UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF PREVENTION PESTICIDES AND TOXIC SUBSTANCES

Date: August 18, 2005

TXR: 0051861

MEMORANDUM

SUBJECT: **COUMAPHOS: Data Evaluation Record of a Developmental Neurotoxicity Study**

PC Code: 036501 Reregistration Case #: 0018
DP Barcode: D289778

FROM: Jess Rowland, Chief
Science Information Management Branch
Health Effects Division (7509C)

TO: Craig Doty
Chemical Review Manager
Special Review and Reregistration Division (7508C)

THRU: Brenda May, Branch Senior Scientist
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Health Effects Division (7509C)

I. Conclusions

Attached is the Data Evaluation Record for Developmental Neurotoxicity (DNT) Study with Coumaphos (MRID No. 45912101). This study is classified Acceptable and may be used for regulatory purposes. It, however, does not satisfy the guideline requirement for a developmental neurotoxicity study in rats (OPPTS 870.6300, §83-6); OECD 426 (draft) at this time pending a comprehensive review of all available positive control data. This classification scheme is applicable only to the Developmental Neurotoxicity studies as determined by DNT Work Group.

II. Action Requested

Review/prepare a Data Evaluation Record for Developmental Neurotoxicity Study with Coumaphos. (MRID No. 45912101).

DATA EVALUATION RECORD

COUMAPHOS

STUDY TYPE: DEVELOPMENTAL NEUROTOXICITY STUDY - RAT; OPPTS 870.6300

MRID 45912101

Prepared for

Health Effects Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
1921 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Toxicology and Hazard Assessment Group
Life Sciences Division
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Task No. 19-2004

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Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

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Template version 11/01

DATA EVALUATION RECORD

TXR#: 0051861

STUDY TYPE: Developmental Neurotoxicity Study - Rat
[OPPTS 870.6300 (§83-6)] OECD 426

PC CODE: 036501

DP BARCODE: D289778
SUBMISSION NO.: S634261

TEST MATERIAL (PURITY): Technical Grade Coumaphos (94.5-94.7%)

SYNONYMS: O-(3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl)O,O-diethyl phosphorothioate

CITATION: Sheets, L. P. (2003) A Developmental neurotoxicity screening study with technical grade Coumaphos in Wistar rats. Bayer Crop Science LP, 17745 South Metcalf Ave., Stilwell, Kansas, 66085-9104. Laboratory report number 200501; April 14, 2003. MRID 45912101. Unpublished

SPONSOR: Bayer Health Care, LLC, Animal Health Division, 12707 Shawnee Mission Parkway, Shawnee, KS 66216-1846.

EXECUTIVE SUMMARY: In a developmental neurotoxicity study (MRID 45912101), Coumaphos (94.5-94.7% a.i., batch # 795-010-112) was administered to 30 parent female Wistar rats/dose in the diet at concentrations of 0, 1.0, 5.0 or 30 ppm from gestation day 0 through postnatal day 21. The average daily intake of Coumophos was 0, 0.09, 0.47, and 2.77 mg/kg/day during gestation and 0, 0.22, 1.06, and 7.40 mg/kg/day during lactation, for the 0, 1.0, 5.0, and 30 ppm groups, respectively. A Functional Operational Battery (FOB) was performed on 30 dams/dose on gestation days 6 and 20, and on 10 dams/dose on lactation days 11 and 21. On postnatal day 4, litter sizes were reduced to yield four males and four females (as closely as possible). Offspring representing at least 20 litters/dose were allocated for detailed clinical observations (abbreviated FOB), assessment of motor activity, assessment of auditory startle response habituation, assessment of learning and memory, opthalmology, and neuropathology at study termination (day 75 of age). On postnatal day 21, the whole brain was collected from 10 pups/sex/dietary level for micropathologic examination and morphometric analysis. [These 21 day old pups were flushed with sodium nitrite followed by fixation with 1% gluteraldehyde and 4% paraformaldehyde.] The remaining pups in this set were sacrificed on postnatal day 21 for

measurement of cholinesterase activity. Brain, erythrocyte, and plasma cholinesterase activities were measured in offspring (20/dose group) on days 4 and 21 and in dams (10/dose group) on postnatal day 21. Pup physical development was assessed by body weight, and sexual maturation of females was assessed by age at vaginal opening. Maturation of males was assessed by age at completion of balano-preputial separation.

In dams during gestation and lactation, no treatment-related effects on mortality, clinical signs, body weight, body weight gain, food consumption, or FOB parameters were noted.Cholinesterase activity was inhibited ($p \leq 0.05$) in maternal animals at 5.0 and 30 ppm. Inhibitions were 21% and 78% at 5.0 ppm, for plasma and erythrocyte cholinesterase activities, respectively, and 68%, 85%, and 36% at 30 ppm, for plasma, erythrocyte and brain cholinesterase activities, respectively.

For maternal systemic toxicity, the NOAEL is 30 ppm (2.77 mg/kg/day), the highest dose tested. A LOAEL was not established.

For maternal cholinesterase inhibition the NOAEL is 1.0 ppm (0.09 mg/kg/day). The LOAEL is 5.0 ppm (0.47 mg/kg/day) based on inhibition of plasma and erythrocyte cholinesterase activities.

In offspring, treatment had no adverse effects on survival, clinical signs, body weight, birth weight, body weight or body weight gain pre- or post-weaning, food consumption, developmental landmarks, pupil constriction, FOB parameters, motor/locomotor, auditory startle response, passive avoidance/latency, learning and memory/latency tests or ophthamology. At necropsy, there were no treatment-related gross or micropathological effects and no effect on absolute or relative brain weight. Brain morphometry revealed statistically significant decreases in the parietal cortex thickness (7%) and cerebellum height (4%) in male offspring at the highest dose (30 ppm) on PND 21 No other treatment-related changes were seen in either sex at any time period at any dose. Cholinesterase activity was not affected at any dose level in offspring on PND 4. For the day 21 offspring, all three cholinesterase activities were affected in high-dose animals. Only plasma cholinesterase activity was inhibited significantly ($p \leq 0.05$, 30% inhibition) in high-dose males; whereas, plasma, erythrocyte, and brain cholinesterase activities were inhibited ($p \leq 0.05$) 27%, 33%, and 8%, respectively, in high-dose females.

