 10/50, 20/50, 34/50; fatty change: 9/50, 8/50, 15/50, 19/50; mixed cell foci: 5/50, 4/50, 14/50, 34/50 Kidney: nephropathy severity (1.4, 1.4, 1.6, 2.3)	None	None
Neoplastic effects	None	None	None	None
Other findings	None	Mammary gland: fibroadenoma, adenoma, or carcinoma (combined): 32/50, 24/50, 11/50, 16/50	Liver: fatty change: 19/50, 17/50, 5/50, 6/50; clear cell foci: 6/50, 5/50, 2/50, 0/50; adenoma or carcinoma (combined): 25/50, 30/50, 27/50, 16/50	None
Level of evidence of carcinogenic activity	No evidence	No evidence	No evidence	No evidence

Genetic toxicology

Salmonella typhimurium gene mutation: Negative in strains TA98, TA100, TA1535, and TA1537 with and without S9

Chinese hamster ovary cells *in vitro*

Sister chromatid exchanges: Positive with and without S9

Chromosomal aberrations: Negative with and without S9

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

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

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

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

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

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

NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Technical Report on 4,4′-thiobis(6-*t*-butyl-*m*-cresol) on June 22, 1993, are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing NTP studies:

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

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SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On June 22, 1993, the draft Technical Report on the toxicology and carcinogenesis studies of 4,4′-thiobis(6-*t*-butyl-*m*-cresol) (TBBC) received public review by the National Toxicology Program Board of Scientific Counselors Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Mr. J.D. Cirvello, NIEHS, introduced the toxicology and carcinogenesis studies of TBBC by discussing the uses of the chemical and rationale for study, describing the experimental design, reporting on survival and body weight effects, and commenting on compound-related nonneoplastic lesions in rats and mice. The proposed conclusions were *no evidence of carcinogenic activity* of 4,4′-thiobis(6-*t*-butyl-*m*-cresol) in male or female F344/N rats or male or female B6C3F$_1$ mice.

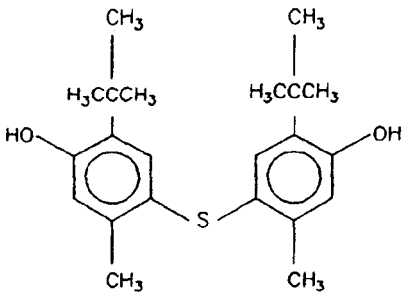
Mr. Beliczky, a principal reviewer, agreed with the proposed conclusions. He asked if the literature had been reviewed as most of the references were from the 1950's. Mr. Cirvello said a literature search had been done in 1992. Mr. Beliczky questioned the reference to the NIOSH Permissible Exposure Limit because the levels that were mentioned as either total dust or respirable dust are generally referred to as nuisance dust, those dusts which are physiologically inactive or inert. He did not think one could call TBBC inert or physiologically inactive. He commented that the nomination for review by the NTP was referenced to a 1978 study at Harvard and wanted to note that this epidemiological study had been funded by the United Rubber Worker's Joint Occupational Health Program.

Dr. Zeise, the second principal reviewer, agreed in principle with the proposed conclusions. She pointed out that, while the liver in male rats is clearly a target organ for toxicity, the data are unclear as to whether or not the liver is a target organ for carcinogenicity. She said the incidence of hepatocellular adenoma would be statistically significant if the historical control incidence at the study laboratory were used instead of the concurrent controls. She said there should be consideration given to changing the conclusion in male rats to "equivocal evidence of carcinogenic activity." Mr. Cirvello commented that if one looks at the overall historical control database, there were three studies from other laboratories with control values as high as those recorded in male rats in the high-dose group in the present study.

