NTP Studies of Tris(Chloropropyl)phosphate [TCPP]: Experimental Design and Materials and Methods in the Three-month and Two-year Feed Studies

Three-month Studies	Two-year Studies
Study Laboratory	
Battelle (Columbus, Ohio)	Same as 3-month studies
Strain and Species	
Rats: Hsd:Sprague Dawley® SD®	Rats: Same as 3-month studies
Mice: B6C3F1/N	Mice: Same as 3-month studies
Animal Source	
Rats: Envigo (formerly Harlan Laboratories, Inc., Indianapolis, IN)	Same as 3-month studies
Mice: Taconic Biosciences, Inc. (Germantown, NY)	
Time Held Before Studies	
F_0 female rats: 5 days	F_0 female rats: 5 days
Mice: 15 (females) or 16 (males) days	Mice: 12 (females) or 13 (males) days
Average Age When Studies Began	
F_0 female rats: 11 to 12 weeks	F_0 female rats: 11 to 15 weeks
Mice: 6 to 7 weeks	Mice: 5 to 6 (females) or 6 (males) weeks
Date of First Exposure	
F_0 female rats: August 7, 2009	F_0 female rats: October 28, 2011
F_1 rats: September 13 (males) or 14 (females), 2009	F_1 rats: December 12, 2011
Mice: September 10 (females) or 11 (males), 2009	Mice: October 24 (females) or 25 (males), 2011
Duration of Exposure	
F_0 female rats: GD 6 through LD 21	F_0 female rats: GD 6 through LD 28
F_1 rats: Perinatal plus 3 months	F_1 rats: Perinatal plus 2 years
Mice: 3 months	Mice: 2 years
Date of Last Exposure	
F_0 female rats: September 13, 2009	F_0 female rats: December 12, 2011
F_1 rats: December 16 (males) or 17 (females), 2009	F_1 rats: December 12 (males) or 17 (females), 2013
Mice: December 14 (females) or 15 (males), 2009	Mice: October 24 (females) or 29 (males), 2013
Necropsy Dates	
F_1 rats: December 16 (males) or 17 (females), 2009	F_1 rats: December 9-12 (males) or 12-17 (females), 2013
Mice: December 14 (females) or 15 (males), 2009	Mice: October 21-24 (females) or 24-25, 28-29 (males), 2013
Average Age at Necropsy	
F_1 rats: 17 weeks	F_1 rats: 109 (males) or 109 to 110 (females) weeks
Mice: 19 to 20 (females) or 20 (males) weeks	Mice: 109 to 111 (females) or 110 to 111 (males) weeks

Three-month Studies	Two-year Studies
Size of Study Groups	
F_0 female rats: 20 (0, 2,500, 10,000, 40,000 ppm) or 8 (5,000, 20,000 ppm)	F_0 female rats: 38
F_1 rats: 10/sex (0, 2,500, 5,000, 10,000, 20,000 ppm)	F_1 rats (2-year study): 50/sex F_1 rats (internal dosimetry): 10/sex
Mice: 10/sex	Mice (2-year study): 50/sex Mice (internal dosimetry): 20/sex
Method of Distribution	
Animals were distributed randomly into groups of approximately equal initial mean body weights	Same as 3-month studies
Animals per Cage	
F_0 female rats: 1 (with litter)	F_0 female rats: 1 (with litter)
F_1 rats: 5 (males) or 5 (females)	F_1 rats: 2 (males) or up to 4 (females)
Mice: 1 (males) or up to 5 (females)	Mice: 1 (male) or up to 4 (females)
Method of Animal Identification	
F_0 female rats: Cage card and tail marking with permanent pen	Rats: Same as 3-month studies
F_1 rats (pups): Limb tattoo	
F_1 rats (3-month): Cage card and tail tattoo	
Mice: Tail tattoo	Mice: Cage card and tail tattoo
Diet	
Rats: Irradiated NIH-07 meal feed (perinatal phase) or irradiated NTP-2000 meal feed (postweaning) (Zeigler Brothers, Inc, Gardners, PA), available ad libitum either untreated or supplemented with tris(2-chloroisopropyl) phosphate, changed twice weekly	Rats: Same as 3-month studies
Mice: Irradiated NTP-2000 meal feed (Zeigler Brothers, Inc., Gardners, PA), available ad libitum either untreated or supplemented with TCPP, changed twice weekly	Mice: Same as 3-month studies
Water	
Tap water (Columbus municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available ad libitum	Same as 3-month studies
Cages	
Rats: Solid polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly (dams and litters through PND 4) or twice weekly (group housed rats), rotated every 2 weeks following the perinatal phase	Same as 3-months studies
Mice: Solid polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly (males) or twice weekly (females), rotated every 2 weeks	Same as 3-month studies