For offspring systemic toxicity, the NOAEL is 5.0 ppm (0.47 mg/kg/day). The LOAEL is 30 ppm (2.77 mg/kg/day) based on morphometric changes in the brain of PND21 males.

For offspring cholinesterase inhibition, the NOAEL is 5.0 ppm (0.47 mg/kg/day). The LOAEL is 30 ppm (2.77 mg/kg/day) based on inhibition of plasma, erythrocyte and brain cholinesterase activities

This study is classified **Acceptable** and may be used for regulatory purposes, however it does not satisfy the guideline requirement for a developmental neurotoxicity study in rats (OPPTS 870.6300, §83-6); OECD 426 (draft) at this time pending a comprehensive review of all available positive control data.

COMPLIANCE: Signed and dated Flagging, GLP, Quality Assurance, and Data Confidentiality statements were provided.

7

I. MATERIALS AND METHODS:

A. MATERIALS:

1. **Test material:** Technical grade Coumaphos

Description:	Cream-colored, slightly yellowish to light grey powder or lumps
Lot/Batch #:	795-010-112
Purity:	94.5-94.7 % a.i.
Compound Stability:	Confirmed for 32 days, frozen
CAS # of TGAI:	56-72-4

2. **Vehicle and/or positive control:** acetone solvent in the diet

3. **Test animals (P):**

Species:	Rat
Strain:	Wistar Hannover Crl:WI(Glx/BRL/Han) IGS BR
Age at study initiation:	females: at least 12 wks; males: at least 15 weeks (breeders only)
Wt. at study initiation:	164.0-229.0 g
Source:	Charles River Laboratories, Raleigh, NC
Housing:	Individually or with litter in stainless steel grid or plastic cages
Diet:	Purina Mills Rodent Lab Chow 5002, *ad libitum*
Water:	Tap water, *ad libitum*
Environmental conditions:	**Temperature:** 19-25°C **Humidity:** 30-70% **Air changes:** 10-15/hour **Photoperiod:** 12 hrs dark/12 hrs light
Acclimation period:	At least 6 days

B. PROCEDURES AND STUDY DESIGN:

1. **In life dates**: Start: August 20, 2001; End: November 27, 2001

2. **Study schedule:** The maternal animals were mated and assigned to study. The test substance was administered to the maternal animals (30/dose group) from gestation day 0 through lactation day 21. Pups were weaned onto control diets on postnatal day 21, after which time maternal animals were killed. F_1 pups remained on study up to postnatal days 70-80.

3. **Mating procedure:** Females were paired 1:1 with males of the same strain and source. Each female was examined daily during the mating period to identify sperm cells in a vaginal smear or the presence of a copulatory plug. The day that sperm or a plug was found was designated gestation day 0. After successful mating, each pregnant female was placed into an individual cage with a solid bottom and bedding, where the dam was maintained through gestation and lactation.

4. **Animal assignment:** Mated females and offspring were allocated as shown in Table 1 using an animal allocation program written in SAS. For offspring, four sets of animals (designated sets A-D) were utilized for assessment at each age. Randomly-selected pups (10/sex/dose) were designated as Set D and were perfused with fixative and brains were collected for histopathological examination and morphometric analysis.

   Sixteen pups/sex/group were allocated on postnatal day 4 to each of the following: motor activity, acoustic startle habituation, passive avoidance, water maze, detailed observational battery, and sacrifice and brain examination on postnatal day 21. At approximately 50-60 days of age, a minimum of 10 offspring/sex/dose level were given an opthalmoscopic examination. On day 70-80, animals were sacrificed by perfusion and brain weights recorded.

   Brain, erythrocyte, and plasma cholinesterase activities were measured in offspring on postnatal days 4 (extra pups from litter reduction) and 21 (Set D) and in dams on lactation day 21. On postnatal day 4, samples were collected from approximately 20 pups/dietary level that were randomly selected for litter size reduction; samples from male and female pups within a litter were pooled to provide adequate samples for blood measures. On day 21, samples were collected from 10 dams/dose group and 5-10 pups/dose group from set D.

TABLE 1. Study design

Experimental parameter	Dose (ppm in diet)			
	0	1.0	5.0	30
	Dose (mg/kg/day) during gestation			
	0	0.09	0.47	2.77
	Dose (mg/kg/day) during lactation			
	0	0.22	1.06	7.40
Maternal animals				
	No. of maternal animals assigned			
FOB (GD 6.20)	30	30	30	30
FOB (LD 11, 21)	10	10	10	10
Erythrocyte, Plasma, and Brain Cholinesterase Activity (LD 21)	10	10	10	10
Offspring				
Set A — Motor activity (PND 13, 17, 21, 58-62)	16/sex	16/sex	16/sex	16/sex
Set B — Acoustic startle habituation (PND 22, 36-40, 58-62)	16/sex	16/sex	16/sex	16/sex
Set C — Passive avoidance (PND 22, 29)	16/sex	16/sex	16/sex	16/sex
Set C — Detailed clinical/FOB (PND 4, 11, 21, 35, 45, 60)	16/sex	16/sex	16/sex	16/sex
Set C — Water maze (PND 58-62, 7 days after first test)	16/sex	16/sex	16/sex	16/sex
Sets A-C — Opthalmologic evaluation (PND 50-60)	10/sex	10/sex	10/sex	10/sex
Sets A-C — Brain Weight (PND 70-80)	10/sex	10/sex	10/sex	10/sex
Set D — Gross necropsy and brain measurements (PND 21)*	10/sex	10/sex	10/sex	10/sex
Set D — Erythrocyte, plasma, and brain cholinesterase activity (PND 21)	10/sex	10/sex	10/sex	10/sex
Culled — Erythrocyte, plasma, and brain cholinesterase activity (PND 4)**	10	10	10	10

*Page 22 of MRID 45912101 states that approval was granted by OPPTS/OPP/HED staff to replace PND 11 with PND 21 for neuropathology for this study.