Dr. Ward, the third principal reviewer, agreed in principle with the proposed conclusions. He said it should be noted that the degree of nephropathy was increased in female rats and there should be a statement that male rats may have been able to tolerate a slightly higher dose. Mr. Cirvello said a statement about the nephropathy should have been included. He said that toxicity and reduction in body weight gain in the prechronic and 2-year studies indicated that the high dose was correct in male rats. Dr. Ward agreed with Dr. Zeise as to the uncertain significance of the liver neoplasms in male rats. Since mixed cell foci were increased more in exposed animals, Dr. Ward said it would be useful to have a morphologic description and an assessment as to whether they are preneoplastic lesions. Dr. S.L. Eustis, NIEHS, said a description would be added to the report, but it was difficult to say whether the foci were preneoplastic. There was no atypia reported, a finding often found in foci induced by hepatocarcinogens.

Mr. Beliczky moved that the Technical Report on 4,4′-thiobis(6-*t*-butyl-*m*-cresol) be accepted with the revisions discussed and with the conclusions as written for male and female rats and mice, *no evidence of carcinogenic activity*. Dr. Bailey seconded the motion, which was accepted unanimously with ten votes.

INTRODUCTION



4,4′-THIOBIS(6-*t*-BUTYL-*m*-CRESOL)

CAS No. 96-69-5

Chemical Formula: $C_{22}H_{30}SO_2$ Molecular Weight: 358.52

Synonyms: 4,4′-Thiobis(6-*t*-butyl-3-cresol); bis(3-*t*-butyl-4-hydroxy-6-methylphenyl)sulfide
Trade names: Santonox; Santowhite Crystals; Sumilizer; Thioalkofen; Yoshinox

CHEMICAL AND PHYSICAL PROPERTIES

4,4′-Thiobis(6-*t*-butyl-*m*-cresol) (TBBC) is a fine, white crystalline powder with a melting point of 161° C and specific gravity of 1.10. This chemical is very soluble in methanol (79 g/mL), soluble in acetone (20 g/mL), less soluble in benzene (5.0 g/mL), and slightly soluble in water (0.08 g/mL) (Lefaux, 1968).

USE AND HUMAN EXPOSURE

TBBC is widely used in the rubber and plastics industries as an antioxidant for polyolefins, polyethylenes, polypropylenes, natural rubber, and latex. TBBC is approved by the U.S. Food and Drug Administration as a constituent of high-pressure polyethylene packaging for foodstuffs, excluding fats, and as a component of polyolefin film packaging in contact with meat or meat food products (Lefaux, 1968). Although the potential exists for the general population to be exposed through contact with polymer products or leaching of TBBC from such products into food, two studies investigating the migration of TBBC from plastic packaging materials indicated no significant exposure from this source (Udhe and Woggon, 1971; Ruedt and Herbolzheimer, 1976). Exposure is also possible via surface water contamination resulting from releases through manufacturing or use operations. No data were found on the environmental occurrence of TBBC.

TBBC reportedly has potential uses as a fungicide against such molds as *Aspergillus niger*, *Penicillium citrinum*, and *Rhizopus nigricans*, and as a preservative for paints, paper, fiber, and leather (Umekawa *et al.*, 1972). However, Hejtmankova *et al.* (1979) found that TBBC did not inhibit *A. niger* or *A. fumigatus*, and only weakly to moderately inhibited seven other strains of fungi.

No recent annual TBBC production or use data were found. Based on a survey conducted by NIOSH from 1981 to 1983, an estimated 12,349 workers are potentially exposed to TBBC in the workplace (NIOSH, 1991). The current Permissible Exposure Limits established by NIOSH for TBBC (as an 8-hour time-weighted average) are 15 mg/m^3 for total dust and 5 mg/m^3 for the respirable fraction.

