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

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
Histopathology		
Histopathology was performed on 0, 2,500, 5,000, and 10,000 ppm rats and 0, 2,500, and 5,000 ppm mice. In addition to gross lesions and tissue masses, the tissues examined included: adrenal gland, bone and marrow, large intestine (cecum, colon, rectum), mandibular or mesenteric lymph node, small intestine (duodenum, jejunum, ileum), spleen, stomach (forestomach and glandular), and thymus. The following tissues were examined only from the 10,000 ppm rats and 5,000 ppm mice: brain, clitoral gland (rats), esophagus, gallbladder (mice), heart, kidney, liver, lung, mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland (rats), prostate gland, salivary gland, skin, testis with epididymis and seminal vesicle, thyroid gland, trachea, urinary bladder, and uterus.	Complete histopathology was performed on 0, 1,000, 2,500, and 5,000 ppm rats and 0, 1,000 and 2,500 ppm mice. In addition to gross lesions and tissue masses, the tissues examined included: adrenal gland, brain, clitoral gland (rats), esophagus, gallbladder (mice), heart, kidney, large intestine (cecum, colon, rectum), liver, lung, mammary gland, mandibular or mesenteric lymph node, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland (rats), prostate gland, salivary gland, skin, small intestine (duodenum, jejunum, ileum), spleen, sternum and vertebra (including marrow), stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thyroid gland, thymus, trachea, urinary bladder, and uterus. Only the following tissues were examined from the 1,000 and 2,500 ppm rats and 1,000 ppm mice: liver and mandibular or mesenteric lymph node. The kidney from the 2,500 ppm rats was also examined.	Complete histopathology was performed on all rats and mice. No histopathology was performed on the clinical pathology group rats or mice or the neurotoxicity group male rats. In addition to gross lesions and tissue masses, the tissues examined included: adrenal gland, bone (including marrow), brain, clitoral gland (rats), esophagus, gallbladder (mice), heart, kidney, large intestine (cecum, colon, rectum), liver, lung, mammary gland with surface skin, mandibular or mesenteric lymph node, nose, ovary, pancreas, parathyroid gland, pharynx, pituitary gland, preputial gland (rats), prostate gland, salivary gland, skeletal muscle, skin, small intestine (duodenum, jejunum, and ileum), spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thyroid gland, thymus, trachea, urinary bladder, and uterus.
Neurotoxicity Evaluations		
None	Male and female 0, 1,000, and 2,500 ppm rats were tested for forelimb and hindlimb grip strength, tail flick, startle response, and foot splay.	Forty male rats per group were designated for neurotoxicity studies. After 3 months of exposure, startle reflex and forelimb and hindlimb grip strength were measured in all 40 animals. Ten males per group were killed and given electrophysiological evaluations; another ten males per group were killed and given whole body perfusion for histopathologic examination. The remaining 20 males per group were fed the control diet for an additional 14-16 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 were then split into two groups of ten and given electrophysiologic and neuropathologic evaluations.

RESULTS

RATS

15-DAY STUDY

All male and female rats receiving diets containing 25,000 ppm 4,4′-thiobis(6-*t*-butyl-*m*-cresol) (TBBC), and three males and four females receiving 10,000 ppm died before the end of the study (Table 2). The majority of these deaths occurred during the second week of the study. The seven surviving 10,000 ppm males had a mean body weight loss of 29% and a final mean body weight 51% lower than those of the controls. The mean body weight gain of the 5,000 ppm males was 71% lower than that of the controls, and the final mean body weight was 22% lower than that of the controls. Surviving females in the 10,000 ppm group had a 27% mean body weight loss and a final mean body weight 43% lower than those of the controls. The 5,000 ppm females had a mean body weight gain 77% lower than that of the controls and a final mean body weight 18% lower than that of the controls. Mean body weight gains, final mean body weights, and feed consumption by males and females receiving 1,000 and 2,500 ppm were generally similar to those of the controls. All rats exposed to 5,000, 10,000, or

TABLE 2
Survival, Body Weights, and Feed Consumption of Rats in the 15-Day Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 2
Male							
0	10/10	145 ± 2	212 ± 4	67 ± 4		15.8	16.2
1,000	10/10	149 ± 3	224 ± 3	75 ± 2	106	16.0	18.8
2,500	10/10	147 ± 4	222 ± 5	74 ± 2	105	15.2	19.6
5,000	10/10	146 ± 2	165 ± 3**	19 ± 5**	78	8.8	11.9
10,000	7/10[d]	145 ± 3	103 ± 4**	−44 ± 3**	49	3.1	6.0
25,000	0/10[e]	149 ± 2	–	–	–	3.4	5.4
Female							
0	10/10	118 ± 3	154 ± 3	36 ± 1		11.9	12.1
1,000	10/10	120 ± 2	156 ± 2	36 ± 2	101	12.3	10.9
2,500	10/10	118 ± 2	157 ± 2	39 ± 1	102	11.9	12.3
5,000	10/10	118 ± 2	127 ± 1**	8 ± 2**	82	7.8	8.1
10,000	6/10[f]	121 ± 2	88 ± 4**	−35 ± 4**	57	2.2	3.4
25,000	0/10[g]	117 ± 2	–	–	–	1.1	4.8

** Significantly different (P≤0.01) from the control group by Williams' or Dunnett's test
a Number of animals surviving at 15 days/number initially in group
b Weights are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study. No final mean body weights were calculated for groups with 100% mortality.
c Feed consumption is expressed as grams per animal per day.
d Day of death: 11, 14, 14
e Day of death: 9, 9, 9, 10, 11, 11, 11, 12, 12, 13
f Day of death: 12, 13, 15, 15
g Day of death: 7, 8, 8, 9, 10, 11, 11, 11, 15, 15

25,000 ppm TBBC consumed markedly less feed than did the control groups. Rats exposed 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) and 85, 220, 325, or 270 mg per kg per day (females). Approximate doses for rats exposed to 25,000 ppm cannot be calculated due to early deaths. Since the reduction in feed consumption was evident from the beginning of the study when no signs of toxicity were apparent, reduced feed consumption appeared to be due to poor feed palatability.