**Samples from male and female pups within a litter were pooled to provide adequate samples for blood measures.

5. **Dose selection rationale:** Dose levels were chosen based on the results from a two-generation reproduction study in Sprague-Dawley rats (Bayer Report 74460; MRID 43061701), in which Coumaphos was administered in the diet at levels of 0, 1.0, 5.0, and 25 ppm beginning 10 weeks before mating. At 1.0 ppm, parental females had a marginally decreased erythrocyte cholinesterase activity ($p \leq 0.05$, 13% decrease). At 5.0 ppm, parental females had decreased erythrocyte cholinesterase activity ($p \leq 0.05$; 70% decrease), and at 25 ppm, decreases (≤ 0.05) of 68%, 95%,and 29% were noted for plasma, erythrocyte, and brain cholinesterase activities, respectively. There were no other compound-related findings in the dams. Effects in the offspring were limited to cholinesterase activity inhibition ($p \leq 0.05$) at the 25 ppm dose level on PND 21. Decreases of 38%, 43%, and 1% (NS) were noted for male offspring, and decreases of 44%, 40%, and 8% were noted for female offspring for plasma, erythrocyte, and brain cholinesterase activities, respectively.

In a developmental toxicity study in Charles River rats (MRID# 00131684), coumaphos was administered by gavage at 0, 1, 5, or 25 mg/kg/day day 6 to 15 of gestation. Three dams showed tremors and 2 of these showed addition mild clinical signs to cholinergic-type signs at 25 mg/kg/day. No cholinesterase analysis were conducted on dams.

Based on the results of the reproduction study, the doses selected for the developmental neurotoxicity study were 0, 1.0, 5.0, and 30 ppm. The 30 ppm level was selected to produce some overt maternal toxicity in addition to cholinesterase inhibition. The 1.0 ppm level was selected to be an overall NOEL in the pups, with possible slight cholinesterase activity inhibition in the dams. The 5.0 ppm dose level was selected as an intermediate dose to assist in establishing compound-related effects.

6. **Dosage administration:** Coumaphos was administered to parent female Wistar rats in the diet at levels of 0, 1.0, 5.0 or 30 ppm from gestation day 0 through postnatal day 21. The test substance intake was 0, 0.09, 0.47, and 2.77 mg/kg/day, respectively, for analytically-determined concentrations of 0, 1.1, 5.3, and 31 ppm in the diet during gestation. The test substance intake was 0, 0.22, 1.06, and 7.40 mg/kg/day, respectively, for analytically-determined concentrations of 0, 1.1, 5.3, and 31 ppm in the diet during lactation.

7. **Dosage preparation and analysis:** Detailed descriptions of feed preparations and test diet analysis were not provided; however, information from the study report is as follows. Acetone was used as the solvent to dissolve the test article for mixing in the diet and was allowed to evaporate before the feed was given to the animals. The control diet was similarly prepared, excluding the test substance. Concentrations of the test substance in the diet were measured by liquid chromatography four times (weeks 1, 2, 3, and 6) during the in-life phase of the study. Homogeneity of Coumaphos in the diet was determined using feed mixed for week one of the study (utilizing concentrations of 1.0 and 30 ppm), and stability data were determined by analyzing 1.0 and 30 ppm diets on days 0, 7, 14, and 32 under freezer storage conditions and on days 0 and 7 at room temperature.

Results:

Homogeneity analysis: was determined from nine samples of ration from each level of the 1.0 and 30 ppm diets. The mean concentrations were 0.968 ppm (CV=4.1%) for the 1.0 ppm diet and 29.5 ppm (CV=2.3%) for the 30 ppm test diet. The coeffieients of variation of 4.1 and 2.3% suggest that the Coumaphos was adequately distributed.

Stability analysis: At nominal concentrations of 1.0 ppm and 30 ppm, Coumaphos is stable in the diet for at least 7 days at room temperature (Day 7: 1.0 ppm diet 101% of initial; 30 ppm diet 86.9% of initial) and 32 days at freezer conditions (Days 7-32: 1.0 ppm diet 103-115% of initial; 30 ppm diet 89.6-92.7% of initial).

Concentration analysis: The 1.0, 5.0, and 30 ppm dietary levels averaged 108%, 106%, and 103% of the nominal concentration, respectively.
The analytical data indicated that the concentration, stability, and homogeneity of Coumaphos in the diets was adequate.

C. OBSERVATIONS:

1. In-life observations:

a. **Maternal animals:** Once daily checks for mortality or moribundity and daily cage-side observations were conducted for maternal animals.

Thirty dams per group were observed (by observers blind to the treatment group) outside the home cage during the gestation dosing period (days 6 and 20) and at least 10 dams/group were observed during the lactation dosing period (days 11 and 21). The following functional observations were recorded.

	Functional observations–Maternal animals
X	Signs of autonomic function, including: 1) Ranking of degree of lacrimation and salivation, with range of severity scores from none to severe 2) Presence or absence of piloerection and exophthalamus, 3) Ranking or count of urination and defecation, including polyuria and diarrhea 4) Pupillary function such as constriction of the pupil in response to light, or a measure of pupil size 5) Degree of palpebral closure, e.g., ptosis.
X	Description, incidence, and severity of any convulsions, tremors, or abnormal movements.
X	Description and incidence of posture and gait abnormalities.
X	Description and incidence of any unusual or abnormal behaviors, excessive or repetitive actions (stereotypies), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance, red or crusty deposits around the eyes, nose, or mouth, and any other observations that may facilitate interpretation of the data.

Individual maternal body weight and food consumption data were recorded on gestation days 0, 6, 13, and 20, and lactation days 0, 4, 7, 14, and 21. Food consumption measurements may have included consumption by the pups, especially during lactation week 3.

From gestation day 20, dams were checked daily for evidence of parturition. They were permitted to deliver and rear offspring until postnatal day 21. Numbers of live and dead offspring were recorded during parturition.

b. **Offspring:**

1. **Litter observations:** Daily throughout lactation, offspring were examined cage-side for gross signs of mortality or morbidity.

On day 4 postpartum, litters were standardized to a maximum of 8 pups/litter (4/sex/litter, as nearly as possible); excess pups were killed and discarded. If there were more than 23 acceptable litters for any dietary level, the surplus litters were sacrificed on PND 4 after weighing without routine necropsy.

2. **Developmental landmarks:** Beginning on postnatal day 38, male offspring were examined daily for balanopreputial separation. Beginning on postnatal day 29, female offspring were examined daily for vaginal patency. The age of onset was recorded.

3. **Detailed observations:** Offspring were examined for clinical signs once daily during the preweaning period and once weekly after weaning by observers who were aware of the treatment groups. Individual offspring body weight data were recorded on postnatal days 0, 4, 11, 17, and 21 and once weekly thereafter. Individual food consumption was measured weekly from the week of postnatal day 28.