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

The disposition of [^{14}C]-labeled TBBC was studied in male F344/N rats (Birnbaum *et al.*, 1983). TBBC was administered by single oral gavage doses of 5, 50, or

500 mg TBBC/kg body weight in corn oil or in Emulphor:ethanol and by intravenous injection of 5 mg/kg in Emulphor:ethanol:water. Following oral exposure, TBBC was incompletely absorbed (the percentage absorbed was not determined) and there was a dose-related decrease in the rate of absorption. When administered *in situ* via luminal perfusion of 4, 49, or 500 mg/kg body weight, TBBC absorption in the small intestine was directly proportional to dose, suggesting that retention of the compound in the stomach was responsible for the apparent dose-related decline in absorption. Following intravenous administration of 5 mg/kg, very low percentages of total dose administered were detected rapidly in liver, adipose tissue, skin, muscle, and blood. The highest percentage of total dose was found in the liver, which had 2% after 15 minutes, 0.5% after 2 hours, and 0.4% after 1 day. The initial rate of clearance from liver and skin was very rapid, followed by a slower terminal decay phase. A slow rate of clearance was also observed in adipose tissue. Twenty-four hours after treatment, the parent compound accounted for most of the residual radioactivity in liver and adipose tissue; chronic exposure to TBBC could result in some accumulation of unmetabolized compound at these sites. More than half of the administered compound was excreted the first day, primarily through the bile into the feces; less than 2% was excreted into the urine. All radioactivity in the bile was in the form of metabolites of TBBC, the major metabolite being a glucuronide conjugate. A later study (Smith *et al.*, 1985) identified the major metabolite of TBBC in bile as the monoglucuronic acid conjugate.

To evaluate the effects of age on the glucuronidation of TBBC, male F344 rats 2.5, 16, and 26 months old were administered 5 mg [^{14}C]-labeled TBBC/kg intravenously. Urine and feces were collected for 3 days (Borghoff *et al.*, 1988). Bile was also collected for 6 hours after intravenous doses of 5 or 25 mg/kg. The 26-month-old animals excreted significantly less TBBC-derived radioactivity in bile, feces, and urine than both of the younger groups. The percentage of the dose eliminated in bile as a glucuronide also decreased with age. After 30 minutes of bile collection following a 5 mg/kg dose, 8% had been eliminated as a glucuronide by the 2.5-month-old group, 5.6% by the 16-month-old group, and 4.4% by the 26-month-old group. When the 26-month-old animals were given 25 mg/kg, elimination as glucuronide was only 2% of the dose. *In vitro* studies using TBBC as a substrate demonstrated that hepatic uridine diphosphate glucuronyl transferase activity decreased in aging animals. Further, the hepatic concentration of uridine diphosphate glucuronyl acid (UDPGA) also decreased in animals from 2.5 to 28 months of age. Thus, the decrease in the ability of the aging rats to conjugate and excrete TBBC may be caused by a decrease in both the activity of the conjugating enzyme and the availability of UDPGA.

Humans

No information on the absorption, distribution, metabolism, or excretion of TBBC in humans was found in the literature.

TOXICITY

Experimental Animals

Few published studies on the toxicity of TBBC exist. In acute oral toxicity studies in rats, the LD_{50} varies from 5,000 to 7,000 mg/kg depending on the purity of the test material (personal communication cited in Birnbaum *et al.*, 1983). Details are not given except that rats exhibited severe diarrhea preceding death. In the previously discussed disposition studies (Birnbaum *et al.*, 1983), TBBC administered by gavage in either Emulphor:ethanol or corn oil (5, 50, or 500 mg/kg) caused mild inflammation, congestion, hemorrhage, and mucosal erosion of the stomach in rats. These findings were dose related and detectable as early as 1 hour after administration of 500 mg/kg. Studies in which rats ingested TBBC in feed for 30 or 90 days were performed by E.I. du Pont de Nemours & Co. and the results are summarized briefly by Lefaux (1968). In the 30-day study, groups of six male and six female rats were fed diets containing 500 or 2,500 ppm TBBC. The 500 ppm group displayed no signs of toxicity, whereas at 2,500 ppm, rats exhibited growth retardation and increased liver weights. In the 90-day study, rats were fed diets containing 50 or 500 ppm TBBC, and the only effects noted were decreased feed consumption and slight growth retardation in 500 ppm males. Monsanto Chemical Company conducted 3-month feed studies using the same doses of TBBC (50 or 500 ppm) and obtained similar results; the only sign of toxicity was growth retardation in animals receiving 500 ppm (McCormick, 1972).

