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.

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

15-Day Studies	13-Week Studies	2-Year Studies
Study Laboratory		
American Biogenics Corporation (Woburn, MA)	American Biogenics Corporation (Woburn, MA)	Battelle Columbus Laboratories (Columbus, OH)
Strain and Species		
Rats: F344/N Mice: $B6C3F_1$	Rats: F344/N Mice: $B6C3F_1$	Rats: F344/N Mice: $B6C3F_1$
Animal Source		
Frederick Cancer Research Center (Frederick, MD)	Frederick Cancer Research Center (Frederick, MD)	Taconic Farms (Germantown, NY)
Time Held Before Studies		
Rats: 14 days (males) or 15 days (females) Mice: 13 days (males) or 14 days (females)	Rats: 15 days Mice: 22 days	11 days
Average Age When Studies Began		
Rats: 44 days Mice: 43 days	Rats: 43 days Mice: 50 days	Rats: 43 days Mice: 39 days
Date of First Dose		
Rats: 29 December (males) or 30 December (females) 1983 Mice: 3 January (males) or 4 January (females) 1984	Rats: 1 August 1984 Mice: 15 August 1984	Rats: 29 December 1986 (special studies and 15-month interim) or 22 December 1986 (2-year study) Mice: 19 January 1987
Duration of Dosing		
15 days	92-94 days	104 weeks
Date of Last Dose		
Rats: 12 January (males) or 13 January (females) 1984 Mice: 17 January (males) or 18 January (females) 1984	Rats: 2 November 1984 Mice: November 1984	Rats: 12 December 1988 Mice: 9 January 1989
Necropsy Dates		
Rats: 12 January (males) or 13 January (females) 1984 Mice: 17 January (males) or 18 January (females) 1984	Rats: 31 October to 2 November 1984 Mice: 14 to 16 November 1984	Rats: 15-Month interim evaluation and clinical pathology - 21-22 March 1988 Terminal - 19-21 December 1988 Mice: 15-Month interim - 18-19 April 1988 Terminal - 16-20 January 1989

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol) (continued)

15-Day Studies	13-Week Studies	2-Year Studies
Average Age at Necropsy		
Rats: 59 days Mice: 57 days	Rats: 135 days Mice: 141 days	15-Month interim evaluation and clinical pathology - 71 weeks Terminal - 111 weeks
Size of Study Groups		
10 males and 10 females	Same as 15-day studies	Rats: 115 males and 75 females Mice 80 males and 80 females
Method of Distribution		
Animals randomized from weight classes into cage groups using a computer-generated list of random numbers; cages randomized into test groups from another computer-generated list of random numbers	Same as 15-day studies	Animals randomized from weight classes into cage groups and dose groups using a partitioning algorithm
Animals per Cage		
5	Rats: 5 Mice: 1	Rats: 5 Mice: 1
Method of Animal Identification		
Ear punch	Same as 15-day studies	Rats: Neurological - ear tag Clinical pathology - toe clip Terminal - toe clip Mice: Toe clip
Diet		
NIH-07 open formula meal diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed daily	Same as 15-day studies	Same as 15-day studies, changed twice weekly
Maximum Storage Time for Feed		
108 days post-milling	120 days post-milling	Same as 13-week studies
Water Distribution		
Tap water (Woburn municipal supply) via automatic watering system (Hardco, Cincinnati, OH), available *ad libitum*	Same as 15-day studies	Tap water (Columbus municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol) (continued)