Diarrhea was observed in two 25,000 ppm males on day 3 of the study and in the eight remaining 25,000 ppm males on days 6, 7, or 8. Diarrhea occurred in three 25,000 ppm females on day 2 and was observed in other females exposed to 25,000 ppm from day 6 onward. Male and female rats exposed to 5,000 or 10,000 ppm TBBC began to experience diarrhea midway or late into the study. No clinical signs were observed in male or female rats receiving 1,000 or 2,500 ppm TBBC. Statistically significant changes in absolute or relative organ weights reflected decreased final mean body weights or stress and were not considered to be directly related to chemical administration (Table F1).

Since no 25,000 ppm rats survived, hematology parameters were measured only in rats receiving 10,000 ppm or less (Table G1). Leukocyte counts in all exposed females were slightly but significantly greater than those of the controls. Segmented neutrophil counts were significantly higher in the 10,000 and 25,000 ppm male and female groups. This increase was not accompanied by an increase in immature forms, suggesting that this was not an inflammatory response but rather to a shift in the total blood pool distribution without an absolute increase.

Significantly lower reticulocyte counts occurred in male rats receiving 10,000 and 5,000 ppm TBBC and in females receiving 10,000 ppm. In males, this decrease was accompanied by a decrease in nucleated erythrocytes. The slightly lower reticulocyte counts in rats receiving TBBC were probably related to the debilitation rather than to a primary effect on the bone marrow. Females receiving 5,000 or 10,000 ppm also had a very slight decrease in erythrocyte size compared to controls as indicated by decreased mean erythrocyte volume values. This also was probably related to debilitation.

Microscopic examination was not performed on tissues from 25,000 ppm rats since they died before the end of the study. The principal lesions associated with the ingestion of TBBC occurred in the kidney and glandular stomach of 10,000 ppm rats (Table 3). There was partial to complete necrosis of the tip of the renal papilla in one male and two females and minimal focal or multifocal necrosis of tubule epithelium in the cortex or outer medulla of four males and seven females receiving 10,000 ppm (Plates 1 and 2). Erosion and/or focal necrosis of the mucosal epithelium was also observed in the glandular stomach of several male and female rats in the 10,000 ppm groups. Lymphocyte depletion in the thymus and spleen were also observed in rats receiving 10,000 ppm, but these changes were attributed to severe debilitation and stress. Depletion of hematopoietic cells from the bone marrow was attributed to nutrient deficiency accompanying weight loss.

Because of decreased survival in 10,000 and 25,000 ppm rats in the 15-day study, the high exposure selected for the 13-week study was 5,000 ppm.

TABLE 3
Incidences of Selected Nonneoplastic Lesions in Rats in the 15-Day Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)[a]

Dose (ppm)	0	1,000	2,500	5,000	10,000
Male					
Kidney[b]	10	–[d]	–	10	10
Renal Papillary Necrosis[c]	0	–	–	0	1 (4.0)[e]
Renal Tubule Necrosis	0	–	–	0	4* (1.3)
Glandular Stomach	10	–	–	10	10
Erosion	0	–	–	0	1 (3.0)
Necrosis	0	–	–	0	2 (2.0)
Hemorrhage	0	–	–	0	4* (1.8)
Congestion	0	–	–	0	4* (1.8)
Female					
Kidney	10	–	–	10	9
Renal Papillary Necrosis	0	–	–	0	2 (3.5)
Renal Tubule Necrosis	0	–	–	0	7**(1.0)
Glandular Stomach	10	–	–	10	9
Erosion	0	–	–	0	1 (3.0)
Necrosis	0	–	–	0	3 (2.3)
Hemorrhage	0	–	–	0	2 (2.5)
Congestion	0	–	–	0	5* (2.4)

* Significantly different (P≤0.05) from the control group by the Fisher exact test
** P≤0.01
[a] No histopathology performed on animals receiving 25,000 ppm due to 100% mortality in this group.
[b] Number of animals with organ examined microscopically
[c] Number of animals with lesion
[d] Animals in these groups not examined microscopically
[e] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)

13-WEEK STUDY

All animals survived to the end of the study (Table 4). The final mean body weights of 5,000 ppm males and females were markedly lower than those of the controls; the mean body weight of males receiving 2,500 ppm was slightly but consistently lower than that of the controls throughout the study. Feed consumption by 5,000 ppm rats was markedly lower than that by controls throughout the study. Feed consumption by 2,500 ppm males was somewhat reduced initially, but was similar to or greater than that by the controls after week 4. Rats exposed to 250, 500, 1,000, 2,500, or 5,000 ppm received 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). Since reduction in feed consumption was apparent from the beginning of the study, the reduction would seem more likely to have been caused by decreased feed palatability than by anorexia resulting from toxicity. This conclusion is supported by the fact that diarrhea, the major clinical finding in 5,000 ppm rats, did not appear in the males until day 64 (with the exception of one male in which diarrhea was observed on day 29) or in the females until day 57.