TBBC toxicity was also studied in adult female B6C3F$_1$ mice by administering 10, 100, or 200 mg/kg daily in corn oil by gavage for 14 consecutive days (Munson *et al.*, 1988). No overt toxicity was observed and no marked effects on serum enzymes occurred. The highest exposure group had a 41% increase in total leukocytes with a 31% increase in lymphocytes and a 177% increase in neutrophils. Bone marrow studies revealed a significant (30%) increase in the number of cells/femur in 200 mg/kg mice; macrophage progenitors were significantly increased by 28% and granulocyte-monocyte progenitors were increased by 20%. A dose-related increase occurred in absolute weights of both the spleen and liver, although the histopathology of the spleens of TBBC-treated mice was not different from that of the controls. The livers of mice in the high-dose group had changes described as mild focal hydropic degeneration, mild hepatitis, and a slight increase in the number of Kupffer cells. Hepatic cytochrome P-450 and microsomal protein levels exhibited a dose-related increase, as did enzyme activities of aminopyrine demethylase and aniline hydroxylase.

Immunotoxicologic studies were conducted after administering TBBC in corn oil by gavage at doses of 10, 100, or 200 mg/kg to B6C3F$_1$ mice daily for 14 consecutive days (Holsapple *et al.*, 1988). A 200 mg/kg dose produced a decrease in the peak IgM (44%) and peak IgG (48%) antibody response to *in vivo* challenge with sheep erythrocytes, but had no effect on the delayed hypersensitivity response to challenge with keyhole limpet hemocyanin. At 10 and 200 mg/kg, a significant decrease in the mixed lymphocyte response (MLR) occurred, but doses of 10, 100, or 200 mg/kg produced no effects on the *in vitro* lymphoproliferative responses of spleen cells to optimal concentrations of concanavalin A, phytohemagglutinin, or lipopolysaccharide. A dose-related increase in the basal (unstimulated) DNA synthesis of the spleen cells occurred in both the MLR and the mitogen assays. A significant increase in natural killer cell and serum complement activity was also observed. The increase in natural killer cell activity was significant in mice administered 100 and 200 mg/kg, with the greatest increase at the 100 mg/kg dose; 10 mg/kg TBBC produced a significant (35%) increase in CH50 and at 100 mg/kg a significant (54%) increase occurred. Effects on macrophage function were complex; either an increase or no effect was observed, depending on the parameter measured. Exposure to 10, 100, or 200 mg/kg caused a dose-related increased resistance to challenge with *Streptococcus pneumoniae* and B16F10 melanoma, a decreased resistance to challenge with PYB$_6$ neoplasms, and no effect on the resistance to HSV-2, *Listeria*, or *Plasmodium*. Thus, several parameters reflecting immune function were altered following 14-day gavage exposure to TBBC.

Humans

Two patients with allergic contact dermatitis were found to be patch-test positive to latex gloves made by the same manufacturer. TBBC was the antioxidant used in making the gloves and both patients had a positive patch test reaction to the TBBC itself (Rich *et al.*, 1991). No other information on the toxicity of TBBC in humans was found in the literature.

Reproductive and Developmental Toxicity

Experimental Animals

In a study to evaluate the effects of TBBC on reproduction in female Swiss mice, 485 mg/kg was administered daily by gavage to 50 pregnant mice on days 6 through 15 of gestation (EHRT, 1989). TBBC caused maternal mortality and a decreased rate of survival of pups, but had no effect on the number of viable litters, litter size, pup birth weight, or pup weight gain.

Humans

No information on the reproductive and developmental toxicity of TBBC in humans was found in the literature.

