ENVIRONMENTAL HEALTH RESEARCH & TESTING, INC.

FINAL REPORT

SCREENING OF PRIORITY CHEMICALS FOR REPRODUCTIVE HAZARDS

TRIORTHOCRESYL PHOSPHATE (CAS NO.: 78-30-8)
4,4'-THIOBIS (6-t-BUTYL-m-CRESOL) (CAS NO.: 96-69-5)
13-CIS-RETINOIC ACID (CAS NO.: 4759-48-2)

Contract No.: 200-84-2735
EHRT's Project No.: ETOX-85-1002

Submitted to:
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16. Abstract (Limit: 200 words) Triorthocresyl-phosphate (78308), 4,4'-thiobis(6-t-butyl-m-cresol) (96695), and 13-cis-retinoic-acid (4759482) were screened for their potential to cause adverse reproductive effects using a postnatal mouse screening test. In the first two phases of the study experiments were designed to determine the appropriate dose levels for the different compounds. In the third phase the reproductive effects were studied. Female CD-1-mice were dosed by gavage on 5 consecutive days for phase one and on gestation days six through 15 for the second and third phases of the study. The predicted median lethal dose for triorthocresyl-phosphate was 515mg/kg/day and for 4,4'-thiobis it was 485mg/kg/day. The 13-cis-retinoic-acid (4759482) was administered at 85mg/kg/day, due to the limited amount of chemical available. All animals received a constant volume of 10 milliliters/kilogram/day. Triorthocresyl-phosphate cause an increased maternal mortality and a decreased number of viable litters but had no effect on litter size or birth weight gain. 4,4'-Thiobis(6-t-butyl-m-cresol) caused increases in maternal mortality and a decreased percent of pup survival while not affecting the number of viable litters, the litter size, birth weight or weight gain of the pups. 13-cis-Retinoic-acid had no effect on maternal mortality, the number of viable litters, litter size, or pup birth weight.

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SCREENING OF PRIORITY CHEMICALS FOR REPRODUCTIVE HAZARDS

Contract No.: 200-85-2735

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QUALITY ASSURANCE

FINAL REPORT STATEMENT

PROJECT NUMBER ETOX-85-1002 was inspected on the following dates: 8/6/85, 8/14/85, 9/13/85, 1/13/86, and 3/5/87 by the Quality Assurance Unit, according to EHRT's Standard Operating Procedures and Good Laboratory Practice Regulations. All findings were reported to the Study Director and Management on the following dates: 8/6/85, 8/14/85, 9/13/85, 1/13/86, and 3/5/87. Action has been taken in response to all items listed by Quality Assurance. It was concluded that the Final Report accurately reflects the raw data for this project. This study has been conducted in compliance with Good Laboratory Practice Regulations.

Karl B. Bailey 4-22-87

QUALITY ASSURANCE UNIT DATE

Q. A. INSPECTIONS

PROJECT NO. ETOX-85-1002

EVENT	DATE INSPECTED	REPORTED TO STUDY DIRECTOR	INITIALS
Report Review	8/6/85	8/6/85	WB
Dose Preparation	8/14/85	8/14/85	WB
Sacrifice	9/13/85	9/13/85	WB
Dose Preparation	1/13/85	1/13/85	WB
Final Report	3/5/87	3/5/87	WB

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SPONSOR: NIOSH INITIATION DATE: July 8, 1985

MATERIALS: Priority Chemicals TERMINATION DATE: January 30, 1986

SUBJECT: Screening of Priority Chemicals for Reproductive Hazards

Contract No. 200-84-2735

EHRT Project No. ETOX-85-1002

Final Report

SUMMARY

The purpose of this contract is to screen selected chemicals, including National Toxicology Program (NTP) priority compounds, for the potential to cause adverse reproductive effects using a post-natal mouse screening test (Chernoff and Kavlock, 1982[1], 1983[2]).

The screen consists of three experimental phases. Phases I and II are range-finding studies designed as a method to identify the predicted LD_{10}, to be used in the Phase III. Phases I and II are done in experimental blocks of six (6) chemicals using nonpregnant female mice in Phase I and pregnant mice in Phase II. Phase III is done in experimental blocks of three (3) chemicals. Treatments are administered by gavage on five (5) consecutive days (Phase I) or on gestation days 6-15 (Phase II and III).

In Phase III, reproductive hazard potential is evaluated by consideration of: maternal body weights (measured at randomization, on days 6 - 15 and day 17 of gestation, and on days 0 and 3 postpartum); maternal mortality and signs of toxicity; pup counts at birth (live and dead); pup weights (recorded at birth and on Day 3 postpartum); and offspring survival from birth to Day 3 postpartum.

This report presents the results for the three phases of testing for triorthocresyl phosphate, 4,4'-thiobis (6-t-butyl-m-cresol), and 13-cis-retinoic acid.

In Phase I, triorthocresyl phosphate and 4,4'-thiobis (6-t-butyl-m-cresol) were administered on 5 consecutive days at 10, 100, or 1000 mg/kg/day. A Phase I was not performed with 13-cis-retinoic acid due to inadequate supply of the chemical. All three animals died in the 1000 mg/kg/day group for triorthocresyl phosphate. 4,4'-thiobis (6-t-butyl-m-cresol) did not produce chemical related mortality. The dose levels for Phase II were determined from Appendix 1. Dose range number 4 was selected for triorthocresyl phosphate. Dose range number 1 was selected for 4,4'thiobis (6-t-butyl-m-cresol). Due to high mortality in the initial Phase II for 4,4'-thiobis (6-t-butyl-m-cresol), a repeat of Phase II was conducted using lower dose levels. The dose range for 13-cis-retinoic acid in Phase II testing was recommended by the NIOSH Project Officer.

Based on the probit analysis of the mortality data from Phase II and the Statistical Analysis System (SAS) at NIOSH, the dose

selected (LD_{10}) for the Phase III reproduction study were 515 mg/kg/day for triorthocresyl phosphate, 485 mg/kg/day for 4,4'-thiobis (6-t-butyl-m-cresol). The dose level (85 mg/kg/day) for 13-cis-retinoic acid was selected by the Project Officer.

INTRODUCTION

Continuing concern exists for potential reproductive hazards from exposure to various chemicals in the workplace environment. The identification of these potential reproductive hazards and the use of these data to establish criteria for Federal standards are major objectives of the National Institute for Occupational Safety and Health (NIOSH). The enormous number of chemicals which are in common use, coupled with new legislation (e.g., the Toxic Substances Control Act) designed to ensure appropriate control of industrial chemicals, has severely taxed the resources available to use traditional reproductive hazard protocols in testing. The identification and prioritization of chemicals in need of more detailed, traditional reproductive hazard testing is essential to a timely study of such a large number of chemicals. The purpose of this contract is to screen selected chemicals, including National Toxicology Program (NTP) priority compounds, for the potential to cause adverse reproductive effects using a postnatal mouse screening test (Chernoff and Kavlock, 1982[1], 1983[2]).

This method screens chemicals for embryonic, fetal, and neonatal toxic responses using pregnant mice treated during major organogenesis (days 6-15 of gestation). Dosing is stopped four days prior to expected parturition, since postnatal maternal toxicity may compound neonatal results. The number of live-born pups, birth weights, and growth and survival to 3.5 days of age are the primary response variables measured. A reduction in the

average litter size as noted within 12 hours postpartum could be due to embryonic resorptions, perinatal death, and/or cannibalization of pups by the dam. All of these events can be associated with an embryotoxic, fetotoxic, or teratogenic action. Less pronounced forms of developmental toxicity may be evidenced by decreased birth weights or by a failure of pups to survive and grow at normal rates during the 72 hours after the initial observation (litters are initially observed and weighed within 12 hours of birth and then again after 72 hours--observation time periods are then approximately 12 and 84 hours post partum). Survival and growth over this period may be sensitive indicators of organ function failure, e.g., cardiac, renal, pulmonary or other systems. The postnatal mouse test may be a sensitive screen for these functional deficits, which are not evaluated in conventional teratology tests.

MATERIALS AND METHODS

The objective of this program was to assess the reproductive hazard potential of priority chemcials in the CD-1 mouse.

TEST ARTICLE

The following summary lists the three (3) test articles used in this program and the appropriate receipt and identification information. Chemicals wer obtained for the NTP by the Radian Corporation and supplied to EHRT as coded chemicals for testing.