TABLE 4
Survival, Body Weights, and Feed Consumption of Rats in the 13-Week Feed Study of 4,4'-Thiobis(6-t-Butyl-m-Cresol)

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 13
Male							
0	10/10	142 ± 4	359 ± 7	220 ± 7		16.3	14.9
250	10/10	140 ± 4	382 ± 6	243 ± 7	107	16.5	15.8
500	10/10	138 ± 5	378 ± 6	240 ± 7	105	16.1	16.1
1,000	10/10	139 ± 3	368 ± 5	230 ± 6	103	15.8	14.1
2,500	10/10	138 ± 4	351 ± 7	213 ± 7	98	15.2	16.7
5,000	10/10	134 ± 5	217 ± 3**	82 ± 3**	60	10.0	12.1
Female							
0	10/10	109 ± 3	209 ± 8	99 ± 7		11.2	9.9
250	10/10	108 ± 3	204 ± 5	96 ± 6	98	11.4	9.0
500	10/10	108 ± 3	200 ± 2	93 ± 3	96	11.5	9.8
1,000	10/10	107 ± 3	201 ± 3	94 ± 4	96	11.8	9.5
2,500	10/10	109 ± 3	200 ± 3	91 ± 3	96	11.9	9.3
5,000	10/10	106 ± 3	153 ± 5**	48 ± 3**	73	8.5	8.3

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

A significant increase in absolute and relative liver weights occurred in females that received 5,000 ppm TBBC (Table F2). The relative, but not absolute, liver weight of 2,500 ppm males was significantly increased. As in the 15-day study, other significant differences in absolute or relative organ weights were considered due to much lower final mean body weights and not to organ-specific toxicity.

Serum alkaline phosphatase levels were significantly higher in 2,500 and 5,000 ppm males and were slightly higher in the females exposed to 5,000 ppm (Table G2). Males and females exposed to 2,500 or 5,000 ppm TBBC had significantly higher serum alanine aminotransferase levels. The increased activity of γ-glutamyl transpeptidase in rats exposed to 5,000 ppm was not considered to be biologically significant.

Hematocrit and hemoglobin concentrations in male rats exposed to 1,000, 2,500, and 5,000 ppm were significantly lower than those of the controls; these results suggest a mild anemia. However, considering the diarrhea and unthriftiness that occurred in these animals, possible dehydration could be masking larger decreases, including decreases in erythrocyte counts, or could account for the absence of changes in hematocrit or hemoglobin values in females. Since reticulocyte counts in male rats were not higher than those of the controls, the anemia in the male rats was considered nonresponsive. Mean erythrocyte volume was significantly lower in males that received 1,000 or 2,500 ppm TBBC and in males and females that received 5,000 ppm; this effect is usually associated with a disturbance in hemoglobin production and has commonly been observed with anemias of chronic inflammation or iron deficiency.

Total leukocyte counts were significantly higher in 5,000 ppm females and slightly increased in 5,000 ppm males. Male and female rats that received 5,000 ppm also exhibited significantly higher segmented neutrophil counts. Band neutrophil counts were significantly higher in all exposed female groups than in controls; the largest increase occurred in 5,000 ppm rats. These changes in leukocyte parameters are consistent with an inflammatory response.

Results of three neurotoxicity trials in 0, 1,000, and 2,500 ppm rats demonstrated a significant dose-related increase in forelimb and hindlimb grip strength (Table H1). Foot splay, tail flick, and startle response reflexes were unaffected by exposure to TBBC.

The principal lesions associated with the administration of TBBC for 13 weeks occurred in the liver and kidney, primarily in 2,500 and 5,000 ppm males and females (Table 5). The lesions in the liver consisted of scattered individual cell necrosis, individual or aggregates of enlarged Kupffer cells with abundant yellow-tan pigmented cytoplasm (Kupffer cell hypertrophy), focal accumulations of similar macrophages in or adjacent to the portal areas, and a slight increase in small bile ductules in the portal areas (Plate 3). By electron microscopy, the pigmented material in the cytoplasm of Kupffer cells was amorphous to finely granular and light to moderately electron dense with a scattering of irregular, highly electron-dense bodies. While the more abundant amorphous substance was not membrane bound, many of the smaller electron-dense bodies were partially surrounded by a plasma membrane. The cytoplasm of the Kupffer cells stained strongly positive with PAS, weakly to strongly by the Ziehl-Neelsen method for acid-fast material, and inconsistently weakly positive by Perl's iron method. While not observed by the study pathologist, enlargement of centrilobular hepatocytes, relative to the periportal hepatocytes, in the 5,000 ppm group was also observed by the Pathology Working Group. This finding is consistent with hepatocellular hypertrophy and with the higher activities of serum enzymes in the 2,500 and 5,000 ppm groups.

The kidney lesions consisted of focal, segmental degeneration and necrosis of the proximal tubule epithelium, primarily in the outer stripe of the outer medulla, and extensive pigmentation of the proximal convoluted tubule epithelium (Plate 4). The degeneration and necrosis were characterized by faintly stained, pale cells with little cytoplasmic or nuclear detail, suggestive of cytolysis and karyolysis. The pigmentation was characterized by pale, yellow-red discoloration of the epithelial cytoplasm.