4. **Neurobehavioral evaluations:** Observations and the schedule for those observations are summarized as follows from the report.

5. **Functional observational battery (FOB):** On postnatal days 4, 11, 21, 35, 45, and 60, a total of 16 offspring/sex/group (one male or one female from each litter) was examined outside the home cage in an FOB assessment by observers blind to the treatment groups. On postnatal days 4 and 11, the animals were not evaluated in the open field, unless deemed necessary by the observer. Otherwise, methods were similar to the procedures used for the dams.

	FUNCTIONAL OBSERVATIONS- Offspring
X	Signs of autonomic function, including: 1) Ranking of degree of lacrimation and salivation, with range of severity scores from none to severe 2) Presence or absence of piloerection and exophthalamus, 3) Ranking or count of urination and defecation, including polyuria and diarrhea 4) Pupillary function such as constriction of the pupil in response to light, or a measure of pupil size 5) Degree of palpebral closure, e.g., ptosis.
X	Description, incidence, and severity of any convulsions, tremors, or abnormal movements.
X	Description and incidence of posture and gait abnormalities.
X	Description and incidence of any unusual or abnormal behaviors, excessive or repetitive actions (stereotypies), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance, red or crusty deposits around the eyes, nose, or mouth, and any other observations that may facilitate interpretation of the data.

6. **Motor activity testing:** Motor activity was evaluated in 16 rats/sex/dose on days 13, 17, 21, and 60. Animals were placed individually in figure-eight mazes and were continuously monitored over a 1-hour period. An automated activity monitoring system collected data over successive 10-minute intervals by recording infra-red light source break frequency within the maze. Motor activity was measured as the number of beam interruptions that occurred during the test session, and locomotor activity was measured by eliminating consecutive counts for a given beam. Therefore, only one interruption of a given beam was counted for locomotor activity until the rat relocated in the maze and interrupted a different beam. Habituation was evaluated as a decrease in activity over consecutive test-session intervals.

8

7. **Auditory startle habituation:** Auditory startle reflex habituation was performed on 16 offspring/sex/dose on postnatal days 22, 38 and 60, using an automated system.

Animals were acclimated for 5 minutes to background noise and were then presented with the startle stimulus at 10-second intervals. The startle stimulus consisted of 50-millisecond bursts of white noise at approximately118 dB. Peak response amplitude (g force exerted on the platform) and latency (msec) measurements were recorded for each animal's individual response curve. Response amplitude was defined as the maximum value of the average curve minus the baseline (body weight). Latency to peak was defined as the time, in msec, following onset of the stimulus when the peak response amplitude occurred.

8. **Learning and memory testing:**

PASSIVE AVOIDANCE CONDITIONING: On postnatal days 22 and 29, learning and short- and long-term retention were assessed in a passive avoidance test of 16 offspring/sex/dose. Testing was done in individual isolation cubicles each with a single shuttle cage. Each cubicle was insulated to attenuate sound and had a fan for ventilation. Each 7 x 7 inch shuttle cage was separated into two equal-sized compartments by a centrally-located sliding door. The two compartments were identical except that the walls in one compartment were lined with black film (dark side) and the walls in the other compartment were not lined and this compartment was illuminated with a high-intensity lamp. The lamp was switched on at the beginning of each trial and remained on until the rat crossed into the dark compartment or the trial ended. The cage floor was constructed of a stainless steel grid and the movement of the rat from the light to dark side was detected by a photocell. Rats were placed individually into the shuttle cage facing toward the light. After 20 seconds, the light was switched on and the door separating the compartments was opened. When the rat crossed into the dark side, the door closed, a brief, mild shock (0.5 sec, 0.5mA) was delivered, and the light was switched off. If the rat failed to cross to the dark side within 180 seconds, it was returned to the holding cage and assigned a latency time of 180 sec. The procedure was repeated until the rat either remained in the bright side for 180 seconds for two consecutive trials or until 15 trials had elapsed (whichever occurred first). Rats that failed to reach criterion performance within 15 trials or failed to cross during the first two acquistion trials were excluded from the retention phase of the experiment.

WATER MAZE: Learning and memory testing was performed in 16 offspring/sex/dose on postnatal days 60 and again seven days later using an M-water maze. Only rats that demonstrated acquisition on the first test occasion were tested for retention seven days later. The water maze was made of opaque Plexiglas with 5-inch wide corridors. The walls were 16-inches high with approximately 7.5 inches of water. The maze was filled with water at 22±1°C. For each test trial, the rat was placed at the base of the M-maze stem, between the two lateral arms. On the learning trial (first trial), the rat was required to enter both arms of the maze before being provided access to the exit ramp to escape the water and was then removed from the maze. The initial arm chosen on the learning trial was designated the incorrect goal during the subsequent trials (15 maximum). Rats failing to make a correct goal choice within 60-seconds in any given trial were led to the correct goal with the exit ramp

and then removed from the water. The inter-trial interval was approximately 15 seconds. Each rat was required to reach a criterion of 5 consecutive error-free trials to stop the test session. Latency (in seconds) to choose the correct goal or the maximum 60-second interval was recorded for each trial, as well as the number of errors (incorrect turns) during each trial.

9. **Opthalomology:**

At postnatal days 50-60, indirect opthalmoscopy was performed on 10 offspring/sex/dose (that had been selected for perfusion) following dilation with a mydriatic agent.

10. **Postmortem observations:**

Cholinesterase measurements: Blood and brain samples were collected from 10 dams/dose group on lactation day 21 and from 10 pups/sex/dose group on postnatal day 21. Blood and brain samples were also collected from 20 pups/dose group (selected from extra pups at postnatal day 4 litter size reduction), and blood samples from male and female offspring within a litter were pooled to provide adequate samples for blood measurements. Blood samples were collected from the orbital plexus of dams and offspring on postnatal day 21 and by decapitation of offspring on postnatal day 4 for determination of plasma and erythrocyte cholinesterase activities. Brain tissues were collected immediately after blood collection for the determination of brain cholinesterase activity.

a. **Maternal animals:** Maternal animals were sacrificed by carbon dioxide inhalation on postnatal day 21. Adult females were not routinely subjected to a gross necropsy. Maternal animals found moribund were sacrificed. Those found moribund or dead were subjected to a macroscopic necropsy, with possible collection of tissues at the discretion of the study director.

b. **Offspring:** The offspring selected for brain weight or neuropathological evaluation were sacrificed on postnatal day 21 or 70-80. These animals were subjected to postmortem examinations as described below.