Test Article Identification Information:

CHEMICAL	SUPPLIER	CAS No.	Date Received
Triorthocresyl Phosphate	Radian Corp.	78-30-8	3/6/85
4,4'-Thiobis (6-t-Butyl-m-Cresol	Radian Corp.	96-69-5	3/6/85
13-Cis-Retinoic Acid	Radian Corp.	4759-48-2	3/6/85

Test Article Preparation and Administration - Phases I, II and III:

Each test article was mixed in corn oil at a concentration which provided the desired amount of test compound to the animals in a standard volume of 10 ml/kg body weight. In Phase II, due to the viscosity of the 9600 mg/kg dose level of 4,4'-thiobis (6-t-butyl-m-cresol), the test chemical was administered at a volume of 15 ml/kg. For each test article, appropriate amounts of the compound were weighed on a pan balance (accurate to 0.1 mg). The vehicle was then added and the resulting test solution

mixed on a magnetic stirrer until suspended. The daily dosing solutions were prepared and color coded by the chemistry staff at study initiation and were stored refrigerated in amber glass vials to prevent photodegradation. All dosing concentrations were verified as accurate by the chemistry staff. The animal technicians dosing the animals were not aware of the identity of the test article name and were blind to the dose levels. Each test article was identified by a number and a corresponding color. This technique allowed for nonbias of all clinical observations.

On the day of dosing, an aliquot was allowed to warm to room temperature prior to dosing. The test dosing solution was delivered directly into the stomach via a 20 gauge 1 1/2 inch stainless steel gavage needle attached to a 1 cc syringe. The animals were dosed once daily for five (Phase I) or ten (Phase II and III) consecutive days. Oral intubation was selected as the route of administration by the sponsor. The dosing mixtures were thoroughly vortexed just prior to and intermittently during dosing.

A vehicle control group was employed only during the Phase III. Mice in the vehicle control group received only corn oil and served as the common control group for the test compounds being evaluated with this block of chemicals.

Purity and Stability:

Test compounds used in this study were obtained from Radian Corporation, Austin, Texas. The compilation of stability, toxicity, storage, and safe handling information was sent by Radian Corporation to EHRT.

The vehicle was Mazola Corn Oil (Corn Products Company, Englewood Cliffs, New Jersey). The corn oil was analyzed prior to each phase for peroxides and the results are presented in Appendix 2.

Prior to and upon completion of the dosing interval, the EHRT Analytical Chemistry Division analyzed dosing solutions to document concentration and chemical stability. The results of the analysis are presented in Appendix 2.

Safety Precautions:

Personnel working with the test materials or in the animal rooms wore disposable gloves, masks, and other appropriate clothing such as laboratory coats and/or uniforms. All animal wastes resulting from this study were disposed of in a landfill, and carcasses in a high temperature incinerator.

Laboratory personnel practiced good sanitation and health habits. No illness or other condition of personnel that may have adversely affected the study was reported.

EXPERIMENTAL METHODS

Test Animals and Husbandry:

Virgin female (Phase I) and primigravida (Phase II and III) specific pathogen free (SPF) CD-1 albino mice, six to eight weeks of age, were obtained from Charles River Breeding Laboratories, Inc. (Kingston, New York). Upon receipt, all animals were individually examined for general physical condition. Body weights were measured within the next two days after receipt. This strain of mouse was selected by the sponsor.

The mice were individually housed in polycarbonate shoe box cages with stainless steel tops and hardwood bedding (Ab-Sorb--Dri, Lab Products, Maywood, N.J.). Cages were sanitized and fresh bedding was supplied at least once during the study. Purina Certified Rodent Chow #5002 and fresh water were available ad libitum by water bottle. The mice were maintained on a 12-hour light/dark cycle in a temperature controlled room (72°± 3 F). Hepafiltered air was supplied at a rate of at least 10 room air changes per hour. Room temperature and humidity were recorded at least once each day.

Prior to study initiation, the mice were quarantined for five days in the room in which the study was to be conducted. This shortened quarantine period was used in Phase I to parallel that used in Phases II and III with pregnant mice. During this period, observations were performed twice daily for mortality and general physical appearance.

Assignment to Treatment Groups:

Based on the observations during quarantine, clinically acceptable mice were randomly assigned to treatment groups, using a computer-generated randomization program. Each group consisted of three (3) virgin females (Phase I), four (4) mated females (Phase II) or fifty (50) mated females (Phase III). Each animal received a unique, permanent identification number using an ear punch numbering system. In addition, each group of mice was assigned a color-coded card which displayed the corresponding project number, individual animal numbers and group number.

Clinical Observations:

During the dosing period, animals were observed twice daily for signs of toxicity. The observations were conducted once in the morning and once in the afternoon. In addition, mortality checks were performed once in the morning (prior to dosing) and again in the afternoon. All animals which succumbed during the dosing phase were examined for evidence of dosing error. The following criteria were used in determining dosing error deaths: 1) compound in the thoracic cavity; 2) compound in the lungs; and/or 3) a hole in the esophagus. All other deaths were assumed to be treatment-related. Observation for signs of toxicity and mortality were also performed once on the days the animals were not dosed (7 days post treatment for Phase I, 2 days post treatment for Phase II).

On gestation day 17, all surviving Phase II mice were sacrificed and uteri were examined. Each female was classified

as pregnant or nonpregnant. Pregnant mice were noted as having live fetuses or no live fetuses.

For Phase III (reproductive phase), litter box bedding was not changed from gestation day 17 throughout the post partum observation period. Beginning on day 18 of gestation and continuing until all litters were delivered, females were observed twice daily for evidence of labor and delivery of litters. Observations were made before 9:00 a.m. and after 4:00 p.m. The day of delivery was recorded to the nearest half day.

The time, date, and gestation day was recorded when labor, fetuses, or other evidence of delivery was observed. Females were not disturbed if they were in labor or if they had not finished cleaning newborn pups. The delivery of pups was considered complete if the pups were clean and dry.

After delivery was complete, the dam was removed and weighed then placed temporarily in a holding container. The pups were removed from the cage and the nesting material was probed for dead fetuses and parts of cannibalized fetuses. The number of live and dead pups, and the weight of all live pups were recorded. The pups of a litter were weighed together to give a litter weight and the average individual weight of the litter calculated. The live pups were then returned to the nesting box and the dam was returned last. Dead fetuses were discarded.

Any female that did not show signs of delivering a litter by gestation day 22 was sacrificed and the uterus examined for evidence of pregnancy. If there was no gross evidence of

pregnancy, the uterus was placed in a 10 percent ammonium sulfide solution to make early implantation sites visible. Based on the presence of early implantation sites the dams were classified as having been either pregnant or never pregnant.

Following the observations made on Day 0 post partum, females and litters were not disturbed until post partum Day 3. At that time, the maternal body weight, and the number of live and dead neonatal mice, and the total litter weight of live pups were recorded. Females and litters were then sacrificed and discarded.

Body Weights:

Body weights for the Phase I animals were recorded at the time of randomization, on days 1 and 5 of treatment and on days 3 and 7 after the final dose. Phase II and III animal body weights were recorded at the time of randomization, on days 6 through 15 of gestation and on day 17 of gestation. Additional body weights measured for Phase III were recorded on post partum day 0 and post partum day 3. Body weight changes were calculated for each phase of the study.

Termination:

All surviving mice were sacrificed by carbon dioxide asphyxiation following the collection of terminal body weights.

STATISTICAL ANALYSES

An overall test for homogeneity of variance (Bartlett's test and F-test) was performed on the weight data of each group following randomization. Average body weight per group and average body weight change per group were calculated for treatment and control groups. Probit analysis of mortality and morbidity data generated in Phase II of the range finding study was used to determine the predicted LD_{10} for the Phase III.

An IBM PC was used to compile all the raw data. At the completion of Phase III a diskette with this information was delivered to the Project Officer. The statistics on these data were performed by Dr. Burg at the NIOSH facility in Cincinnati, Ohio.

The following statistical procedures were used for analyzing maternal data:

- Random weights (Table 21) ANOVA (2-tail)
  (all groups, pregnant dams)
  (all groups, viable litters only)
- Survival (Table 19) Fisher's Exact Test (one-tail)
  (each group vs control, all dams)
  (each group vs control, pregnant only) (Table 20B)
- Weight gains (Table 22) Mann-Whitney U-Test (2-tail)
  (each animal, day 6 to day 0 postnatal)
  (each group vs control; viable litters only)

- Proportion of viable litters Fisher's Exact Test (Table 23) (one-tail)

  (each group vs control, pregnant only)

- Survival of pups Chi-Square Test (one-tail)

  Table 23 (each group vs control)

The Mann-Whitney U-test (2-tail) was used to compare each group to the concurrent control.

- Number live pups/litter (day 0, day 3) Table 23
- Length of gestation (Table 24)
- Average pup weight (day 0, day 3) Table 25
- Average wt. gain/litter (day 3 - day 0) Table 25

The p-value listed is not corrected for multiple comparisons.

RESULTS - RANGE FINDING

(PHASE I)

In Phase I (range finding), triorthocresyl phosphate and 4,4'-thiobis (6-t-butyl-m-cresol) were evaluated. The two test chemicals were administered to three (3) groups of mice, using three mice per group. Doses of 10, 100, and 1000 mg/kg/day was administered over a five-day period (Monday through Friday). Dosing was by oral intubation once per day in a standard volume of 10 ml/kg.