15-Day Studies	13-Week Studies	2-Year Studies
Cages		
Polycarbonate, (Suburban Surgical Co., Inc., Wheeling, IL), changed twice weekly	Same as 15-day studies except cages were changed twice weekly for rats.	Polycarbonate (Lab Products, Inc., Garfield, NJ), changed twice weekly (rats) or weekly (mice)
Bedding		
SaniChip® hardwood chips (P.J. Murphy Forest Products Corp., Rochelle Park, NJ), changed twice weekly	Same as 15-day studies	BetaChip® hardwood chips (Northeastern Products, Inc., Warrensburg, NY) until 22 May 1988; SaniChip® (P.J. Murphy Forest Products Corp., Montville, NJ) thereafter; changed twice weekly (rats) or weekly (mice)
Cage Filters		
Nonwoven filter sheets, DuPont (Snow Filtration Co., Cincinnati, OH), changed biweekly	Same as 15-day studies	Spun-bonded polyester, DuPont 2024 (Snow Filtration Co., Cincinnati, OH), changed biweekly
Racks		
Stainless steel, changed biweekly	Stainless steel, changed biweekly	Stainless steel (Lab Products, Inc., Maywood, NJ), changed biweekly
Animal Room Environment		
Average temperature: 18.6° C (male rats), 18.5° C (female rats), 18.4° C (mice) Relative humidity: 35% to 51% Fluorescent light: 12 hours/day Room air: 12 to 16 changes/hour	Average temperature: 21.7° C (rats), 17.8° C (mice) Relative humidity: 41% to 60% Fluorescent light: 12 hours/day Room air: 12 changes/hour	Average temperature: 22.5° C (rats), 22.2° C (mice) Relative humidity: 40% to 56% (rats), 45% to 58% (mice) Fluorescent light: 12 hours/day Room air: minimum of 10 changes/hour
Doses		
0, 1,000, 2,500, 5,000, 10,000, or 25,000 ppm in feed, available *ad libitum*	Rats: 0, 250, 500, 1,000, 2,500, or 5,000 ppm in feed, available *ad libitum* Mice: 0, 100, 250, 500, 1,000, or 2,500 ppm in feed, available *ad libitum*	Rats: 0, 500, 1,000, or 2,500 ppm in feed, available *ad libitum* Mice: 0, 250, 500, or 1,000 ppm in feed, available *ad libitum*

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of 4,4'-Thiobis(6-*t*-Butyl-*m*-Cresol) (continued)

15-Day Studies	13-Week Studies	2-Year Studies
Type and Frequency of Observation		
Observed twice daily; animals were weighed initially, weekly, and at the end of the studies; and clinical observations were recorded daily. Feed consumption was recorded daily by cage.	Observed twice daily; animals were weighed initially, weekly, and at the end of the studies; clinical observations were recorded weekly. Feed consumption was recorded daily by cage (rats) and daily by animal (mice).	Observed twice daily; animals were weighed and clinical observations were recorded initially, weekly for 13 weeks, monthly thereafter, and at the end of the studies. Feed consumption was recorded monthly by cage (rats) or by animal (mice).
Method of Sacrifice		
Anesthesia with methoxyflurane followed by exsanguination by cardiac puncture	Same as 15-day studies	Carbon dioxide asphyxiation or pentobarbital anesthesia with exsanguination and transcardial perfusion (neurotoxicity evaluation rats)
Necropsy		
Necropsy performed on all animals. Organs weighed were brain, gastrointestinal tract, heart, right kidney, liver, lung, spleen, right testis, and thymus.	Necropsy performed on all animals. Organs weighed were brain, heart, right kidney, liver, lung, spleen, right testis, and thymus.	Necropsy performed on all animals. Organs weighed were brain, gastrointestinal tract, right kidney, liver, and spleen.
Clinical Pathology		
Blood was collected from all animals surviving to the end of the studies by cardiac puncture for hematology. *Hematology:* hematocrit, hemoglobin, erythrocytes, mean erythrocyte volume, mean erythrocyte hemoglobin, mean erythrocyte hemoglobin concentration, reticulocytes, leukocyte counts, and nucleated erythrocytes	Blood was collected from all animals from the orbital sinus for hematology and by cardiac puncture from rats for clinical chemistry. *Hematology:* hematocrit, hemoglobin, erythrocytes, mean erythrocyte volume, reticulocytes, leukocyte differentials, and nucleated erythrocytes *Clinical chemistry:* (rats) urea nitrogen, creatinine, alkaline phosphatase, alanine aminotransferase, and γ-glutamyltranspeptidase	Blood was collected from the orbital sinus and urine was collected from up to 15 male and female rats per group (slated only for clinical pathology evaluation). Blood was also collected from the orbital sinus of 10 male and female rats and mice at 3, 9, and 15 months into the 2-year study. *Hematology:* hematocrit, hemoglobin, erythrocytes, mean erythrocyte volume, mean erythrocyte hemoglobin, mean erythrocyte hemoglobin concentration, platelets, reticulocytes, leukocyte differentials, and nucleated erythrocytes *Clinical chemistry:* urea nitrogen, creatinine, sodium, potassium, chloride, calcium, direct bilirubin (15-month rats and mice), total bilirubin, alkaline phosphatase, alanine aminotransferase, sorbitol dehydrogenase, and bile salts (rats and 15-month mice) *Urinalysis:* creatinine, alkaline phosphatase, lactate dehydrogenase, *N*-acetyl-β-*D*-glucosaminidase, volume, and β-galactosidase