Both the size and number of macrophages were increased in the mesenteric lymph nodes of male and female rats exposed to 2,500 or 5,000 ppm TBBC (Table 5).

Dose selection rationale: The exposure levels selected for the 2-year rat study were 500, 1,000, and 2,500 ppm. A high dose of 5,000 ppm was not included because of reduced body weights and the degree of liver and kidney toxicity observed in 5,000 ppm males and females in the 13-week study.

TABLE 5
Incidences of Selected Nonneoplastic Lesions in Rats in the 13-Week Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Dose (ppm)	0	250	500	1,000		2,500		5,000	
Male									
Liver[a]	10	–[c]	–	10		10		10	
Bile Duct Hyperplasia[b]	0	–	–	1	(1.0)[d]	2	(1.5)	10**	(2.0)
Kupffer Cell Hypertrophy	0	–	–	0		6**	(1.0)	10**	(3.7)
Necrosis	0	–	–	1	(1.0)	3	(1.0)	10**	(1.0)
Lymph Node, Mesenteric	10	–	–	10		10		10	
Macrophage Hyperplasia	0	–	–	1	(2.0)	2	(1.0)	10**	(3.2)
Kidney	10	–	–	10		10		10	
Necrosis	0	–	–	0		0		9**	(1.3)
Pigmentation	0	–	–	0		2	(1.0)	10**	(1.1)
Female									
Liver	10	–	–	10		10		10	
Bile Duct Hyperplasia	0	–	–	0		1	(1.0)	10**	(1.7)
Kupffer Cell Hypertrophy	0	–	–	0		10**	(1.6)	10**	(3.6)
Necrosis	0	–	–	0		1	(1.0)	10**	(1.1)
Lymph Node, Mesenteric	10	–	–	10		10		10	
Macrophage, Hyperplasia	0	–	–	0		3	(1.7)	10**	(2.9)
Kidney	10	–	–	10		10		10	
Necrosis	0	–	–	0		0		9**	(1.8)
Pigmentation	0	–	–	0		3	(1.0)	10**	(1.0)

** Significantly different ($P \leq 0.01$) from the control group by the Fisher exact test
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Animals in these groups not examined microscopically
[d] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)

2-YEAR STUDY

Survival

Estimates of survival probabilities for male and female rats receiving TBBC in feed for 2 years are presented in Table 6 and in Kaplan-Meier survival curves (Figure 1). Survival rates of exposed rats were similar to those of the controls.

Body Weights, Feed Consumption, and Clinical Findings

Throughout most of the study, the mean body weights of 2,500 ppm male rats were approximately 3% lower than those of the controls and the final mean body weight was 5% lower than that of the controls. Mean body weights of 500 and 1,000 ppm males were similar to those of the controls during the study, but the final mean body weights of these groups were 5% and 6% lower than that of the controls, respectively. The mean body weights of 2,500 ppm females began to decrease 12 weeks into the study and at week 65 was 14% lower than that of the controls. The final mean body weight, however, was 6% lower than that of the controls (Figure 2 and Tables 7 and 8). Exposure levels of 500, 1,000, or 2,500 ppm TBBC resulted in a daily ingestion of 20, 40, or 100 mg/kg body weight for males or 20, 45, or 120 mg/kg body weight for females. Feed consumption by male and female rats was similar to that by controls (Tables J1 and J2). The behavior and general health and appearance of exposed male and female rats were similar to those of controls.

TABLE 6
Survival of Rats in the 2-Year Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

	0 ppm	500 ppm	1,000 ppm	2,500 ppm
Male				
Animals initially in study	60	60	60	60
15-month interim evaluation[a]	10	10	10[e]	10
Natural deaths	9	8	6	9
Moribund	23	14	22	23
Animals surviving to study termination	18	28	22	18
Percent probability of survival at end of study[b]	36	56	42	36
Mean survival (days)[c]	614	637	633	620
Survival analysis[d]	P=0.506	P=0.049N	P=0.540N	P=1.000N
Female				
Animals initially in study	60	60	60	60
15-month interim evaluation[a]	10	10	10	10
Natural deaths	5	5	2	6
Moribund	11	14	16	16
Animals surviving to study termination	34	31[f]	32	28
Percent probability of survival at end of study	68	62	64	56
Mean survival (days)	663	651	645	644
Survival analysis	P=0.202	P=0.559	P=0.711	P=0.195

[a] Censored from survival analyses
[b] Kaplan-Meier determinations based on the number of animals alive on the first day of terminal sacrifice
[c] Mean of all deaths (uncensored, censored, and terminal sacrifice)
[d] The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the exposed columns. A lower mortality in an exposure group is indicated by N.
[e] Three male rats exposed to 1,000 ppm were killed moribund prior to the 15-month interim evaluation.
[f] Includes one animal that died the last week of the study