Carcinogenicity

Experimental Animals

A report by Draganov *et al.* (1974) suggests that TBBC may be a neoplasm promoter. When Yoshida sarcomas were transplanted to rats, neoplasm development was enhanced if TBBC was administered orally for 10 days at a dose of 80 mg/kg daily, beginning 5 days after transplantation. No other data were provided in the report.

Humans

No information on the potential carcinogenicity of TBBC in humans was found in the literature.

GENETIC TOXICITY

TBBC was tested for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 with a preincubation protocol in the presence and absence of S9; no mutagenic activity was observed in any of these four strains (Zeiger *et al.*, 1987). There are no other published data on the genotoxicity of this compound.

STUDY RATIONALE

The National Cancer Institute nominated TBBC for study as a representative of the sulfur-containing class of antioxidants used in rubber processing. A study that was recent at the time of nomination demonstrated an excess of several types of cancer among a cohort of 13,570 rubber workers (Monson and Fine, 1978). In addition, the presence of TBBC in plastic food wraps and containers was viewed as a possible hazard to the general population.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF 4,4'-THIOBIS(6-*T*-BUTYL-*M*-CRESOL)

4,4'-Thiobis(6-*t*-butyl-*m*-cresol) was obtained in one lot (12) from Monsanto Industrial Chemical Company (Akron, OH). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO), (Appendix I).

The chemical, a white powdered solid, was identified as 4,4'-thiobis(6-*t*-butyl-*m*-cresol) (TBBC) by infrared, ultraviolet/visible, and nuclear magnetic resonance spectroscopy. Purity was determined by elemental analyses, Karl Fischer water analysis, functional group titration, thin-layer chromatography, and gas chromatography. Analyses of the chemical for carbon, hydrogen and sulfur were in agreement with theoretical values for TBBC. Functional group titration indicated a purity of 100% ± 3%. Thin-layer chromatography using two systems indicated a major spot and two trace impurities. Gas chromatography using one system indicated two impurities with a total area of 0.7% relative to the major peak area that eluted before the major peak. A second system indicated one impurity that eluted before the major peak and had an area of 0.39% relative to the major peak. The overall purity was determined to be approximately 99%. Subsequent analysis by the analytical chemistry laboratory indicated a purity of approximately 99%.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared weekly by mixing 4,4'-thiobis(6-*t*-butyl-*m*-cresol) with feed (Table I1). Homogeneity and stability studies of the 250 and 25,000 ppm dose formulations were performed by the analytical chemistry laboratory. For the homogeneity and stability studies, dose formulations were analyzed by high performance liquid chromatography. Homogeneity was confirmed at the 100 and 10,000 ppm concentrations, and stability was established at these concentrations for at least 3 weeks at –20° C when stored in the dark and for 3 days when exposed to air and light.

Periodic analyses of the dose formulations of TBBC were conducted at the study laboratory and analytical chemistry laboratory using high-performance liquid chromatography. During the 15-day studies, only the initial formulation was analyzed (Table I2). During the 13-week and the 2-year studies, the dose formulations were analyzed every 6 to 10 weeks (Tables I3 and I4). In the 2-year studies, 93% (86/92) of the formulations were within 10% of the target concentrations. Results of the periodic referee analyses performed by the analytical chemistry laboratory were in good agreement with the results obtained by the study laboratory (Table I5).

15-DAY STUDIES

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Frederick Cancer Research Center (Frederick, MD). At receipt, the rats and mice were 6 weeks old. Animals were quarantined for 13 to 15 days before exposure began. At this time, two males and two females of each species were randomly selected and evaluated for evidence of disease. Groups of 10 male and 10 female rats and mice were fed diets containing 0, 1,000, 2,500, 5,000, 10,000, or 25,000 ppm TBBC. Feed and water were available *ad libitum*. Rats and mice were housed five per cage. Clinical findings were recorded daily for rats and mice. Feed consumption was recorded daily by cage. The animals were weighed initially, weekly, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

At the end of the 15-day studies, blood was collected from all animals by cardiac puncture for hematology analyses. The parameters measured are listed in Table 1. A necropsy was performed on all rats and mice. The brain, gastrointestinal tract, heart, right kidney, liver, lung, spleen, right testis, and thymus were weighed. Tissues for microscopic examination were embedded in paraffin, sectioned to a thickness of 4 to 6 μm, and stained with hematoxylin and eosin. Histopathologic examinations were performed on 0, 2,500, 5,000, and 10,000 ppm rats and 0, 2,500, and 5,000 ppm mice. Table 1 lists the tissues and organs examined microscopically.