Phase I was not required for cis-retinoic acid. The dose levels for Phase II were specified by the Project Officer on the basis of existing developmental toxicity data.

Body Weights:

Average body weights and average body weight changes for each dose level are presented in Table 1 for tiorthocresyl phosphate, and in Table 3 for 4,4'-thiobis (6-t-butyl-m-cresol).

Mortality:

Tables 2 and 4 present the number of deaths for triorthocresyl phosphate and 4,4'-thiobis (6-t-butyl-m-cresol) respectively. Triorthocresyl phosphate produced three deaths in the 1000 mg/kg of body weight/day group. These deaths did not appear to be caused by dosing trauma. 4,4'-Thiobis (6-t-butyl-m-cresol) produced one death in the 10 mg/kg/day group; this death appeared to be caused by dosing trauma.

Clinical Signs:

Triorthocresyl phosphate produced signs of systemic toxicity and death in the 1000 mg/kg/day dose level. Signs of toxicity included languid behavior, rough haircoat, squinted eyes, excessive urination, pale extremeties, hunched posture, labored respiration, pale eyes, and stained fur in the ano-genital area. One animal was found dead during the morning observation of treatment day 2; a second animal during the morning observation of treatment day 3; and the third animal during the afternoon observation of treatment day 4. No deaths or toxic signs were noted in the 10 and 100 mg/kg/day dose levels. None of the deaths appeared to be caused by dosing trauma.

4,4'-Thiobis (6-t-butyl-m-cresol) produced no signs of toxicity or death. One animal in the 10 mg/kg/day dose group was found dead on treatment day 4. This death appeared to be caused by dosing trauma.

Conclusion:

Because triorthocresyl phosphate produced three deaths in the 1000 mg/kg/day dose level, the Phase II dose levels selected from Appendix 1 were 45, 85, 160, 305, and 575 mg/kg/day (dose range 4).

4,4'-Thiobis (6-t-butyl-m-cresol) did not produce chemical related mortality when administered at dose levels of 10, 100, and 1000 mg/kg/day. Therefore, the dose levels for the Phase II selected from Appendix 1 were 600, 1200, 2400, 4800, and 9600 mg/kg/day (dose range 1).

PHASE 1

Test Chemical Identification: Triorthocresyl Phosphate
CAS No. 78-30-8

TABLE 1

AVERAGE BODY WEIGHTS AND BODY WEIGHT CHANGES (G)
MEAN ± STANDARD DEVIATION

	DOSE LEVEL (mg/kg)		
	10	100	1000
TREATMENT DAYS			
1	24.5 ± 0.91	25.6 ± 1.55	24.7 ± 1.10
5	24.8 ± 1.74	24.7 ± 1.22	All Dead
POST TREATMENT DAYS			
3	25.7 ± 1.54	25.1 ± 1.13	All Dead
7	26.7 ± 1.79	25.8 ± 0.80	All Dead
CHANGES			
TD 5 - TD 1	+0.3	-0.9	All Dead
PTD 7 - TD5	+1.9	+1.1	All Dead
PTD 7 - TD1	+2.2	+0.2	All Dead

TD - TREATMENT DAY
PTD - POST TREATMENT DAY

TABLE 2

MORTALITY (Number of Deaths)

DOSE LEVEL (mg/kg)	DAYS						
	1	2	3	4	5	6-12	TOTAL
10	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0
1000	0	1	1	1	-	-	3

PHASE I

Test Chemical Identification: 4,4'-Thiobis (6-t-Butyl-m-Cresol)
CAS No. 96-69-5

TABLE 3

AVERAGE BODY WEIGHTS AND BODY WEIGHT CHANGES (G)
MEAN ± STANDARD DEVIATION

	DOSE LEVEL (mg/kg)		
	10	100	1000
TREATMENT DAYS			
1	24.8 ± 0.78	26.2 ± 1.40	25.3 ± 1.21
5	25.3 ± 0.95	27.0 ± 0.68	26.8 ± 0.47
POST TREATMENT DAYS			
3	27.7 ± 0.96	27.7 ± 0.98	26.7 ± 0.93
7	26.3 ± 0.89	27.1 ± 1.03	26.2 ± 0.52
CHANGES			
TD 5 - TD 1	+0.5	+0.8	+1.5
PTD 7 - TD 5	+1.0	+0.1	-0.6
PTD 7 - TD1	+1.5	+0.9	+0.9

TABLE 4

MORTALITY (Number of Deaths)

DOSE LEVEL (mg/kg)	DAYS						
	1	2	3	4	5	6-12	TOTAL
10	0	0	0	1a	0	0	1
100	0	0	0	0	0	0	0
1000	0	0	0	0	0	0	0

a - Animal appeared to die from dosing trauma.

RESULTS - RANGE FINDING

(PHASE II)

In the Phase II (range finding) experimental block triorthocresyl phosphate, 4,4'-thiobis (6-t-butyl-m-cresol) and 13-cis-retinoic acid were evaluated. The high dose level (9600 mg/kg) of 4,4'-thiobis (6-t-butyl-m-cresol) was highly viscous and therefore, administered at a volume of 15 ml/kg. Because of the high mortality in the initial Phase II for 4,4'-thiobis (6-t-butyl-m-cresol), a repeat of Phase II was conducted using lower dose levels. Because no Phase I was performed on 13-cis-retinoic acid, the dose levels were selected for Phase II by the NIOSH Project Officer. The results for each chemical evaluated are discussed below:

Body Weights:

Average body weights and average body weight changes for each test chemical can be found as follows: triorthocresyl phosphate, Tables 7 and 8; 4,4'-thiobis (6-t-butyl-m-cresol), Tables 10, 11, 13 and 14; 13-cis-retinoic acid, Tables 16 and 17.

Mortality and Pregnancy Status:

The number of animals dead per group/number of animals per group and the pregnancy status of all animals found dead or sacrificed are presented in Table 9 (triorthocresyl phosphate); Table 12, (4,4'-thiobis (6-t-butyl-m-cresol),initial; Table 15, (4,4'-thiobis (6-t-butyl-m-cresol), repeat; and Table 18 (13-cis-retinoic acid).

Clinical Signs:

When triorthocresyl phosphate was administered to primigravida mice over a ten day period (gestation day 6 through gestation day 15), signs of systemic toxicity and death were produced. Systemic signs were as follows:

Dose Level: (mg/kg)	
45	No chemical related signs of systemic toxicity were noted at this dose level.
85	One animal at this dose level exhibited a rough haircoat, hunched posture, rapid respiration, languid behavior, squinted eyes, and was noted as being underweight. These signs were noted starting on treatment day 7 and continued throughout the study. All other animals appeared normal.
160	All animals at this dose level appeared normal.
305	All animals at this dose level appeared normal.
575	Systemic signs noted at this dose level included: hunched posture, languid behavior, rough haircoat, stained fur (ano-genital area), squinted eyes, rapid respiration, and labored respiration. One animal was found dead at the afternoon observation of treatment day 10. This death did not appear to be caused by dosing trauma.

When 4,4'-thiobis (6-t-butyl-m-cresol) was administered to primigravida mice over a ten day period (gestation day 6 through gestation day 15), signs of systemic toxicity and death were produced. Systemic signs were as follows:

4,4'-Thiobis (6-t-Butyl-m-Cresol)

(Initial Study)

Dose Level (mg/kg)	
600	Systemic signs noted at this dose level included: stained fur (ano-genital area), rough haircoat, hunched posture, languid behavior, squinted eyes, labored respiration, and death. One animal was found dead during the afternoon observation of treatment day 6 and one found dead during the morning observation of treatment day 7. On treatment day 10, one animal was found dead during the morning observation. None of these deaths appeared to caused by dosing trauma.
1200	Signs at this dose level included: languid behavior, labored respiration, squinted eyes, stained fur (ano-genital area), rapid respiration, tremors, and death. On treatment day 3, one animal was found dead during the morning observation and one animal was found dead during the afternoon observation. The remaining two animals were found dead during the morning observation of treatment day 4. None of these deaths appeared to caused by dosing trauma.

Dose Level: (mg/kg)	
2400	Systemic signs in this group included: stained fur (ano-genital area), hunched posture, rough haircoat, languid behavior, lacrimation, squinted eyes, rapid respiration, labored respiration, tremors, and death. On treatment day 3, one animal was found dead during the morning observation and one animal was found dead during the afternoon observation. On treatment day 4, the remaining two animals were found dead; one in the morning and one in the afternoon. None of these deaths appeared to be caused by dosing trauma.
4800	Signs of toxicity in this group included: stained fur (ano-genital area), hunched posture, rough haircoat, stained fur (mouth and chest area), rapid respiration, wheezing, squinted eyes, languid behavior, labored respiration, tremors, and death. One animal was found dead during the afternoon observation of treatment day 3; a second animal was found dead during the morning observation of treatment day 4; a third animal was found dead during the afternoon observation of treatment day 5; and the fourth animal was found dead during the morning observation of treatment day 6. None of these deaths appeared to be caused by dosing trauma.