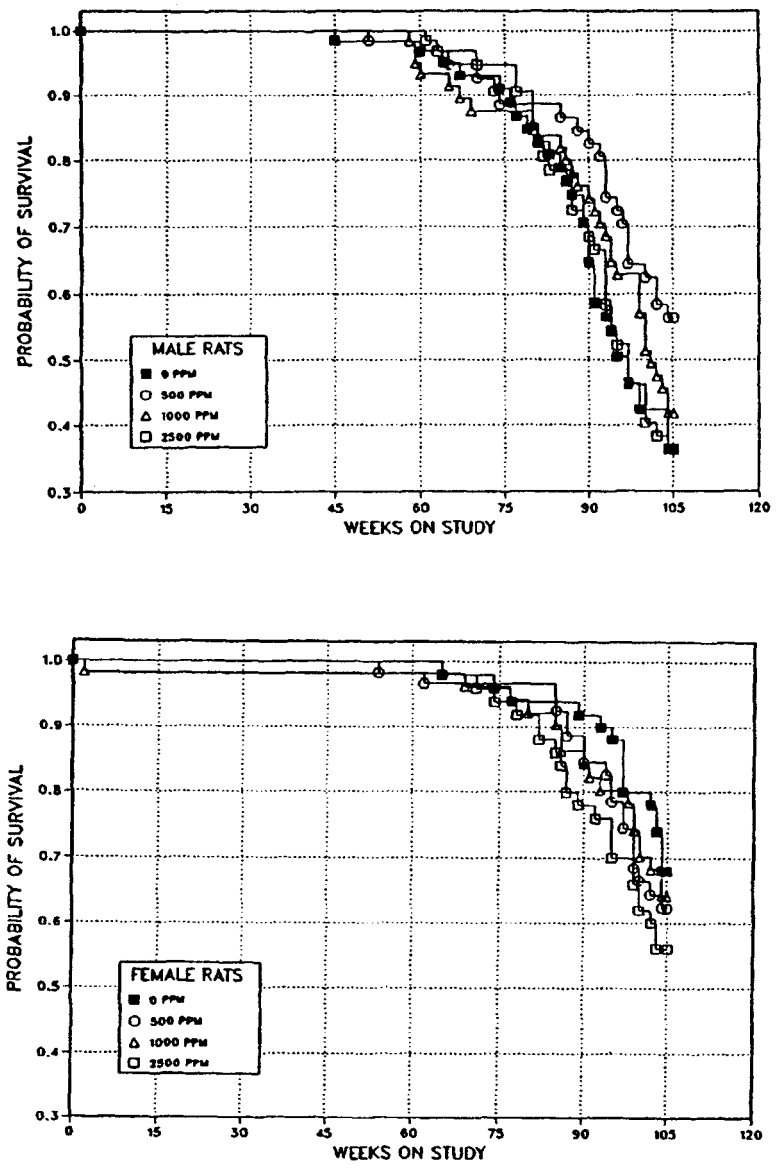

FIGURE 1
Kaplan-Meier Survival Curves for Male and Female Rats Administered 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol) in Feed for 2 Years

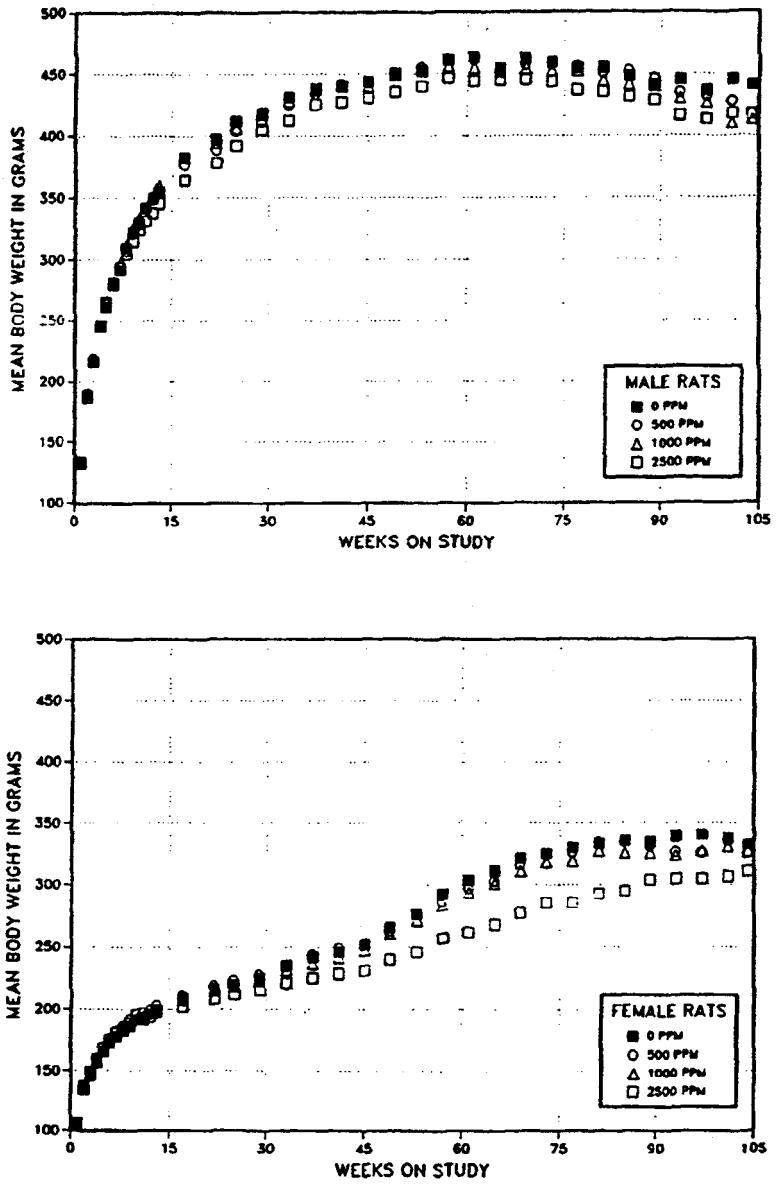

FIGURE 2
Growth Curves for Male and Female Rats
Administered 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol) in Feed for 2 Years