At postnatal day 21, up to 10 pups/sex/group were sacrificed by intraperitoneal injection of pentobarbital (50 mg/kg) and perfused via the left ventricle with a sodium nitrite flush followed by fixation with 1% gluteraldehyde and 4% paraformaldehyde. The brain was collected, weighed, and post-fixed with 10% buffered formalin. Anterior to posterior cerebrum and cerebellum length were measured by an individual not blind to treatment using a Vernier caliper. Brains from all dose groups were embedded in paraffin and were sectioned for control and high-dose animals. Tissues were sectioned at 5 µm and stained with hematoxylin and eosin and luxol fast blue/cresyl violet. Eight coronal sections from control and high-dose animals were examined microscopically.

The following brain morphometric measurements were performed:

Frontal cortex thickness (dorsal portion of the cerebral cortex within the coronal section passing through the region of the optic chiasm)

Parietal cortex thickness (dorsolateral portion of the cerebral cortex within the coronal section taken through the optic chiasm)

Caudate putamen horizontal width (coronal section taken at the level of the optic chiasm)

Corpus callosum (thickness at the midline)

Hippocampal gyrus (greatest dorsal-ventral thickness)

Cerebellum (roof of the fourth ventricle to the dorsal surface)

On postnatal day 70-80, 10 animals/sex/group were euthanized by carbon dioxide asphyxiation, underwent a gross necropsy and the brains were removed and weighed (fresh weight); the animals were then discarded. Another 10 rats/sex/dose were were sacrificed by intraperitoneal injection of pentobarbital (50 mg/kg) and perfused via the left ventricle with a sodium nitrite flush followed by fixation with 1% gluteraldehyde and 4% paraformaldehyde. The brain, spinal cord, both eyes with optic nerves, peripheral nerves, gasserian ganglion, gastrocnemius muscle, and both forelimbs were collected, weighed (brain only), and post-fixed with 10% buffered formalin. Anterior to posterior cerebrum and cerebellum length were measured by an individual not blind to treatment using a Vernier caliper.

The following central and peripheral nervous system tissues were dissected and preserved in paraffin (CNS tissues) or plastic (PNS tissues): eight coronal sections of the brain, cervical, thoracic, and lumbar sections of the spinal cord, the cauda equina, eyes, optic nerves, gastrocnemius muscle, dorsal root ganglia and fibers, and gasserian ganglion. Tissues from all dose groups were embedded; however, only control and high-dose tissues were examined unless effects warranted examination of low- and mid-dose samples. Paraffin-embedded tissues were sectioned at 5 µm and stained with hematoxylin and eosin. Plastic-embedded tissues were sectioned at 2-3 µm and stained with a modified Lee's stain.

Detailed morphometric evaluation of the neocortex, hippocampus, and cerebellum was conducted as follows:

Frontal cortex thickness (dorsal portion of the cerebral cortex within the coronal section passing through the region of the optic chiasm)

Parietal cortex thickness (dorsolateral portion of the cerebral cortex within the coronal section taken through the optic chiasm)

Caudate putamen horizontal width (coronal section taken at the level of the optic chiasm)
Corpus callosum (thickness at the midline)

11

Hippocampal gyrus (greatest dorsal-ventral thickness)

Cerebellum (roof of the fourth ventricle to the dorsal surface)

D. DATA ANALYSIS:

1. **Statistical analyses:** Continuous data were initially analyzed for equality of variance using Bartlett's test. Group means with equal variances were further analyzed with ANOVA, followed by Dunnett's test if significance was identified with the ANOVA. Group means with unequal variances were analyzed by Kruskal-Wallis ANOVA followed by the Mann-Whitney U test for between-group comparisons. The level of significance was set at $p \leq 0.05$, except for Bartlett's test which was set at $p \leq 0.001$.

   Motor and locomotor activity were analyzed with ANOVA, followed by Dunnett's test if significance was attained with ANOVA. Acoustic startle peak amplitude data were analyzed by ANOVA followed by Dunnett's test if significance was observed with the ANOVA. The response amplitude data for each block of 10 trials were subjected to a Repeated-Measures ANOVA, using the test block as the repeated measure. Passive avoidance latency data were analyzed with a Wilcoxon Test for time to failure. The number of trials to criterion were analyzed with Kruskal-Wallis and Wilcoxon tests for the acquisition phase and Fisher's exact test for retention. The number of rats failing to meet the criterion level of performance in the acquisition phase was treated as incidence data. Water maze data were analyzed by a univariate ANOVA followed by Dunnett's test. The number of trials to criterion and the number of errors were analyzed with Kruskal-Wallace and Wilcoxon test for the acquisition phase and Fischer's exact test for retention. The number of rats failing to meet the criterion level of performance in the water maze learning phase was treated as incidence data. Micropathology frequency data were analyzed by Chi-Square followed by Fisher's Exact Test if significance was identified with the Chi-Square.

2. **Indices:**

   a. **Reproductive indices:** The following reproductive indices were calculated from breeding and parturition records of animals in the study:

      Mating index = (Number of inseminated females/Number of females co-housed with males) × 100

      Fertility index = (Number of pregnant females/Number of inseminated females) × 100

   b. **Offspring viability indices:** The following viability (survival) indices were calculated from lactation records of litters in the study:

      Live birth index = (Number of live offspring per litter /Total number of offspring born per litter) × 100

Viability index = (Number of live offspring at PND 4 per litter/Number of live offspring born per litter) × 100

Lactation index = (Number of live offspring on Day 21 per litter/Number of live offspring on PND 4 after litter size reduction) × 100

3. **Positive and historical control data:** Positive control/methodology validation and historical control studies (MRID Nos. 45441302, 45464602, and 45441303) are under review.

II. RESULTS:

A. PARENTAL ANIMALS:

1. **Mortality and clinical and functional observations:** No dams were found dead or were sacrificed in moribund condition during gestation or lactation. There were no treatment-related clinical signs observed during gestation or lactation. During gestation there were areas of hair loss in one or three dams at each dose-level, including the controls. The severity and incidence of hair loss was similar across all groups and is considered incidental to treatment.