Dose Level (mg/kg)	
9600	Systemic signs at this dose level included: stained fur (ano-genital area), rough haircoat, hunched posture, rapid respiration, squinted eyes, labored respiration, prostrate, and death. One animal was found dead during the afternoon observation of treatment day 3; and three found dead during the morning observation of treatment day 4. None of these deaths appeared to be caused by dosing trauma.

4,4'-Thiobis (6-t-Butyl-m-Cresol)

(Repeated Study))

Because of the high mortality in the initial Phase II for 4,4'-thiobis (6-t-butyl-m-cresol), a repeat of Phase II was conducted using lower dose levels. The dose levels used were 100, 310, 400, 500, and 630 mg/kg of body weight/day. Due to a miscalculation, the low dose group of the repeated study was dosed at 100 mg/kg of body weight/day instead of 250 mg/kg of body weight/day. When the mistake was discovered on the first day of dosing, the Project Officer was contacted. It was his decision to continue dosing at the low dose level.

Dose Level (mg/kg)	
100	One animal at this dose level exhibited hunched posture, rough haircoat, languid behavior, labored respiration, and a swollen leg. This animal was found dead after

Dose Level (mg/kg)	
100	dosing on treatment day 4. This death appeared to be caused by dosing trauma. All other animals appeared normal.
310	All animals at this dose level appeared normal throughout the study.
400	One animal exhibited a rough haircoat and one animal had stained fur in the ano-genital area. These were the only deviations from normal noted at this dose level.
500	One animal was found dead during the morning observation of treatment day 4. This death appeared to be caused by dosing trauma. Another animal was found dead during the afternoon observation of treatment day 10 and a third animal was found dead during the morning observation of post-treatment day 2. Neither of these deaths appeared to be caused by dosing trauma. Clinical observations in this group included: hunched posture, languid behavior, rough haircoat, labored respiration, squinted eyes, and tremors.
630	One animal was found dead during the morning observation of treatment day 8. This death did not appear to be caused by dosing trauma. All surviving animals appeared normal throughout the treatment period.

When 13-cis-retinoic acid was administered to primigravida mice over a ten day period (gestation day 6 through gestation day

15), signs of systemic toxicity were produced. Systemic signs were as follows:

Dose Level (mg/kg)	
5	All animals at this dose level appeared normal
10	One animal appeared hyperactive on treatment day 7. All other animals appeared normal.
20	All animals at this dose level appeared normal.
40	All animals at this dose level appeared normal.
80	Systemic signs noted at this dose level included: rough haircoat, hunched posture, stained fur (ano-genital area), languid behavior, and labored respiration. One animal was found dead during the afternoon observation of treatment day 6. This death appeared to be caused by dosing trauma.

Conclusion:

Based on the mortality data, probit analysis using the Statistical Analysis System used by NIOSH, the predicted LD_{10} for triorthocresyl phosphate was 515 mg/kg and 485 mg/kg for 4,4'-thiobis (6-t-butyl-m-cresol). The dose level for the 13-cis-retinoic acid (85 mg/kg) was selected by the Project Officer.

PHASE II

Test Chemical Identification: Triorthocresyl Phosphate
CAS No. 78-30-8

TABLE 5

AVERAGE MATERNAL BODY WEIGHTS IN GRAMS
(Mean ± Standard Deviation)
(Pregnant Animals Only)

TREATMENT	DOSE LEVEL (mg/kg of body weight/day)				
DAY	45	85	160	305	575
1	28.96 ± 0.98	27.94 ± 1.89	29.50 ± 2.02	28.34 ± 1.36	29.36 ± 1.93
n	3	3	4	2	3
2	29.77 ± 1.51	28.23 ± 1.15	29.61 ± 1.88	27.83 ± 0.49	30.43 ± 3.07
n	3	3	4	2	3
3	29.54 ± 1.22	28.21 ± 1.58	30.43 ± 2.29	28.01 ± 0.30	29.50 ± 3.55
n	3	3	4	2	3
4	29.78 ± 1.91	29.34 ± 1.61	31.02 ± 1.86	28.23 ± 1.06	28.74 ± 3.80
n	3	3	4	2	3
5	30.01 ± 2.02	30.64 ± 0.99	31.95 ± 1.93	28.97 ± 0.55	29.67 ± 4.92
n	3	3	4	2	3
6	30.49 ± 2.16	32.49 ± 1.27	34.13 ± 2.09	29.35 ± 3.21	30.69 ± 6.24
n	3	3	4	2	3
7	31.67 ± 2.51	34.29 ± 0.99	35.33 ± 1.87	29.87 ± 4.20	30.82 ± 7.08
n	3	3	4	2	3
8	32.67 ± 3.63	36.01 ± 1.10	37.60 ± 1.94	31.33 ± 5.90	31.57 ± 7.98
n	3	3	4	2	3
9	33.92 ± 4.37	38.19 ± 1.24	39.15 ± 1.77	31.77 ± 7.25	31.83 ± 8.30
n	3	3	4	2	3
10	35.17 ± 5.47	40.60 ± 1.33	41.99 ± 1.79	32.76 ± 8.85	32.21 ± 9.33
n	3	3	4	2	3
POST TREATMENT DAY					
2	37.82 ± 7.92	45.93 ± 1.42	48.17 ± 1.77	36.05 ±12.46	33.43 ±12.53
n	3	3	4	2	3

n - number of pregnant animals in each dose level

PHASE II

Test Chemical Identification: Triorthocresyl Phosphate
CAS No. 78-30-8

TABLE 6

AVERAGE MATERNAL BODY WEIGHT CHANGES IN GRAMS
(PREGNANT ANIMALS ONLY)

	Dose Level (mg/kg)				
Triorthocresyl Phosphate	45	85	160	305	575
Treatment day 10-treatment day 1	+6.21	+12.66	+12.49	+4.42	+2.85
Post-treatment day 2-treatment day 10	+2.65	+ 5.33	+ 6.18	+3.29	+1.22
Post-treatment day 2-treatment day 1	+8.86	+17.99	+18.67	+7.71	+4.07

TABLE 7
MORTALITY AND
PREGNANCY STATUS

	DOSE LEVEL (mg/kg)				
	45	85	160	305	575
Number of Animals Treated	4	4	4	4	4
Animals Found Dead	0	0	0	0	1
Ammonium Sulfide Positive	-	-	-	-	-
Ammonium Sulfide Negative	-	-	-	-	1
Animals Sacrificed	4	4	4	4	3
Live Pups	2	3	4	1	1
Ammonium Sulfide Positive	1	-	-	1	2
Ammonium Sulfide Negative	1	1	-	2	-

PHASE II
(Initial Study)

Test Material Identification: 4,4'-Thiobis (6-t-Butyl-m-Cresol)
CAS No. 96-69-5

TABLE 8

AVERAGE MATERNAL BODY WEIGHTS IN GRAMS
(Mean ± Standard Deviation)
(Pregnant Animals Only)

TREATMENT	DOSE LEVEL (mg/kg of body weight/day)				
DAY	600	1200	2400	4800	9600
1	29.31 ± 0.27	28.97 ± 1.47	29.12 ± 2.06	28.76 ± 1.35	28.23 ± 0.69
n	2	3	3	3	2
2	29.43 ± 0.66	25.73 ± 0.68	27.21 ± 3.21	28.98 ± 1.42	29.01 ± 0.30
n	2	3	3	3	2
3	29.75 ± 0.47	24.93 ± 0.55	27.34 ± 1.90	26.56 ± 1.51	27.20 ± 0.85
n	2	3	2	3	2
4	30.59 ± 0.98	All Dead	25.28 ± --	25.30 ± --	All Dead
n	2	0	1	1	0
5	31.67 ± 1.54	All Dead	All Dead	All Dead	All Dead
n	2	0	0	0	0
6	31.10 ± 0.93	All Dead	All Dead	All Dead	All Dead
n	2	0	0	0	0
7	30.98 ± 2.55	All Dead	All Dead	All Dead	All Dead
n	2	0	0	0	0
8	32.02 ± 5.57	All Dead	All Dead	All Dead	All Dead
n	2	0	0	0	0
9	31.59 ± 5.39	All Dead	All Dead	All Dead	All Dead
n	2	0	0	0	0
10	27.10 ± --	All Dead	All Dead	All Dead	All Dead
n	1	0	0	0	0
POST TREATMENT DAY					
2	25.60 ± --	All Dead	All Dead	All Dead	All Dead
n	1	0	0	0	0

n - number of pregnant animals in each dose level

PHASE II
(Initial Study)