Three-month Studies	Two-year Studies
Bedding	
Irradiated Sani-Chips® (P.J. Murphy Forest Products Corporation, Montville, NJ), changed with cage changes	Same as 3-month studies
Rack Filters	
Spun-bonded polyester (Snow Filtration Company, Cincinnati, OH), changed every 2 weeks	Same as 3-month studies
Racks	
Stainless steel (Lab Products, Inc., Seaford, DE), changed and rotated every 2 weeks	Same as 3-month studies
Animal Room Environment	
Temperature: 72°F ± 3°F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Same as 3-month studies
Exposure Concentrations	
Rats: 0, 2,500, 5,000, 10,000, 20,000, or 40,000 (F_0 females only) ppm in feed	Rats: 0, 2,500, 5,000, 10,000, or 20,000 ppm in feed
Mice: 0, 1,250, 2,500, 5,000, 10,000, or 20,000 ppm in feed	Mice: 0, 1,250 (males only), 2,500, 5,000, or 10,000 (females only) ppm in feed
Type and Frequency of Observation	
F_0 female rats: Observed twice daily. Weighed on GDs 5, 6, 9, 12, 15, 18, and 21 and on LDs 1, 4, 7, 14, and 21. Feed consumption was measured continuously from GD 6 to LD 21. Parturition checks recorded from GD 21 through GD 25.	F_0 female rats: Observed twice daily. Weighed on arrival, GDs 5, 6, 9, 12, 15, 18, and 21 and on LDs 1, 4, 7, 10, 14, 17, 21, 24, and 28. Feed consumption was measured on GDs 6, 9, 12, 15, 18, and 21 and on LDs 1, 4, 7, 10, 14, 17, 21, 24, and 28. Parturition checks recorded from GD 21 through GD 25.
F_1 rats: Observed twice daily. Litter data (litter count by sex, litter weights by sex, and litter observations) were recorded on PND 1. Daily litter counts were performed throughout the lactation period. Pups were weighed on PNDs 4, 7, 14, and 21, on study day 1, weekly thereafter, and at study termination. Clinical observations were recorded on study day 1, weekly thereafter, and at study termination. Feed consumption was recorded weekly.	F_1 rats: Observed twice daily. Litter data (litter count by sex, litter weights by sex, and litter observations) were recorded on PND 1. Pups per litter were recorded on PNDs 2 and 3. Pups were weighed on PNDs 4, 7, 10, 14, 17, 21, 24, and 28, on study day 1, weekly for the first 13 weeks, every 4 weeks thereafter, and at study termination. Clinical observations were recorded every 4 weeks and at study termination. Feed consumption was recorded weekly.
Mice: Observed twice daily. Weighed initially, weekly thereafter, and at study termination. Clinical observations were recorded initially, weekly thereafter, and at study termination. Feed consumption was recorded weekly.	Mice: Observed twice daily. Weighed initially, weekly for the first 13 weeks, every 4 weeks thereafter, and at study termination. Clinical observations were recorded every 4 weeks and at study termination. Feed consumption was recorded weekly for 13 weeks, and every 4 weeks thereafter.
Method of Euthanasia	
Carbon dioxide	Same as 3-month studies
Necropsy	
Necropsies were performed on all F_1 rats and all mice.	Same as 3-month studies