TABLE 7
Mean Body Weights and Survival of Male Rats in the 2-Year Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Weeks on Study	0 ppm Av. Wt. (g)	0 ppm No. of Survivors	500 ppm Av. Wt. (g)	500 ppm Wt. (% of controls)	500 ppm No. of Survivors	1,000 ppm Av. Wt. (g)	1,000 ppm Wt. (% of controls)	1,000 ppm No. of Survivors	2,500 ppm Av. Wt. (g)	2,500 ppm Wt. (% of controls)	2,500 ppm No. of Survivors
1	134	60	135	100	60	134	100	60	132	99	60
2	188	60	190	101	60	189	100	60	186	99	60
3	215	60	218	101	60	218	101	60	216	100	60
4	245	60	246	100	60	246	100	60	246	100	60
5	261	60	264	101	60	266	102	60	265	102	60
6	281	60	280	100	60	282	100	60	280	100	60
7	292	60	295	101	60	298	102	60	294	101	60
8	310	60	307	99	60	312	101	60	305	98	60
9	323	60	321	100	60	325	101	60	315	98	60
10	329	60	332	101	60	335	102	60	325	99	60
11	342	60	341	100	60	343	100	60	332	97	60
12	350	60	349	100	60	351	100	60	338	97	60
13	355	60	358	101	60	360	102	60	346	97	60
17	383	60	377	99	60	383	100	60	365	95	60
22	397	60	389	98	60	395	99	60	382	95	60
25	412	60	405	98	60	406	99	60	392	95	60
29	419	60	412	98	60	417	100	60	405	97	60
33	431	60	425	99	60	428	99	60	413	96	60
37	438	60	434	99	60	437	100	60	425	97	60
41	441	60	442	100	60	440	100	60	428	97	60
45	444	59	444	100	60	440	99	60	431	97	60
49	451	59	451	100	60	449	100	60	436	97	60
53	453	59	455	101	59	453	100	60	440	97	60
57	461	59	462	100	59	456	99	60	447	97	60
61	464	58	462	100	58	455	98	56	445	96	59
65[a]	454	47	453	100	47	452	100	48	445	98	48
69	462	46	458	99	47	455	98	46	446	96	48
73	459	46	458	100	44	453	99	46	444	97	48
77	455	43	457	100	44	453	100	46	437	96	46
81	455	41	451	99	44	445	98	44	436	96	42
85	448	39	453	101	43	442	99	43	431	96	39
89	442	35	447	101	42	441	100	40	429	97	35
93	445	28	435	98	40	430	97	37	416	94	32
97	437	24	432	99	33	427	98	33	413	95	25
101	446	21	428	96	31	410	92	27	418	94	20
104	441	20	417	95	29	413	94	23	417	95	18
Mean for weeks											
1-13	279		280	100		281	101		275	99	
14-52	427		424	99		422	100		409	96	
53-104	451		452	99		442	98		433	96	

[a] Interim evaluation occurred.

TABLE 8
Mean Body Weights and Survival of Female Rats in the 2-Year Feed Study of 4,4′-Thiobis(6-*t*-Butyl-*m*-Cresol)

Weeks on Study	0 ppm		500 ppm			1,000 ppm			2,500 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	107	60	107	100	60	107	100	60	107	100	60
2	134	60	137	102	60	136	101	60	137	102	60
3	146	60	148	102	60	147	101	59	149	102	60
4	156	60	159	102	60	158	101	59	160	102	60
5	165	60	169	102	60	166	101	59	168	102	60
6	173	60	176	102	60	174	101	59	175	101	60
7	178	60	182	103	60	178	101	59	180	101	60
8	182	60	186	102	60	184	101	59	184	101	60
9	186	60	192	103	60	188	101	59	189	102	60
10	192	60	196	103	60	192	100	59	191	100	60
11	192	60	197	103	59	194	101	59	193	101	60
12	196	60	200	102	60	196	100	59	193	99	60
13	199	60	203	102	60	198	100	59	197	99	60
17	209	60	211	101	60	208	99	59	202	97	60
22	216	60	219	101	60[b]	215	100	59	209	97	60
25	219	60	223	102	60	218	100	59	212	97	60
29	225	60	228	101	60	223	99	59	216	96	60
33	235	60	236	100	60	232	99	59	221	94	60
41	246	60	249	101	60	241	98	59	228	93	60
45	252	60	253	100	60	248	98	59	231	92	60
49	266	60	263	100	60	261	98	59	240	90	60
53	277	60	277	100	60	272	98	59	246	89	60
57	293	60	287	98	59	284	97	59	257	88	60
61	304	60	297	98	59	294	97	59	262	86	60
65[a]	312	49	303	97	48	301	97	49	268	86	49
69	322	49	316	98	48	312	97	48	278	86	49
73	326	49	324	100	48	319	98	48	286	88	48
77	330	47	327	99	48	320	97	48	286	87	47
81	334	47	335	100	48	328	98	46	293	88	46
85	336	47	335	100	46	326	97	45	295	88	43
89	335	47	331	99	44	327	98	43	304	91	39
93	339	46	327	96	42	324	96	41	305	90	38
97	341	40	327	96	38	328	96	40	305	90	35
101	338	40	336	100	33	331	98	35	307	91	31
104	333	37	326	98	32	327	98	32	311	94	28
Mean for weeks											
1-13	170		173	102		171	101		171	101	
14-52	234		236	101		231	99		220	94	
53-104	323		318	98		314	97		286	89	

[a] Interim evaluation occurred.
[b] The number of animals weighed for this week is less than the number of animals surviving.