Test Material Identification: 4,4'-Thiobis (6-t-Butyl-m-Cresol)
CAS No. 96-69-5

TABLE 9

AVERAGE BODY WEIGHT CHANGES IN GRAMS
(PREGNANT ANIMALS ONLY)

	Dose Level (mg/kg)				
3-EThoxy-1-Propanol	600	1200	2400	4800	9600
Treatment day 10 - treatment day 1	-2.21	0a	0a	0a	0a
Post-treatment day 2 - treatment day 10	-1.50	0a	0a	0a	0a
Post-treatment day 2 - treatment day 1	-3.71	0a	0a	0a	0a

a - all animals of these dose levels were found dead by treatment day 6

TABLE 10

MORTALITY AND
PREGNANCY STATUS

	DOSE LEVEL (mg/kg/day)				
	600	1200	2400	4800	9600
Number of Animals Treated	4	4	4	4	4
Animals Found Dead	3	4	4	4	4
Ammonium Sulfide Positive	1	3	3	3	2
Ammonium Sulfide Negative	2	1	1	1	2
Animals Sacrificed	1	0	0	0	0
Live Pups	0	-	-	-	-
Ammonium Sulfide Positive	1	-	-	-	-
Ammonium Sulfide Negative	0	-	-	-	-

PHASE II
(Repeat Study)

Test Chemical Identification: 4,4'-Thiobis (6-t-Butyl-m-Cresol)
CAS No. 96-69-5

TABLE 11

AVERAGE MATERNAL BODY WEIGHTS IN GRAMS
(Mean ± Standard Deviation)
(Pregnant Animals Only)

TREATMENT	DOSE LEVEL (mg/kg of body weight/day)				
DAY	100	310	400	500	630
1	30.30 ± --	27.87 ± 2.27	28.40 ± 3.25	24.00 ± --	28.58 ± 2.29
n	1	3	2	1	4
2	31.30 ± --	30.07 ± 3.35	30.15 ± 2.76	25.20 ± --	30.23 ± 2.98
n	1	3	2	1	4
3	31.40 ± --	31.00 ± 3.74	31.40 ± 3.54	23.80 ± --	30.98 ± 2.52
n	1	3	2	1	4
4	31.80 ± --	31.27 ± 3.55	32.05 ± 2.90	24.90 ± --	31.78 ± 2.30
n	1	3	2	1	4
5	33.00 ± --	31.93 ± 3.23	33.00 ± 2.83	26.10 ± --	32.75 ± 2.63
n	1	3	2	1	4
6	34.60 ± --	34.50 ± 3.63	35.15 ± 1.91	26.10 ± --	35.10 ± 2.83
n	1	3	2	1	4
7	36.40 ± --	36.63 ± 4.19	37.50 ± 0.85	24.80 ± --	37.80 ± 2.94
n	1	3	2	1	4
8	37.80 ± --	37.67 ± 4.22	40.10 ± 0.99	25.70 ± --	39.13 ± 2.86
n	1	3	2	1	3
9	38.80 ± --	40.13 ± 4.57	42.60 ± 0.14	25.10 ± --	41.20 ± 3.06
n	1	3	2	1	3
10	41.90 ± --	44.03 ± 5.03	44.85 ± 0.21	24.00 ± --	45.03 ± 2.74
n	1	3	2	1	3
POST TREATMENT DAY					
2	46.60 ± --	51.20 ± 7.27	51.35 ± 0.78	23.10 ± --	51.33 ± 4.02
n	1	3	2	1	3

n - number of pregnant animals in each dose level

PHASE II
(Repeat Study)

Test Chemical Identification: 4,4'-Thiobis (6-t-Butyl-m-Cresol)
CAS No. 96-69-5

TABLE 12

AVERAGE MATERNAL BODY WEIGHT CHANGES (IN GRAMS)

	Dose Level (mg/kg)				
4,4'-Thiobis (6-t-Butyl-m-Cresol)	100	310	400	500	630
Treatment day 10-treatment day 1	+11.60	+16.16	+16.45	+ 0.00	+16.45
Post-treatment day 2-treatment day 10	+ 4.70	+ 7.17	+ 6.50	- 0.90	+ 6.30
Post-treatment day 2-treatment day 1	+16.30	+23.33	+22.95	- 0.90	+22.75

TABLE 13

MORTALITY AND
PREGNANCY STATUS

	DOSE LEVEL (mg/kg/day)				
	100	310	400	500	630
Number of Animals Treated	4	4	4	4	4
Animals Found Dead	1	0	0	3	1
Ammonium Sulfide Positive	-	-	-	1	1
Ammonium Sulfide Negative	1a	-	-	2a	-
Animals Sacrificed	3	4	4	1	3
Live Pups	1	3	2	-	3
Ammonium Sulfide Positive	-	-	-	-	-
Ammonium Sulfide Negative	2	1	2	1	-

a - One animal of each of the two dose levels appeared to have died from dosing trauma.

PHASE II

Test Chemical Identification: 13-Cis-Retinoic Acid
CAS No. 4759-48-2

TABLE 14

AVERAGE MATERNAL BODY WEIGHTS IN GRAMS
(Mean ± Standard Deviation)
(Pregnant Animals Only)

TREATMENT	DOSE LEVEL (mg/kg of body weight/day)				
DAY	5	10	20	40	80
1	28.80 ± 1.81	29.18 ± --	27.96 ± 0.90	28.54 ± 0.21	29.54 ± 2.18
n	2	1	4	3	2
2	29.27 ± 0.75	28.06 ± --	28.11 ± 1.15	28.74 ± 1.12	29.61 ± 2.45
n	2	1	4	3	2
3	28.83 ± 1.32	29.18 ± --	28.17 ± 1.08	28.55 ± 0.56	29.36 ± 2.23
n	2	1	4	3	2
4	29.15 ± 0.81	30.24 ± --	28.46 ± 1.13	28.93 ± 0.48	28.06 ± 0.42
n	2	1	4	3	2
5	30.32 ± 1.19	31.82 ± --	29.64 ± 0.91	30.01 ± 0.27	27.81 ± 2.02
n	2	1	4	3	2
6	32.23 ± 1.03	33.44 ± --	30.98 ± 0.99	31.43 ± 0.34	28.00 ± 3.68
n	2	1	4	3	2
7	33.63 ± 1.15	35.18 ± --	32.10 ± 0.97	33.25 ± 0.52	32.20 ± --
n	2	1	4	3	1
8	35.68 ± 1.53	36.98 ± --	34.02 ± 1.28	34.81 ± 0.31	34.06 ± --
n	2	1	4	3	1
9	37.57 ± 1.99	39.46 ± --	35.60 ± 1.47	36.79 ± 0.67	35.66 ± --
n	2	1	4	3	1
10	40.82 ± 2.55	41.70 ± --	37.55 ± 1.59	39.19 ± 0.71	37.82 ± --
n	2	1	4	3	1
POST TREATMENT DAY					
2	47.45 ± 2.62	46.74 ± --	43.12 ± 2.37	44.95 ± 0.58	44.08 ± --
n	2	1	4	3	1

n - number of pregnant animals in each dose level

PHASE II

Test Chemical Identification: 13-Cis-Retinoic Acid
CAS No. 4759-48-2

TABLE 15

AVERAGE MATERNAL BODY WEIGHT CHANGES IN GRAMS
(PREGNANT ANIMALS ONLY)

	Dose Level (mg/kg)				
13-Cis-Retinoic Acid	5	10	20	40	80
Treatment day 10-treatment day 1	+12.02	+12.52	+ 9.59	+10.65	+ 8.28
Post-treatment day 2-treatment day 10	+ 6.63	+ 5.04	+ 5.57	+ 5.76	+ 6.26
Post-treatment day 2-treatment day 1	+18.65	+17.56	+15.16	+16.41	+14.54

TABLE 16
MORTALITY AND
PREGNANCY STATUS

	DOSE LEVEL (mg/kg)				
	5	10	20	40	80
Number of Animals Treated	4	4	4	4	4
Animals Found Dead	0	0	0	0	1
Ammonium Sulfide Positive	-	-	-	-	1a
Ammonium Sulfide Negative	-	-	-	-	-
Animals Sacrificed	4	4	4	4	3
Live Pups	2	1	4	3	1
Ammonium Sulfide Positive	-	-	-	-	-
Ammonium Sulfide Negative	2	3	-	1	2

a - death appeared to be caused by dosing trauma.

RESULTS - REPRODUCTIVE PHASE III

Triorthocresyl Phosphate, 4,4'-Thiobis (6-t-Butyl-m-Cresol), and 13-Cis-Retinoic Acid

Based on the results of the Statistical Analyses System (SAS) at NIOSH and probit analyses of the mortality data in Phase II, the predicted LD_{10} for triorthocresyl phosphate was 515 mg/kg/day and for 4,4'-thiobis (6-t-butyl-m-cresol) was 485 mg/kg/day. 13-cis-retinoic acid was dosed at 85 mg/kg/day which was the maximum dose permitted by the available chemical. The vehicle control group received corn oil. All animals were dosed at a constant volume of 10 ml/kg/day.