13-WEEK STUDIES

The 13-week studies were conducted to evaluate the cumulative toxic effects of repeated exposure to TBBC and to determine the appropriate exposure levels to be used in the 2-year studies.

Male and female F344/N rats and B6C3F$_1$ mice were obtained from the Frederick Cancer Research Center (Frederick, MD). On receipt, the rats and mice were 29 days old. The rats were quarantined for 15 days and the mice for 22 days before exposure began. Before initiation of the studies, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. At the end of the studies, serologic analyses were performed on five male and five female control rats and mice using the protocols of the NTP Sentinel Animal Program (Appendix L).

Groups of 10 male and 10 female rats were fed diets containing 0, 250, 500, 1,000 2,500, or 5,000 ppm TBBC. Groups of 10 male and 10 female mice were fed diets containing 0, 100, 250, 500, 1,000, or 2,500 ppm TBBC. Feed and water were available *ad libitum*. Rats were housed five per cage and mice were housed individually. Clinical findings were recorded weekly. Feed consumption was recorded daily by cage for rats and daily by animal for mice. The animals were weighed initially, weekly, and at the end of the studies. Further details of study design and animal maintenance are summarized in Table 1.

During the final eight days of the 13-week study in rats, males and females receiving 0, 1,000, and 2,500 ppm were tested for forelimb and hindlimb grip strength, startle response, tail flick, and foot splay. See Appendix H for detailed methods.

Two days before the end of the 13-week studies, blood was collected from the orbital sinus of all rats and mice for hematology analyses. At the end of the 13-week studies, blood was collected from all rats by cardiac puncture for clinical chemistry analyses. The hematology and clinical chemistry parameters measured are listed in Table 1. A necropsy was performed on all animals. The brain, heart, right kidney, liver, lung, spleen, right testicle, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm, and stained with hematoxylin and eosin. A complete histopathologic examination was performed on 0, 1,000, 2,500, and 5,000 ppm rats and 0, 1,000, and 2,500 ppm mice. Table 1 lists the tissues and organs routinely examined.

2-YEAR STUDIES

Study Design

Groups of 115 male and 75 female rats were fed diets containing 0, 500, 1,000, or 2,500 ppm TBBC (Table 1). Fifteen male and 15 female rats from each group were evaluated at 3, 9, and 15 months for alterations in hematology, clinical chemistry, and urinalysis parameters and then discarded. An additional 10 male and 10 female rats from each group were also evaluated at 15 months for alterations in hematology and clinical chemistry parameters; these animals received complete necropsy and histopathology examinations.

Forty of the 115 male rats in each exposure group were designated for neurotoxicity evaluation at 3 and 6 months (Appendix H). At 3 months, startle reflex and fore- and hindlimb grip strength were measured in all 40 animals. Ten males per group received electrophysiologic evaluations, including measurements of sciatic nerve conduction time following various frequencies of electrical stimulation and contractile tension of the gastrocnemius muscle following various frequencies of electrical stimulation or following graded electrical stimulation. An additional 10 males per group received whole body perfusion for histopathologic examination of the left quadriceps muscle and left sciatic nerve and of teased nerve preparations of the sciatic nerve. The remaining 20 male rats in each group were fed the control diet for 13 additional weeks to determine the reversibility of TBBC-induced changes. At 6 months, grip strength tests were repeated in all 20 rats per group. These 20 rats were then split into two groups of 10 and given electrophysiologic and neuropathologic evaluations as described above.