2. **Body weight and food consumption:** Selected group mean body weights and food consumption values for pregnant or nursing dams are summarized in Table 2. There were no significant treatment-related effects on body weight or body weight gain during gestation or lactation.

   There were no treatment-related effects on food consumption during gestation or lactation. There was an isolated statistical ($p \leq 0.05$, 13%) increase in food consumption for high-dose dams during week 2 of lactation. This observation is considered incidental to treatment.

TABLE 2. Selected mean (±SD) maternal body weight and food consumption[a]

Observations/study interval	Dose (ppm)			
	0	1.0	5.0	30
Gestation (n= 27-29)				
Body wt. Gestation day 0 (g)	193.8±2.42	194.2±2.40	196.5±2.60	194.0±2.97
Body wt. Gestation day 6 (g)	212.7±2.59	208.9±3.57	217.2±2.84	214.8±3.09
Body wt. Gestation day 13 (g)	237.6±4.02	238.9±2.90	245.3±3.32	240.1±3.48
Body wt. Gestation day 20 (g)	298.1±4.64	297.6±4.21	304.5±4.22	298.6±5.01
Wt. gain gestation days 0-20 (g)	104.3±3.11	103.4±2.65	108.0±2.27	104.6±3.09
Food consumption gestation days 0-6 (g/day)	17.2±0.86	16.1±0.56	17.1±0.38	17.8±0.55
Food consumption gestation days 6-13 (g/day)	18.5±0.58	18.6±0.42	19.8±0.42	19.5±0.48
Food consumption gestation days 13-20 (g/day)	20.9±0.42	20.7±0.52	21.6±0.40	21.0±0.41
Lactation (n=21-29)				

TABLE 2. Selected mean (±SD) maternal body weight and food consumption[a]				
Observations/study interval	Dose (ppm)			
	0	1.0	5.0	30
Body wt. lactation day 0(g)	226.0±3.36	228.0±3.23	233.4±3.82	224.2±3.52
Body wt. lactation day 4 (g)	245.4±3.06	247.9±3.12	246.9±3.79	238.6±4.03
Body wt. lactation day 7 (g)	254.7±3.15	254.6±4.47	256.7±3.03	251.8±3.47
Body wt. lactation day 14(g)	275.1±3.79	271.1±5.37	272.0±3.68	260.7±5.11
Body wt. lactation day 21(g)	263.2±4.69	261.5±5.83	268.6±3.16	264.2±3.72
Food consumption lactation days 0-7 (g/day)	42.0±2.93	37.5±1.38	36.2±1.41	48.1±3.64
Food consumption lactation days 7-14 (g/day)	52.0±0.96	50.6±1.07	51.5±0.95	58.8*±2.04 (13%)
Food consumption lactation days 14-21 (g/day)	67.6±1.51	63.9±1.02	65.3±1.66	70.8±2.34

[a]Data obtained from Tables 3 & 4 pages 60-63 and Tables 6 & 7 pages 66-69. MRID 45912101. *p≤ 0.05. Number in parentheses is % difference compared to control, calculated by reviewer.

3. **Reproductive performance:** There were no treatment-related effects on fertility, gestation indices or gestation length. Results for the maternal animals are summarized in Table 3.

TABLE 3. Reproductive Performance [a]				
Observation	Dose (ppm)			
	0	1.0	5.0	30
Number Mated	30	30	30	30
Mating Index (%)	100.0	100.0	100.0	100.0
Fertility Index (%)	96.7	90.0	90.0	96.7

[a]Data obtained from Table 1, pages 56-57. MRID 45912101.

4. **Maternal postmortem results:** Results for the cholinesterase studies in dams are presented in Table 15 below, along with the results for the offspring. For dams, a dose-related decrease in cholinesterase activity was noted in mid- and high-dose animals on lactation day 21. Percent inhibition (*p≤0.05) compared to controls was as follows:

Plasma: 6% at 1.0 ppm, 21%* at 5.0 ppm, and 68%* at 30 ppm
Erythrocyte: 16% at 1.0 ppm, 78%* at 5.0 ppm, and 85%* at 30 ppm
Brain: 4% at 1.0 ppm, 6% at 5.0 ppm, and 36%* at 30 ppm

B. OFFSPRING:

1. **Viability and clinical signs:** Litter size and viability (survival) results from pups during lactation are summarized in Table 4. There was no treatment-related effect on the number of litters, live litter size, number of stillborn pups, live birth index, or viability index.

TABLE 4. Litter size and viability [a]				
Observation	Dose (ppm)			
	0	1.0	5.0	30
Number of Litters*	21	23	21	23
Total number born	247	257	242	257
Number born live	246	254	240	256
Number born dead	1	3	2	1
Mean No. of viable pups:				
Day 0	12	11	12	11
Day 4 [b]	12	11	11	11
Day 4 [c]	8	8	8	8
Day 21	8	8	8	8
Live birth index (%)	100	99.6	100	99.4
Viability index	98.5	97.9	98.4	99.7
Lactation index	99.4	98.9	98.8	99.5

[a] Data obtained from Table 9, pages 73-75, MRID 45912101.
[b] Before standardization (litter size reduction).
[c] After standardization (litter size reduction).
*If there were more than 23 acceptable litters for any dietary level, the surplus litters were sacrificed on PND 4 after weighing without routine necropsy.

2. **Body weight**: Body weights were comparable at birth across all dose groups, and there were no differences in body weight or body weight gain pre-weaning or post-weaning. Selected mean pre-weaning pup body weight data are presented in Table 5, and selected mean post-weaning offspring body weight data are presented in Table 6.