Mortality and Survival:

A mortality table showing all deaths by individual treatment days is presented in Table 17. The mortality rate for all animals (pregnant and nonpregnant) was statistically significant ($p \leq 0.001$, Fishers Exact-one tail) in the triorthocresyl phosphate (19/50) and 4,4'-thiobis (6-t-butyl-m-cresol) (22/50) groups when compared to the control group (0/47). The mortality rate for 13-cis-retinoic acid (0/48) did not differ from the control group.

A maternal status and survival table showing all pregnant animals except accidental deaths are presented in Tables 18A and 18B. There was significantly decreased survival of pregnant dams in the triorthocresyl phosphate (58%) and the 4,4'-thiobis (6-t-butyl-m-cresol) (42%) groups when compared to the control group. No pregnant animals died of toxicity in the 13-cis-retinoic acid group.

Clinical Observations:

Three animals in the vehicle control group, corn oil, were found dead. All three of these deaths appeared to be caused by dosing trauma. All other control animals appeared normal throughout the study. Triorthocresyl phosphate produced clinical signs of toxicity and death. Clinical signs noted in this group included: hunched posture, languid behavior, wheezing, labored respiration, rapid respiration, rough haircoat, tremors, squinted eyes, blood-like stains and fur stained (ano-genital area), prostrate, and pale extremeties. Nineteen animals in this group died; all animals died of toxicity. 4,4'-thiobis (6-t-butyl-m-cresol) also produced clinical signs of toxicity and death. Signs of toxicity included: prostration, rapid respiration, languid behavior, rough haircoat, squinted eyes, stained fur (ano-genital area), tremors, labored respiration, and hunched posture. Twenty two animals died in this dose group; all died of toxicity. Two animals appeared to die from dosing trauma in the 13-cis-retinoic acid group. Clinical signs noted in this group included: languid behavior, fur stained and bloody discharge (ano-genital area), rapid respiration, squinted eyes, and one animal was noted as having alopecia. All surviving animals appeared normal by post treatment day 3.

Maternal Body Weights:

Summaries of mean maternal body weights and body weight changes measured at the designated intervals during the study are presented in Tables 19 and 20. No group differences were found for randomization weights. 13-Cis-retinoic acid produced a decrease in the maternal body weight on postpartum days 0 and 3. 4,4'-Thiobis (6-t-butyl-m-cresol) increased the maternal body weight on postpartum day 0 and while it decreased the body weight on postpartum day 3. There was no effect of triorthocresyl phosphate on the postpartum maternal body weight.

Reproductive Index and Litter Data:

The reproductive index, average number of live pups/litter (day 0 and day 3) and percent postnatal survival are presented in Table 21. Triorthocresyl phosphate decreased the reproductive index as measured by the number of females producing viable litters/number of surviving females that were ever pregnant. 4,4'-Thiobis (6-t-butyl-m-cresol) and 13-cis-retinoic acid did not affect the reproductive index.

None of the three test chemicals affected the average number of pups/litter on day 0 while 4,4'-thiobis (6-t-butyl-m-cresol) and 13-cis-retinoic acid caused a reduced survival of the pups on day 3. All three test chemicals produced a significant decrease in the survival of the pups as measured as the percent survival.

Duration of Gestation:

Average gestation length is presented by group in Table 22. The length of gestation was significatnly increased by 4,4'-thiobis (6-t-butyl-m-cresol) ($p<0.001$) and triorthocresyl phosphate ($p<0.05$). 13-Cis-retinoic acid did not affect the length of gestation.

Weight Data:

The average weight of each live pup/litter (day 0 and day 3), and the change in pup weight between day 3 and day 0 are presented in Table 23. The average pup weight/litter was reduced by triorthocresyl phosphate, increased by 13-cis-retinoic acid (day 3) and not affected by 4,4'-thiobis (6-t-butyl-m-cresol). The weight gain between day 0 and day 3 was increased by 4,4'-thiobis (6-t-butyl-m-cresol) and unaffected by the other two test chemicals.

Conclusion:

Triorthocresyl Phosphate

Triorthocresyl Phosphate caused an increased maternal mortality (42%), a decreased number of viable litters (47.4%), no effect on litter size and birth weight gain by the pups and a decrease in the percent survival of the pups on day 3. Using the ranking system of Hardin (1987[4]) with a maximum score of 22 and the values for high maternal mortality, the following values should be subtracted: viable litters (0); percent pup survival (0); litter size (4); birth weight of pups (0); and weight gain of pups (4). Thus, the priority

score for triorthocresyl phosphate is 14 which indicates an intermediate priority, however, due to the high maternal mortality triorthocresyl phosphate should be retested in the Chernoff/Kavlock test. In summary, triorthocresyl phosphate was positive in the assay.

4,4'-Thiobis (6-t-Butyl-m-Cresol)

4,4'-Thiobis (6-t-butyl-m-cresol) caused an increased maternal mortality and a decreased percent survival of the pups while having no effect on the number of viable litters, the litter size, birth weight of the pups and weight gain of the pups. Using the ranking system of Hardin (1987[4]) with a maximum score of 22 and for high maternal mortality the following values are subtracted: viable litters (6); litter size (4); percent pup survival (0); birth weight of pups (3); pup weight gain (4). Thus, the toal score for 4,4'-thiobis (6-t-butyl-m-cresol) is 5 which low priority with evidence of some reproduction effects. In summary, 4,4'-thiobis (6-t-butyl-m-cresol) reduced the survival of the pups in the Chernoff/Kavlock assay, however due to the high maternal mortality it should be retested at a lower dose.

13-Cis-Retinoic Acid

13-Cis-retinoic acid had no effect upon maternal mortality, number of viable litters, litter size, and pup birth weight. 13-Cis-retinoic acid did decrease the percent of pup survival and weight gain of the pups. Using the ranking system of Hardin (1987[4]) with a maximum score of 22 and for low maternal mortality the following scores should be subtracted: viable litters (4); litter size (2); percent pup survival (0); birth weight of pups (1),

and weight gain of pups (0). Thus, the score for 13-cis-retinoic acid is 15 which represents high priority for conventional developmental toxicity testing. In summary, 13-cis-retinoic acid was positive in the Chernoff/Kavlock preliminary developmental toxicity test.

REFERENCE

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2 Chernoff, N. and Kavlock, R.: A teratology test system which utilizes post natal growth and viability in the mouse, in Short-Term Bioassays in the Analysis of Complex Environmental Mixtures III, Plenum Publishing Co., NY, 1983 pp 417-427.

3 MacKenzie KM: Screening of Priority Chemicals for Potential Reproductive Toxicity, Final Report to NIOSH, Contract No.: 200-82-2542, Hazleton Laboratories America, Inc., Dec., 1983.

4 Hardin BD: A recommended protocol for the Chernoff/Kavlock preliminary developmental toxicity test and a proposed method for assigning priority scores based on the results of that test. Teratog Carcinog Mutagen, in press.

TABLE 17

MORTALITY
NUMBER OF ANIMALS DEAD PER GROUP[a]

DOSE GROUP	TREATMENT DAY										
	1	2	3	4	5	6	7	8	9	10	TOTAL
Vehicle Corn Oil	0	0	0	1[b]	0	0	0	1[b]	0	0	2
Triorthocresyl Phosphate (515 mg/kg/day)	0	1	2	0	1	0	0	0	4	2	10
4,4'-Thiobis (6-t-Butyl-m-Cresol) (485 mg/kg/day)	0	0	0	1	0	0	1	5	4	3	14
13-Cis-Retinoic Acid (85 mg/kg/day)	0	0	0	0	0	1[b]	1[b]	0	0	0	2

DOSE GROUP	POST-TREATMENT DAYS					SUMMARY OF DEATHS		
	1	2	3	4	5	SACRIFICE	TOXICITY	TRAUMA
Vehicle Corn Oil	1[b]	0	0	0	0	47	0	3
Triorthocresyl Phosphate (515 mg/kg/day)	5	4	0	0	0	31	19***	0
4,4'-Thiobis (6-t-Butyl-m-Cresol) (485 mg/kg/day)	1	7	0	0	0	28	22***	0
13-Cis-Retinoic Acid (85 mg/kg/day)	0	0	0	0	0	48	0	2

[a] - Each group contained 50 animals
[b] - The death at each indicated dose level appeared to be caused by dosing trauma
[c] - The results were statistically significant from controls by the Fisher's Exact One-tail Test with *** denoting $p \leq 0.001$. None of the other results in the summary of deaths table had a $p \leq 0.05$.