Groups of 80 male and 80 female mice were fed diets containing 0, 250, 500, or 1,000 ppm TBBC. At 3, 9, and 15 months, groups of 10 male and 10 female mice per group were killed and evaluated for alterations in hematology and clinical chemistry parameters. The 10 male and 10 female mice per group killed at 15 months also received a complete necropsy and histopathologic evaluation.

Source and Specification of Animals

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Taconic Farms (Germantown, NY) for use in the 2-year studies. Rats and mice were quarantined for 11 days before the beginning of the studies. Five male and five female rats and mice were selected for parasite evaluation and gross observation of disease. Serology samples were collected for viral screening. Rats were approximately 43 days old and mice approximately 39 days old at the beginning of the studies. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix L).

Animal Maintenance

Rats were housed five per cage and mice were housed individually. Feed and water were available *ad libitum*. Feed consumption was measured twice weekly by cage. Cages and racks were rotated biweekly. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is provided in Appendix K.

Clinical Examinations and Pathology

All animals were observed twice daily. Clinical findings and body weights were recorded at the beginning of the studies, weekly for 13 weeks, and monthly thereafter. A complete necropsy and microscopic examination were performed on all rats and mice except: the 15 male and 15 female rats per group designated for hematology, clinical chemistry, and urinalysis evaluations at 3, 9, and 15 months; the 10 male and 10 female mice per group designated for hematology and clinical chemistry at 3 and 9 months; and the 40 male rats per group designated for neurotoxicity and neuropathologic evaluations. At the 15-month interim evaluation, the brain, gastrointestinal tract, right kidney, liver, and spleen of rats and mice were weighed. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 μm, and stained with hematoxylin and eosin for microscopic examination. 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 microscopic slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a quality assessment pathologist reviewed the liver of male and female rats, neoplasms of the thyroid gland, mammary gland, and uterus of female rats, neoplasms of the skin, bone, and nose of male rats, the liver of female mice, and neoplasms of the ovary of female mice.

The quality assessment report and slides were submitted to the NTP Pathology Working Group (PWG) chair, who reviewed the selected tissues and any other tissues for which a disagreement in diagnosis between the laboratory and quality assessment pathologists existed. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologist, or lesions of general interest were presented by the chair to the PWG for review. Tissues examined included the skin, bone, and nose of male rats, the liver of male and female rats, the mammary gland, thyroid gland, and uterus of female rats, and the liver and ovary of female mice. The PWG consisted of the quality assessment pathologist and other pathologists experienced in rodent toxicologic pathology. This group examined the tissues without any knowledge of exposure groups or previously rendered diagnoses. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Thus, the final diagnoses represent a consensus of contractor pathologists 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 diagnosed lesions for each tissue type were evaluated separately or combined according to the guidelines of McConnell *et al.* (1986).

Statistical Methods

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missing

were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions as presented in Tables A1, A5, B1, B5, C1, C5, D1, and D4 are given as the number of animals bearing such lesions at a specific anatomic site and the number of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A3, B3, C3, and D3) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., skin, intestine, harderian gland, and mammary gland) before microscopic evaluation, or when neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed.

Analysis of Neoplasm Incidences

The majority of neoplasms in these studies were considered to be incidental to the cause of death or not rapidly lethal. Thus, the primary statistical method used was logistic regression analysis, which assumed that the diagnosed neoplasms were discovered as the result of death from an unrelated cause and thus did not affect the risk of death. In this approach, neoplasm prevalence was modeled as a logistic function of chemical exposure and time. Both linear and quadratic terms in time were incorporated initially, and the quadratic term was eliminated if the fit of the model was not significantly enhanced. The neoplasm incidences of exposed and control groups were compared on the basis of the likelihood score test for the regression coefficient of dose. This method of adjusting for intercurrent mortality is the prevalence analysis of Dinse and Lagakos (1983), further described and illustrated by Dinse and Haseman (1986). When neoplasms are incidental, this comparison of the time-specific neoplasm prevalences also provides a comparison of the time-specific neoplasm incidences (McKnight and Crowley, 1984).