Hematology, Clinical Chemistry, and Urinalysis

Results of hematology evaluations at 3, 9, and 15 months are presented in Tables G3 through G6. Slight but significant decreases in hematocrit levels, hemoglobin concentrations, and erythrocyte counts were observed in one set of 1,000 and 2,500 ppm males at 15 months, but not in the other set. These differences were not observed in males at 3 or 9 months. Similar significant decreases in hematocrit level and hemoglobin concentration occurred in 2,500 ppm females at 9 months; hemoglobin concentrations of 2,500 ppm females were significantly decreased in both sets evaluated at 15 months, but hematocrit levels were similar to those of the controls. Mean erythrocyte hemoglobin counts and concentration in the 2,500 female group were significantly lower than those of the controls at 9 months and in both sets of animals evaluated at 15 months. Platelet counts in 2,500 ppm males and females were slightly but significantly higher than those of the controls at 3 and 9 months, as were the platelet counts of 2,500 ppm males in one set of animals evaluated at 15 months and of 2,500 ppm females in the other set. While the results of the hematology evaluations were somewhat variable, they do suggest a slight chemical-related effect. It is not clear, however, if these differences indicate a direct effect on stem cells in the bone marrow or on circulating erythrocytes, or if they are secondary to other physiological alterations caused by TBBC.

Clinical chemistry results for rats evaluated at 3 and 9 months and for the two sets of rats evaluated at 15 months were generally similar (Tables G3, G4, G5, and G6). Serum activities of alkaline phosphatase, alanine aminotransferase, and sorbitol dehydrogenase in 2,500 ppm males were significantly greater that those of the controls at each evaluation. 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 the controls at each evaluation. These results are consistent with hepatocellular damage caused by TBBC.

Urine volumes of all exposed groups of males and females were significantly lower than those of the controls at 3 months, but not at later evaluations. This is consistent with decreased water or feed intake in the exposed groups, but it is not considered a direct chemical effect. Elevated urine creatinine concentrations at the 3-month evaluation, particularly in exposed groups of male rats, indicate that the urine constituents were more highly concentrated in these groups and are consistent with the volume measurements. Urine specific gravity was not measured, however. The urinary activity of *N*-acetyl-ß-D-glucosaminidase was mildly increased at all evaluations in 2,500 ppm females in comparison to controls. Differences in other urine enzyme activities between exposed and control rats were variable and not considered chemical related.

Neurotoxicity Evaluation

At 3 months, there was no difference in startle reflex between exposed and control male groups and, in contrast to the findings in the 13-week study, there were no differences in forelimb or hindlimb grip strength between exposed and control groups in the first three trials (Table H2). The standard methodology for measuring grip strength consists of three trials. However, eight trials were used in the chronic study, and the grip strength of control groups decreased with subsequent trials, apparently due to fatigue or habituation. Although the grip strength of exposed groups also decreased with repeated trials, the decrement was less than that of the controls. Thus, grip strength in later trials (particularly that of the forelimbs) of each exposed group was significantly greater than controls. The electrophysiologic evaluation revealed no significant inhibitory effects of TBBC on motor nerve excitability or conduction, neuromuscular transmission, or muscle contractility (Tables H4, H5, and H6). Further, there were no microscopic lesions that could be attributed to TBBC observed in the sciatic nerve, quadriceps muscle, or teased nerve preparations of the sciatic nerve.

In the reversibility study, the effects on grip strength observed at 3 months were no longer evident at the 6 month evaluation (Table H3). The results of the remaining neurotoxicity studies at 6 months were similar to those at 3 months (Tables H4, H5, and H6), and there were no significant effects of TBBC on motor nerve excitability or conduction, neuromuscular transmission, muscle contractility, or pathology.

Pathology and Statistical Evaluation

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms and nonneoplastic lesions in the liver, kidney, thyroid gland, uterus, and mammary gland. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix A for male rats and Appendix B for female rats.

Liver: At the 15-month interim evaluation, both the absolute and relative liver weights of 2,500 ppm females were significantly greater than those of the controls (Table F3). Relative liver weights of 2,500 ppm males and 1,000 ppm females were also significantly greater than those of the controls.

The incidence of Kuppfer cell hypertrophy was significantly increased in 2,500 ppm males and females at the 15-month interim evaluation and at the end of the 2-year study (Tables 9, A5, and B5). At 15 months, the incidence of cytoplasmic vacuolization was significantly increased in all exposed groups of males and in 2,500 ppm females. At 2 years, the incidence of cytoplasmic vacuolization was slightly increased in 1,000 and 2,500 ppm males and significantly increased in 1,000 and 2,500 ppm females. Also at 2 years, the incidence of fatty change was significantly increased in 2,500 ppm females. Cytoplasmic vacuolization was characterized by the presence of multiple, small vacuoles, whereas fatty change was indicated by the presence of single, large cytoplasmic vacuoles. In both instances, these changes are presumably the result of lipid accumulation.