TABLE 18A

MATERNAL STATUS

	TEST CHEMICAL			
	Vehicle Corn Oil	Triorthocresyl Phosphate	4,4'-Thiobis (6-t-Butyl-m-Cresol)	13-Cis Retinoic Acid
Number Evaulated	47	50	50	48
Animals Found Dead	0	19	22	0
Pregnant	0	14	19	0
Never Pregnant[c]	0	5	3	0
Animals Delivered Live Pups	31	9	12	28
Animals Sacrificed[a]	16	22	16	20
No Live Pups[b]	2	10	2	3
Never Pregnant[c]	14	12	14	17

[a] - Number of animals sacrificed - no live pups delivered
[b] - Visually dead pups or ammonium sulfide positive
[c] - Ammonium Sulfide Negative

TABLE 18B

MATERNAL SURVIVAL

	PREGNANT ANIMALS		
TEST CHEMICAL	LIVED	DIED	PERCENT SURVIVAL
Vehicle Corn Oil	33	0	100%
Triorthocresyl Phosphate (515 mg/kg/day)	19	14	58%***[a]
4,4'-Thiobis (6-t-Butyl-m-Cresol) (485 mg/kg/day)	14	19	42%***
13-Cis-Retinoic Acid (85 mg/kg/day)	31	0	100%

[a] - The results were statistically significant from controls by the Fisher's Exact One-tail Test with *** denoting $p \leq 0.001$. None of the other results in the Table 18B had a $p \leq 0.05$.

TABLE 19

AVERAGE MATERNAL BODY WEIGHTS IN GRAMS
(MEAN ± STANDARD DEVIATION)

RANDOMIZATION WEIGHTS	VEHICLE CORN OIL	TRIORTHOCRESYL PHOSPHATE 515 mg/kg/day	4,4'-THIOBIS (6-t-BUTYL-m-CRESOL) 485 mg/kg/day	13-CIS RETINOIC ACID 85 mg/kg/day
PREGNANT FEMALES ONLY	26.0 ± 1.16	25.7 ± 1.37	25.9 ± 1.29	26.0 ± 1.42
n[a]	33	19	14	31
GESTATION DAYS				
PREGNANT FEMALES ONLY				
6	28.4 ± 1.31	28.0 ± 1.43	28.4 ± 1.59	28.2 ± 1.92
n	33	19	14	31
7	28.9 ± 1.30	28.5 ± 1.55	28.9 ± 1.54	28.8 ± 1.44
n	33	19	14	31
8	29.5 ± 1.41	28.9 ± 1.64	29.8 ± 1.73	28.8 ± 1.55
n	33	19	14	31
9	30.5 ± 1.57	28.7 ± 2.25	30.5 ± 1.63	29.4 ± 1.53
n	33	19	14	31
10	31.6 ± 1.71	29.1 ± 2.82	32.1 ± 2.15	30.0 ± 1.92
n	33	19	14	31
11	33.2 ± 2.07	30.2 ± 3.65	33.5 ± 1.97	31.4 ± 2.43
n	33	19	14	31
12	34.6 ± 2.66	31.3 ± 4.52	35.3 ± 1.97	32.8 ± 2.98
n	33	19	14	31
13	36.3 ± 3.25	32.0 ± 5.25	36.7 ± 2.10	34.0 ± 3.67
n	33	19	14	31
14	38.1 ± 3.98	33.2 ± 6.14	38.2 ± 2.92	36.1 ± 4.42
n	33	19	14	31
15	40.7 ± 4.89	33.9 ± 6.61	40.5 ± 3.27	38.3 ± 5.27
n	33	19	14	31
17	45.2 ± 6.57	35.6 ± 7.97	44.4 ± 3.77	43.0 ± 7.16
n	33	19	14	31
POSTPARTUM DAY				
0	31.9 ± 1.65	31.8 ± 1.64	33.3 ± 2.93*[b]	30.6 ± 1.95**
n	31	9	12	28
3	35.0 ± 2.70	34.3 ± 3.20	32.4 ± 2.84**	33.5 ± 2.71*
n	31	9	12	28

[a] - n = number of animals

[b] - The postpartum body weights were analyzed statistically by the Mann-Whitney U-test with * denoting $p \leq 0.05$ and ** denoting $p \leq 0.01$.

TABLE 20

AVERAGE MATERNAL BODY WEIGHT CHANGES IN GRAMS
(MEAN ± STANDARD DEVIATION)

	CORN OIL	TRIORTHOCRESYL PHOSPHATE	4,4'-THIOBIS (6-t-BUTYL-m-CRESOL)	13-CIS-RETINOIC ACID
Random Weight Pregnant Only	26.0 ± 1.16	25.7 ± 1.37	25.9 ± 1.29	26.0 ± 1.42
n[a]	33	19	14	31
Random Weight Viable Litters Only	26.0 ± 1.19	26.5 ± 1.21	25.8 ± 1.37	26.0 ± 1.34
n	31	9	12	28
Weight Gain Postpartum Day 3 - Gestation Day 6	+6.5 ± 2.57	+5.4 ± 2.52	+4.2 ± 2.40*[b]	+5.1 ± 2.80**
n	31	9	12	28

[a] - n = Number of animals

[b] - Results were evaluated by the Mann-Whitney U-test with ** denoting p=0.006 and *, p=0.10

TABLE 21

REPRODUCTIVE AND LITTER DATA

	Reproductive Index[a]		Average Number/Litter		Postnatal Survival[b]	
	Ratio	Percent	Day 0 Live	Day 3 Live	Ratio	Percent
VEHICLE						
CORN OIL	31/33	93.9	9.7 ± 3.17	9.5 ± 3.32	295/301	98.0
n[c]			31	31		
TRIORTHOCRESYL PHOSPHATE						
515 mg/kg	9/19	47.4***	9.7 ± 2.74	8.7 ± 2.40	78/87	89.7**
n			9	9		
4,4'-THIOBIS (6-t-BUTYL-m-CRESOL)						
485 mg/kg	12/14	85.7	8.0 ± 3.19	4.7 ± 3.55***	56/96	58.3***
n			12	12		
13-CIS-RETINOIC ACID						
85 mg/kg	28/31	90.3	9.4 ± 2.84	7.2 ± 3.60*	202/263	76.8***
n			28	28		

[a] - Number of females producing viable litters/number of surviving females that were ever pregnant

[b] - Number of pups alive on day 3/number of pups alive on day 0

[c] - n = number of litters used to calculate the mean

* - $p<0.05$

** - $p\leq0.01$

***-$p<0.001$

TABLE 22
DURATION OF GESTATION (IN DAYS)
DAMS THAT PRODUCED VIABLE LITTERS ONLY

	DOSE GROUP			
	Vehicle Corn Oil	Triorthocresyl Phosphate 515 mg/kg/day	4,4'-Thiobis (6-t-Butyl-m-Cresol) 485 mg/kg/day	13-Cis-Retinoic Acid 85 mg/kg/day
Mean	18.1	18.5*[a]	18.6***	18.2
SD[b]	0.44	0.50	0.43	0.42
n[c]	31	9	12	28

[a] - Results were evaluated by the Mann-Whitney U-test with * denoting $p \leq 0.05$ and *** denoting $p \leq 0.001$
[b] - SD = Standard Deviation
[c] - n = number of animals that produced viable litters

TABLE 23

AVERAGE WEIGHT OF EACH LIVE PUP PER LITTER AND
DAY 3 AVERAGE LIVE PUP WEIGHTS MINUS DAY 0 WEIGHTS (G)
MEAN ± STANDARD DEVIATION

DOSE GROUP	AVERAGE WEIGHT/PUP/LITTER (G) DAY 0	DAY 3	CHANGE DAY 3 - DAY 0
Vehicle			
Corn Oil	1.5 ± 0.19[a]	2.0 ± 0.38	+0.4 ± 0.24
n[b]	31	30	30
Triorthocresyl Phosphate			
515 mg/kg	1.3 ± 0.15***	1.7 ± 0.31*	+0.4 ± 0.24
n	9	9	9
4,4'-Thiobis (6-t-Butyl-m-Cresol)			
485 mg/kg	1.4 ± 0.21	1.9 ± 0.27	+0.4 ± 0.32
n	12	10	10
13-Cis-Retinoic Acid			
85 mg/kg	1.5 ± 0.15	2.2 ± 0.25***	+0.7 ± 0.22***
n	26	26	24

a - Results were analyzed by the Mann-Whitney U-test with * denoting $p \leq 0.05$ and *** denoting $p \leq 0.001$.

b - n = number of litters with live pups

APPENDIX 1

Range-Finding Dose Sheet (RFDS)

Phase I

Number of mice dead at:			Then Phase 2
mg/kg/day 10	100	1000	Dose Ranges
-	-	0	1
-	-	1	2
-	-	2	3
-	0	3	4
-	1	3	5
-	2	3	6
0	3	3	7
1	3	3	8
2	3	3	9
3	3	3	10

Phase II

Dose Range No.	Phase II Doses (mg/kg/day)				
1	600	1200	2400	4800	9600
2	200	380	720	1370	2605
3	95	180	340	645	1225
4	45	85	160	305	575
5	20	40	75	140	270
6	10	19	35	67	127
7	5	9	17	31	60
8	2	4	8	15	28
9	1.0	1.9	3.7	6.9	13.2
10	0.5	0.9	1.7	3.3	6.2

APPENDIX 2

DOSING SOLUTIONS

1. Reagents

The test chemicals used in the preparation of the dosing solutions were obtained from Radian Corporation, Austin, Texas. All chemicals were the purest grade commercially available.