Three-month Studies	Two-year Studies
Clinical Pathology	
F_1 rats: At the end of the study, blood was collected from the retroorbital site for hematology, clinical chemistry, and erythrocyte micronuclei determinations.	None
Hematology: red blood cell count, mean corpuscular volume, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, differential white blood cell count, reticulocyte count, and platelet count. Additionally, a qualitative evaluation of morphological features in red blood cells and platelets and a manual hematocrit were performed.	
Clinical Chemistry: alanine aminotransferase, sorbitol dehydrogenase, alkaline phosphatase, total bile acids, creatinine, urea nitrogen, glucose, albumin, total protein, creatine kinase, cholesterol, and triglycerides.	
Mice: At the end of the study, blood was collected from the retroorbital site for hematology and erythrocyte micronuclei determinations.	None
Hematology: red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, white blood cell count, differential white blood cell count, and platelet count. Additionally, a qualitative evaluation of morphological features in red blood cells and platelets and a manual hematocrit were performed.	
Histopathology	
F_1 rats: Complete histopathology was performed on all control, 10,000, and 20,000 ppm rats, and those with gross lesions. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal glands, brain (frontal cortex and basal ganglia, parietal cortex and thalamus, and cerebellum and pons), clitoral glands, esophagus, eyes, femur (including diaphysis with marrow cavity and epiphysis [femoral condyle with epiphyseal cartilage plate, articular cartilage and articular surface]), Harderian gland, heart and aorta, large intestine (cecum, colon, and rectum), small intestine (duodenum, jejunum, and ileum), kidneys, liver (two sections including left lateral lobe and median lobe), lungs and mainstem bronchi, lymph nodes (mandibular and mesenteric), mammary gland and adjacent (inguinal) skin, nasal cavity with tubinates (3 sections), ovaries, pancreas, parathyroid glands, pituitary gland, preputial glands, prostate, salivary glands, seminal vesicles, spleen, stomach (forestomach and glandular), testis with epididymides, thymus, thyroid gland, trachea, urinary bladder, and uterus. Liver and mandibular lymph nodes were also examined in female 10,000 ppm rats. The liver,	F_1 rats: Complete histopathology was performed on all control, 10,000, and 20,000 ppm rats. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal glands, brain (olfactory bulbs, fronto-parietal cortex and basal ganglia, mid-parietal cortex and thalamus, mid-brain with substantia nigra and red nucleus, posterior colliculi, mid-cerebellum including cranial nerve VIII, and posterior medulla), clitoral glands, esophagus, eyes, femur (including diaphysis with marrow cavity and epiphysis [femoral condyle with epiphyseal cartilage plate, articular cartilage and articular surface]), Harderian gland, heart and aorta, large intestine (cecum, colon, and rectum), small intestine (duodenum, jejunum, and ileum), kidneys, liver (two sections including left lateral lobe and median lobe), lungs and mainstem bronchi, lymph nodes (mandibular and mesenteric), mammary gland with adjacent (inguinal) skin, nasal cavity and nasal turbinates (3 sections), ovaries, pancreas, parathyroid glands, pituitary gland, preputial glands, prostate, salivary glands, seminal vesicles, spleen, stomach (forestomach and glandular), testis with epididymides,