In addition to logistic regression, other methods of statistical analysis were used, and the results of these tests are summarized in the appendixes. These methods include the life table test (Cox, 1972; Tarone, 1975), appropriate for rapidly lethal neoplasms, and the Fisher exact test and the Cochran-Armitage trend test (Armitage, 1971; Gart *et al.*, 1979), procedures based on the overall proportion of neoplasm-bearing animals.

Tests of significance included pairwise comparisons of each exposed group with controls and a test for an overall dose-related trend. Continuity-corrected tests were used in the analysis of neoplasm incidence, and reported P values are one sided. The procedures described in the preceding paragraphs were also used to evaluate selected nonneoplastic lesions. For further discussion of these statistical methods, see Haseman (1984).

Analysis of Nonneoplastic Lesion Incidences

Because all nonneoplastic lesions in this study were considered to be incidental to the cause of death or not rapidly lethal, the primary statistical analysis used was a logistic regression analysis in which nonneoplastic lesion prevalence was modeled as a logistic function of chemical exposure and time. For lesions detected at the interim evaluation, the Fisher exact test was used, a procedure based on the overall proportion of affected animals.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between exposed and control groups in the analysis of continuous variables. Organ and body weight data, which have approximately normal distributions, were analyzed using the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Clinical chemistry and hematology data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Average severity values were analyzed for significance using the Mann-Whitney U test (Hollander and Wolfe, 1973).

Historical Control Data

Although the concurrent control group is always the first and most appropriate control group used for evaluation, historical control data can be helpful in the overall assessment of neoplasm incidence in certain instances. Consequently, neoplasm incidences from the NTP historical control database (Haseman *et al.*, 1984, 1985) are included in the NTP reports for neoplasms appearing to show compound-related effects.

Quality Assurance Methods

The 13-week and 2-year studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 2-year studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assurance contractor. Separate audits covering completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and preliminary review draft of this NTP Technical Report were conducted. 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, so all comments had been resolved or were otherwise addressed during the preparation of this Technical Report.

GENETIC TOXICOLOGY

The genetic toxicity of TBBC was assessed by testing the ability of the chemical to induce mutations in various strains of *Salmonella typhimurium* and chromosomal aberrations in cultured Chinese hamster ovary cells. The protocols for these studies and the results are given in Appendix E.

The genetic toxicity studies of TBBC are part of a larger effort by the NTP to develop a database that would permit the evaluation of carcinogenicity in experimental animals from the structure and responses of the chemical in short-term *in vitro* and *in vivo* genetic toxicity tests. These genetic toxicity tests were originally developed to study mechanisms of chemically induced DNA damage and to predict carcinogenicity in animals, based on the electrophilic theory of chemical carcinogenesis and the somatic mutation theory (Miller and Miller, 1977; Straus, 1981; Crawford, 1985).

There is a strong correlation between a chemical's potential electrophilicity (structural alert to DNA reactivity), mutagenicity in *Salmonella*, and carcinogenicity in rodents. The combination of electrophilicity and *Salmonella* mutagenicity is highly correlated with the induction of carcinogenicity in rats and mice and/or at multiple tissue sites (Ashby and Tennant, 1991). Other *in vitro* genetic toxicity tests do not correlate well with rodent carcinogenicity (Tennant *et al.*, 1987; Zeiger *et al.*, 1990), although these other tests can provide information on the types of DNA and chromosome effects that can be induced by the chemical being investigated. Data from NTP studies show that a positive response in *Salmonella* is currently the most predictive *in vitro* test for rodent carcinogenicity (89% of the *Salmonella* mutagens were rodent carcinogens), and that there is no complementarity among the *in vitro* genetic toxicity tests. That is, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. The predictivity for carcinogenicity of a positive response in bone marrow chromosome aberration or micronucleus tests is not yet defined.