2. Vehicle

The vehicle used in the preparation of the dosing solutions was Mazola Corn Oil, Corn Products Company, Englewood Cliffs, New Jersey. The corn oil was analyzed for peroxides by the official method of A.O.C.S. (1972).

3. The dosing solutions were prepared in the Analytical Lab at EHRT, by the same chemist who analyzed them. The amount of chemical to be weighed for the preparation of each dosing solution was calculated by the Lab Manager. The chemicals were weighed by the chemist. The dosing solutions were shaken for 20 minutes on the automatic shaker to ensure complete solubility or homogenous distribution of the compound in the vehicle. Each chemical was color coded and handed to the toxicology department in an amber-colored vial. A specific color tape was attached on the vial. It was labelled with the date of preparation and with a letter

referring to a certain concentration (A = lowest concentration, B = next higher concentration, etc.). For Phase III, weighing of the chemicals was witnessed by a Q.A. officer or by another chemist.

4. Storage

The dosing solutions were usually prepared the day before the beginning of the study Phase. They were stored in a refrigerator (at 4°C) for the entire length of the study Phase. The dosing solutions were vortexed just prior to gavage.

CHEMICAL ANALYSIS

Summary:

Triorthocresyl Phosphate, 4,4'-thiobis (6-t-butyl-m-cresol) and 13-cis-retinoic acid were analyzed by High Pressure Liquid Chromatography (HPLC).

Solubility and Extraction:

Triorthocresyl Phosphate and 4,4'-thiobis (6-t-butyl-m-cresol) were soluble in corn oil. They were extracted from corn oil using acetonitrile. The extraction was done on 5 mL of the corn oil solution and repeated 5 times. The sample was diluted with acetonitrile and injected on the HPLC.

13-Cis-retinoic acid could not be extracted from corn oil. Because of its high sensitivity to light, it decomposed during the extraction procedures. The dosing solutions in corn oil were prepared daily in the dark and stored in amber colored vials. The samples were diluted 1:100 in hexane. The standards used in the analysis of the dosing solutions were spiked with an equivalent amount of corn oil and diluted to volume with hexane.

HPLC

COMPOUND	TRIORTHOCRESYL PHOSPHATE	4',4'-THIOBIS (6-T-BUTYL-M-CRESOL)	13-CIS-RETINOIC ACID
COLUMN	NOVA PAK C_{18}, 5 um	NOVA PAK C_{18}, 5um	NOVA PAK C_{18}, 5 um
DETECTOR	UV	UV	UV
WAVELENGTH	258 nm	279 nm	351 um
PHOTOMETRIC ATTENUATION	0.01 AUFS	0.01 AUFS	0.01 AUFS
SAMPLE SIZE	20 uL	20 uL	10 uL
FLOW RATE	1.0 mL/minute	1.0 mL/minute	1.0 mL/minute
MOBILE SOLVENT	80% ACETONITRILE/ 20% WATER	80% ACETONITRILE/ 20% WATER	70% ACEONITRILE/ 30%(1% AMMONIUM ACETATE)

RESULT OF ANALYSIS - PHASE I

Triorthocresyl phosphate and 4,4'-thiobis (6-t-butyl-m-cresol) were extracted and the percentage found to be as follows:

Dose Level	TRIORTHOCRESYL PHOSPHATE Percent Recovery	TRIORTHOCRESYL PHOSPHATE Analyzed Dose mg/kg	4,4'-THIOBIS (6-T-BUTYL-M-CRESOL) Percent Recovery	4,4'-THIOBIS (6-T-BUTYL-M-CRESOL) Analyzed Dose mg/kg
(L) 10 mg/kg	99.4%	9.9	104.9%	10.5
(M) 100 mg/kg	71.8%	71.8	80.3%	80.3
(M) 1000 mg/kg	83.5%	835.0	90.4%	904.0

Corn Oil Analysis:

Corn Oil was analyzed for peroxide - peroxide value = 3.52 meq/kg

RESULT OF ANALYSIS - PHASE II

Triorthocresyl phosphate and 4,4'-thiobis (6-t-butyl-m-cresol) were extracted and the percent recoveries found to be as follows:

Dose Level	TRIORTHOCRESYL PHOSPHATE Percent Recovery	TRIORTHOCRESYL PHOSPHATE Analyzed Dose mg/kg
45 mg/kg	108.1%	48.6
85 mg/kg	83.4%	70.9
160 mg/kg	82.2%	131.5
305 mg/kg	100.3%	305.9
575 mg/kg	64.8%	372.6

Dose* Level	4,4'-THIOBIS (6-T-BUTYL-M-CRESOL) Percent Recovery	4,4'-THIOBIS (6-T-BUTYL-M-CRESOL) Analyzed Dose mg/kg
100 mg/kg	106%	106.0
310 mg/kg	100%	310.0
400 mg/kg	99%	396.0
500 mg/kg	102%	510.0
630 mg/kg	93%	585.9

*Based on Repeated Study

Corn Oil Analysis: Peroxide Value: 1.76 meq/kg

RESULT OF ANALYSIS - PHASE II

13-CIS-RETINOIC ACID

Date	Dose Level mg/kg	Percent Recovery	Analyzed Dose Level mg/kg
9-2-85	5.0	87.1%	4.4
	10.0	97.2%	9.7
	20.0	109.7%	21.9
	40.0	84.3%	33.7
	80.0	88.9%	71.1
9-3-85	5.0	69.5%	3.5
	10.0	77.0%	7.7
	20.0	65.9%	13.2
	40.0	55.9%	22.4
	80.0	41.2%	33.0
9-4-85	5.0	199.0%	10.0
	10.0	100.0%	10.0
	20.0	114.0%	22.8
	40.0	85.6%	34.2
	80.0	70.4%	56.3
9-5-85	5.0	125.0%	6.3
	10.0	153.0%	15.3
	20.0	118.0%	23.6
	40.0	102.0%	40.8
	80.0	72.3%	57.8
9-6-85	Area of standard came very low so all percent recovery came very high.		
9-7-85	5.0	88.2%	4.4
	10.0	98.1%	9.8
	20.0	75.0%	15.0
	40.0	79.6%	31.8
	80.0	22.1%	17.7
9-8-85	5.0	154.0%	7.7
	10.0	108.0%	10.8
	20.0	32.3%	6.5
	40.0	99.2%	39.7
	80.0	104.0%	83.2

13-CIS-RETINOIC ACID CONTINUED

Date	Dose Level mg/kg	Percent Recovery	Analyzed Dose Level mg/kg
9-9-85	5.0	116.0%	5.8
	10.0	86.3%	8.6
	20.0	70.3%	14.1
	40.0	103.0%	41.2
	80.0	79.3%	63.4
9-10-85	5.0	75.0%	3.8
	10.0	90.6%	9.1
	20.0	74.6%	14.9
	40.0	63.0%	25.2
	80.0	58.9%	47.1
9-11-85	5.0	106.0%	5.3
	10.0	79.3%	7.9
	20.0	101.0%	20.2
	40.0	79.0%	31.6
	80.0	82.6%	66.1

RESULT OF ANALYSIS - PHASE III

Triorthocresyl phosphate and 4,4'-Thiobis (6-t-butyl-m-cresol) were extracted on the day prepared, 6 days after preparation and 13 days after preparation and the results are presented below.

	TRIORTHOCRESYL PHOSPHATE (515 mg/kg)		4,4'-THIOBIS (6-T-BUTYL-M-CRESOL) (485 mg/kg)	
Date	Precent Recovery	Analyzed Dose mg/kg	Percent Recovery	Analyzed Dose mg/kg
01-10-86	106.2%	546.9	96.2%	466.6
01-15-86	91.8%	472.8	76.8%	372.5
01-22-86	92.9%	478.4	86.3%	418.6

13-CIS-RETINOIC ACID
(85 mg/kg)

Date	Percent Recovery	Analyzed Dose Level mg/kg
01-13-86	102.2%	86.9
01-14-86	59.0%	50.2
01-22-86	77.4%	65.8

Corn Oil Analysis: Peroxide Value: 1.79 meq/kg