TABLE 5. Mean (±SD) pre-weaning pup body weights and body weight gain (g) [a]								
Postnatal Day	Dose (ppm)							
	0	1.0	5.0	30	0	1.0	5.0	30
	Males				Females			
0	5.8±0.09	5.8±0.10	6.0±0.09	5.9±0.11	5.5±0.09	5.5±0.10	5.6±0.08	5.6±0.10
4 [b]	9.2±0.23	9.5±0.24	9.5±0.26	9.5±0.27	8.9±0.21	9.1±0.23	9.2±0.25	9.2±0.27
4 [c]	9.2±0.23	9.5±0.25	9.5±0.27	9.5±0.27	9.0±0.20	9.1±0.24	9.1±0.24	9.2±0.27
11	23.4±0.48	23.8±0.58	23.7±0.61	23.7±0.52	23.1±0.38	23.0±0.57	23.1±0.60	23.3±0.50
17	36.7±0.60	36.6±0.80	35.9±0.78	36.1±0.69	35.9±0.50	35.4±0.76	34.8±0.72	35.2±0.67
21	45.9±0.78	46.1±1.08	46.2±0.93	46.3±0.81	45.3±0.71	44.4±1.09	44.7±0.91	45.2±0.79
Weight gain Days 0-4	3.4±0.16	3.6±0.17	3.5±0.22	3.6±0.19	3.4±0.14	3.6±0.17	3.5±0.20	3.7±0.19
Weight gain Days 4-11	14.2±0.36	14.3±0.43	14.2±0.40	14.2±0.33	14.1±0.26	13.8±0.42	14.0±0.44	14.1±0.31
Weight gain Days 4-17	27.5±0.53	27.1±0.69	26.4±0.61	26.7±0.54	27.0±0.45	26.3±0.64	25.6±0.59	26.0±0.52
Weight gain Days 4-21	36.8±0.68	36.7±0.94	36.7±0.71	36.9±0.64	36.3±0.62	35.3±0.95	35.5±0.75	36.0±0.62

[a] Data obtained from Tables12-13, pages 82-90, MRID 45912101.
[b] Before standardization. (n= 21, 23, 21, and 23 males and females each at each dose group).
[c] After standardization to approximately 8 pups/litter.

TABLE 6. Mean (±SD) post-weaning pup body weights (g) [a]								
Postnatal Day (M/F)	Dose (ppm)							
	0	1.0	5.0	30	0	1.0	5.0	30
	Males				Females			
28/29	74.3±12.1	77.6±8.8	73.2±10.4	76.6±7.2	74.8±10.4	75.3±8.8	74.2±7.1	76.0±6.6
35/36	116.2±13.7	120.2±12.5	117.9±10.7	120.0±10.6	109.5.±11.4	110.1±10.2	109.1±8.5	110.8±8.4
42/43	163.8±15.8	167.0±14.0	162.5±12.7	164.3±13.4	134.1±10.9	134.3±10.9	131.3±9.7	132.9±9.2
49/50	203.1±20.6	208.2±16.8	201.4±16.3	201.9±17.3	150.3±12.9	149.6±12.4	147.7±11.0	148.1±10.6
56/57	246.5±22.7	250.3±20.5	239.6±23.3	238.0±19.9	167.7±14.9	166.5±12.8	165.2±12.3	165.0±12.6
63/64	278.9±23.7	283.5±22.4	273.1±22.7	271.1±22.3	180.1±16.1	179.4±13.1	178.2±12.7	176.7±14.0
70/71	307.0±24.4	312.3±24.9	303.3±25.7	298.4±25.0	189.9±17.7	190.6±13.9	189.9±13.8	188.0±15.2

[a] Data obtained from Table15, pages 93-95, MRID 45912101.

There were no treatment-related effects on pre-weaning or post-weaning food consumption.

3. **Developmental landmarks:**

   a. **Sexual maturation:** Preputial separation in males was unaffected by treatment. The mean age for attainment of vaginal opening for females was unaffected by treatment. Although the average age of onset of vaginal patency was statistically increased ($p \leq 0.01$) in the 5 and 30 ppm groups, no dose-response relationship was present and values were reportedly well within the range of historical controls (no data. The data are presented in Table 7.

TABLE 7. Mean (±SD) age of sexual maturation (days) [a]				
Parameter	Dose (ppm)			
	0	1.0	5.0	30
N (M/F)	60/63	67/69	62/63	68/69
Preputial separation (males)	43.5±0.29	43.0±0.20	43.9±0.39	43.3±0.24
Vaginal opening (females)	33.7±0.27	34.0±0.28	35.0**±0.37	35.0**±0.28

[a] Data obtained from Table 14, pages 91-92, MRID 45912101. **$p \leq 0.05$.

   b. **Pupil constriction:** No treatment-related effects were noted. All control and treated rats exhibited pupil constriction on PND 21.

4. **Behavioral assessments:**

   a. **Functional observational battery:** There were no treatment-related effects on offspring at any dose level on any test day (PND 4, 11, 21, 35, 45, or 60).

b. **Motor/locomotor activity:** No treatment-related overall or interval motor or locomotor activity effects were noted. For motor activity in control males and females, habituation was apparent on all four test days in males and females, including PND 13, when activity levels were relatively low. For locomotor activity, habituation was achieved on all test days, except PND 13 when activity was so low during the first interval for males and females that habituation was not evident. Total activity data are presented in Tables 8 and 9.

TABLE 8. Mean (±S.D.) motor activity data (total activity counts for session) [a]

Test Day	Dose (ppm)			
	0	1.0	5.0	30
Males				
PND 13	64±65	54±48	55±51	64±68
PND 17	240±160	297±188	224±107	180±132
PND 21	334±89	392±141	316±106	311±147
PND 60	531±200	562±107	593±141	579±103
Females				
PND 13	62±54	61±50	63±48	51±43
PND 17	150±115	309±175	219±143	265±189
PND 21	326±86	329±101	361±111	320±114
PND 60	723±167	760±235	715±179	755±219

[a] Data obtained from Table 19, pages 181-183, MRID 45912101.
N = 15-16/sex/dose.

TABLE 9. Mean (±S.D.) locomotor activity data (total activity counts for session) [a]

Test Day	Dose (ppm)			
	0	1.0	5.0	30
Males				
PND 13	11±21	5±8	3±4	5±8
PND 17	60±41	79±56	59±36	43±37
PND 21	94±35	106±32	97±33	90±33
PND 60	347±137	380±76	419±130	411±100
Females				
PND 13	6±9	7±9	6±9	5±9
PND 17	41±34	85±60	55±39	69±52
PND 21	101±33	100±32	112±31	97±40
PND 60	465±111	500±175	477±130	498±160

[a] Data obtained from Table 20, pages 184-186, MRID 45912101.
N = 15-16/sex/dose.

c. **Auditory startle reflex:** There were no treatment-related effects in males or females on startle amplitude, latency or habituation at any dietary level on any test day. Also, there were no statistical differences from controls at any dietary level on any test day. Peak amplitude

data are summarized in Table 10 and latency data are summarized in Table 11. Peak amplitude can be seen to show a slight nominal decreased trend in the larger numbered trial blocks compared with lower numbered trial blocks, suggesting some habituation may have occurred, but the latency period did not affected with the trial block or dose level.