Three-month Studies	Two-year Studies
bone marrow, thymus, spleen, and lymph nodes were identified as target organs and examined to a no-effect-level.	thymus, thyroid gland, trachea, urinary bladder, uterus, and vagina with cervix. If neurological signs were present, the spinal cord and nerves (sciatic, tibial, and trigeminal with ganglion) were examined. If neuromuscular signs were present, the thigh muscle was examined.
Mice: Complete histopathology was performed on all control group and 20,000 ppm mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal glands, brain (frontal cortex and basal ganglia, parietal cortex and thalamus, and cerebellum and pons), clitoral glands, esophagus, eyes, femur (including diaphysis with marrow cavity and epiphysis [femoral condyle with epiphyseal cartilage plate, articular cartilage and articular surface]), gallbladder, Harderian gland, heart and aorta, large intestine (cecum, colon, and rectum), small intestine (duodenum, jejunum, and ileum), kidneys, liver (two sections including left lateral lobe and median lobe), lungs and mainstem bronchi, lymph nodes (mandibular and mesenteric), mammary gland and adjacent (inguinal) skin, nasal cavity with turbinates (3 sections), ovaries, pancreas, parathyroid glands, pituitary gland, preputial glands, prostate, salivary glands, seminal vesicles, spleen, stomach (forestomach and glandular), testis with epididymides, thymus, thyroid gland, trachea, urinary bladder, and uterus. Liver was identified as a target organ and examined to a no-effect-level.	Mice: Complete histopathology was performed on all core mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal glands, brain (olfactory bulbs, fronto-parietal cortex and basal ganglia, mid-parietal cortex and thalamus, mid-brain with substantia nigra and red nucleus, posterior colliculi, mid-cerebellum including cranial nerve VIII, and posterior medulla), clitoral glands, esophagus, eyes, femur (including diaphysis with marrow cavity and epiphysis [femoral condyle with epiphyseal cartilage plate, articular cartilage and articular surface]), gallbladder, Harderian gland, heart and aorta, large intestine (cecum, colon, and rectum), small intestine (duodenum, jejunum, and ileum), kidneys, liver (two sections including left lateral lobe and median lobe), lungs and mainstem bronchi, lymph nodes (mandibular and mesenteric), mammary gland with adjacent (inguinal) skin, nasal cavity and nasal turbinates (3 sections), ovaries, pancreas, parathyroid glands, pituitary gland, preputial glands, prostate, salivary glands, seminal vesicles, spleen, stomach (forestomach and glandular), testis with epididymides, thymus, thyroid gland, trachea, urinary bladder, and uterus.
Sperm Motility and Vaginal Cytology	
At the end of the studies, sperm samples were collected from F_1 male rats in the control, 2,500 5,000, and 10,000 ppm groups and from male mice in the vehicle control, 5,000, 10,000, and 20,000 ppm groups for sperm count and motility evaluations. The following parameters were evaluated: epididymal spermatozoal motility and concentration. The left cauda, left epididymis, and left testis were weighed. Vaginal samples were collected for up to 16 consecutive days before the end of the studies from F_1 female rats in the vehicle control, 5,000, 10,000, and 20,000 ppm groups and from female mice in the vehicle control, 5,000, 10,000, and 20,000 ppm groups for vaginal cytology evaluations. Due to poor quality of samples, an assessment of estrous cyclicity could not be made for female rats or mice.	None
Internal Dose Assessment	

Three-month Studies	Two-year Studies
Rats: Maternal plasma, amniotic fluid, and fetal (pooled by litter) tris(2-chloroisopropyl) phosphate concentrations were measured at GD 18; maternal plasma and whole pup concentrations were measured at PND 4 from the 0, 2,500, and 10,000 ppm groups. The samples were not collected following a protocol to stabilize the parent in matrix or to minimize the background contamination which led to highly variable results; hence, these data are not reported here.	Rats: Maternal and pup plasma tris(2-chloroisopropyl) phosphate concentrations were measured at PND 28 using a validated analytical method. Concentrations were also measured in F_1 male and female rats at 3, 6, 12, and 18 months.
Mice: None	Mice: Plasma tris(2-chloroisopropyl) phosphate concentrations were measured at 3, 6, 12, and 18 months using a validated analytical method.

GD = gestation day; LD – lactation day; PND = postnatal day