At 15 months, the incidence of basophilic foci was significantly increased in 2,500 ppm males and these foci were present in all females; the incidences in exposed males and females at terminal sacrifice were similar to those in the controls. Incidences of mixed cell foci were significantly increased in 2,500 ppm males and females at 15 months and in 1,000 and 2,500 ppm males and females at the end of the study; at each time point, the incidence of mixed cell foci in 2,500 ppm females was twice that in 2,500 ppm males. Hepatocyte foci were characterized as basophilic, eosinophilic, clear, or mixed based on cytoplasmic staining properties. These differences in staining properties are generally attributed to variations in the amounts of rough or smooth endoplasmic reticulum, glycogen, or fat. Thus, basophilic foci consist predominantly of cells with greater amounts of rough endoplasmic reticulum, while eosinophilic foci consist of cells with more smooth endoplasmic reticulum. Clear cell foci consist of cells with vacuolated cytoplasm caused by the accumulation of lipid or with clear cytoplasm caused by the accumulation of glycogen. The mixed cell foci consist of cells with either basophilic or eosinophilic cytoplasm and cells with vacuolated or clear cytoplasm.

The incidences of hepatocellular adenoma or carcinoma (combined) in exposed male rats were not significantly greater than that in the control group (Tables 9 and A3).

TABLE 9
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Rats in the 2-Year Feed Study of 4,4'-Thiobis(6-*t*-Butyl-*m*-Cresol)

Dose (ppm)	0	500	1,000	2,500
Male				
15-Month Interim Evaluation				
Liver[a]	10	10	7	10
Kupffer Cell Hypertrophy[b]	0	0	0	10** (1.2)[c]
Cytoplasmic Vacuolization	1 (1.0)	10** (1.1)	7** (1.0)	10** (1.7)
Basophilic Focus	5	2	7	10*
Mixed Cell Focus	1	1	1	5
2-Year Study				
Liver	50	50	50	49
Kupffer Cell Hypertrophy	2 (1.5)	3 (1.0)	2 (1.0)	31** (2.1)
Cytoplasmic Vacuolization	13 (1.2)	11 (1.5)	19 (1.4)	18 (2.0)
Basophilic Focus	18	22	23	22
Mixed Cell Focus	6	14	18*	15*
Clear Cell Focus	2	0	1	1
Eosinophilic Focus	3	7	2	1
Hepatocellular Adenoma				
Overall rates[d]	1/50 (2%)	2/50 (4%)	3/50 (6%)	4/49 (8%)
Adjusted rates[e]	5.6%	7.1%	13.6%	17.0%
Terminal rates[f]	1/18 (6%)	2/28 (7%)	3/22 (14%)	2/18 (11%)
First incidence (days)	729 (T)	729 (T)	729 (T)	625
Logistic regression test[g]	P=0.091	P=0.653	P=0.377	P=0.177
Hepatocellular Carcinoma	0/50 (0%)	1/50 (2%)	0/50 (0%)	1/49 (2%)
Hepatocellular Adenoma or Carcinoma[h]				
Overall rates	1/50 (2%)	3/50 (6%)	3/50 (6%)	5/49 (10%)
Adjusted rates	5.6%	10.7%	13.6%	21.0%
Terminal rates	1/18 (6%)	3/28 (11%)	3/22 (14%)	2/18 (11%)
First incidence (days)	729 (T)	729 (T)	729 (T)	625
Logistic regression test	P=0.056	P=0.472	P=0.377	P=0.100

(continued)

TABLE 9
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Rats in the 2-Year Feed Study of 4,4'-Thiobis(6-*t*-Butyl-*m*-Cresol) (continued)

Dose (ppm)	0		500		1,000		2,500	
Female								
15-Month Interim Evaluation								
Liver	10		10		10		10	
Kupffer Cell Hypertrophy	1	(1.0)	0		5	(1.0)	10**	(2.7)
Cytoplasmic Vacuolization	0		1	(1.0)	1	(1.0)	8**	(1.0)
Basophilic Focus	10		10		10		10	
Eosinophilic Focus	0		0		1		0	
Mixed Cell Focus	0		1		0		10**	
2-Year study								
Liver	50		50		50		50	
Kupffer Cell Hypertrophy	11	(1.2)	10	(1.5)	9	(1.0)	42**	(2.7)
Cytoplasmic Vacuolization	12	(1.3)	10	(1.4)	20*	(1.3)	34**	(2.7)
Fatty Change	9	(1.4)	8	(1.5)	15	(1.3)	19*	(1.5)
Basophilic Focus	37		34		38		36	
Mixed Cell Focus	5		4		14*		34**	
Eosinophilic Focus	5		7		8		4	
Clear Cell Focus	0		1		1		1	
Adenoma	0		0		0		1	

* Significantly different ($P \leq 0.05$) by the Fisher exact test (15-month interim evaluation) or the logistic regression test (terminal sacrifice)
** ($P \leq 0.01$)
(T) Terminal sacrifice
[a] Number of animals with liver examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals (1=minimal; 2=mild; 3=moderate; 4=marked)
[d] Number of animals with neoplasm per number of animals with liver examined microscopically
[e] Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
[f] Observed incidence at terminal kill
[g] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The logistic regression test regards these neoplasms as nonfatal.
[h] Historical incidence for 2-year feed studies with untreated control groups (mean ± standard deviation): 41/1,251 (3.3% ± 3.6%); range 0%-10%