TABLE 10. Auditory startle reflex peak amplitude data (mean g ±S.D.) [a]

	Trial Block	Dose (ppm)			
		0	– 1.0	5.0	30
Males					
PND 22	1	42±10	47±13	48±20	47±15
	2	42±16	46±15	44±16	42±14
	3	42±17	41±16	43±20	38±16
	4	41±35	39±15	38±18	38±21
	5	35±15	39±13	29±9	34±19
	Mean	39±13	42±13	40±15	40±15
PND 38	1	88±42	102±52	82±41	82±40
	2	68±39	103±70	77±44	74±38
	3	64±38	81±60	71±45	63±29
	4	59±42	75±34	53±34	62±34
	5	55±27	76±34	48±26	61±44
	Mean	67±34	88±46	66±34	69±32
PND 60	1	171±94	275±163	244±152	175±109
	2	155±148	193±134	182±121	145±90
	3	124±137	183±111	124±106	125±76
	4	116±103	140±78	120±85	123±82
	5	105±89	133±74	119±72	105±46
	Mean	134±111	185±103	158±93	134±73
Females					
PND 22	1	49±20	46±16	56±23	51±17
	2	48±22	42±20	49±17	48±20
	3	47±24	40±20	46±15	45±14
	4	43±23	37±18	41±15	42±19
	5	42±21	35±17	40±13	37±16
	Mean	46±21	40±17	46±14	45±15
PND 38	1	62±23	51±27	69±40	61±29
	2	55±24	44±25	59±39	51±29
	3	43±21	37±21	45±29	48±26
	4	43±24	39±20	45±21	38±23
	5	43±21	55±24	31±17	40±24
	Mean	49±19	41±21	50±26	47±24
PND 60	1	122±81	109±55	139±115	110±73
	2	112±80	100±78	127±122	90±51
	3	82±21	76±43	103±96	77±55
	4	66±25	68±35	78±41	62±33
	5	60±21	62±35	73±60	73±41
	Mean	88±38	83±41	104±82	82±44

[a] Data obtained from Tables 23-24, pages 205-214, MRID 45912101.
N = 15-16/sex/dose

TABLE 11. Auditory startle latency to peak data (mean msec ±S.D.) [a]

	Trial Block	Dose (ppm)			
		0	1.0	5.0	30
Males					
PND 22	1	45±10	47±7	39±9	44±10
	2	39±9	44±11	38±9	39±8
	3	40±9	43±8	38±9	38±7
	4	42±9	45±7	38±7	37±6
	5	40±7	41±11	39±7	38±7
	Mean	41±5	44±6	38±6	39±6
PND 38	1	36±6	34±4	34±4	36±6
	2	33±4	33±5	35±4	33±4
	3	35±6	34±5	34±4	34±4
	4	38±6	35±5	35±5	35±3
	5	35±4	35±3	37±7	36±4
	Mean	35±3	34±3	35±4	35±3
PND 60	1	38±4	38±4	36±2	39±4
	2	37±6	36±3	39±5	38±5
	3	39±5	36±4	40±6	39±5
	4	39±5	36±4	37±6	37±5
	5	39±6	37±5	36±6	40±4
	Mean	38±4	37±3	38±4	38±4
Females					
PND 22	1	45±11	45±9	39±9	44±10
	2	45±13	43±8	36±6	40±11
	3	41±11	38±6	37±6	39±9
	4	39±8	38±6	37±6	40±9
	5	41±11	39±7	37±6	39±9
	Mean	42±9	40±6	37±4	40±9
PND 38	1	37±6	37±6	35±6	37±4
	2	35±4	38±6	38±6	36±6
	3	38±6	37±7	35±6	38±7
	4	37±5	36±5	36±6	39±5
	5	38±6	38±6	37±6	38±4
	Mean	37±4	37±4	36±4	37±3
PND 60	1	39±4	43±6	38±5	41±6
	2	42±7	42±6	40±5	42±6
	3	42±7	39±6	38±6	42±6
	4	43±5	41±4	39±5	44±7
	5	44±6	37±5	39±5	44±5
	Mean	42±4	40±4	39±3	43±4

[a]Data obtained from Tables 23-24, pages 205-214. MRID 45912101.
N = 15-16/sex/dose

20

d. Learning and memory testing:

Passive avoidance: There were no treatment-related effects, and acquisition and retention were appropriate in control animals. Data are summarized in Table 12.

TABLE 12. Passive avoidance performance at PND 24/31(mean ± S.D.) [a]

Test Day/Parameter		Dose (ppm)			
		0	1.0	5.0	30
Males					
Session 1 (Learning)	Trials to criterion	3.3±1.2	3.3±0.8	3.3±0.4	2.9±0.4
	Latency trial 1 (sec)	54.0±64.4	34.7±39.0	45.6±38.5	63.4±58.8
	Latency trial 2 (sec)	173.8±17.5	172.6±23.5	170.8±21.7	176.7±13.2
	Failed to Learn/No. Tested	3/16	0/16	0/16	2/16
Session 2 (Retention)	Trials to criterion	2.0±0.0	2.0±0.0	2.1±0.3	2.0±0.0
	Latency trial 1 (sec)	180.0±0.0	180.0±0.0	174.0±21.4	180.0±0.0
	Latency trial 2 (sec)	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0
Females					
Session 1 (Learning)	Trials to criterion	3.0±0.0	2.9±0.3	3.1±0.6	2.9±0.3
	Latency trial 1 (sec)	39.8±33.6	56.0±53.2	40.0±42.3	53.9±50.8
	Latency trial 2 (sec)	180.0±0.0	180.0±0.0	180.0±0.0	180.0±0.0
	Failed to Learn/No. Tested	0/16	2/16	1/16	1/16
Session 2 (Retention)	Trials to criterion	2.3±0.6	2.1±0.3	2.1±0.5	2.1±0.4
	Latency trial 1 (sec)	166.9±40.1	170.8±33.1	180.0±0.0	167.4±39.5
	Latency trial 2 (sec)	175.9±16.3	180.0±0.0	174.6±20.9	180.0±0.0

[a] Data obtained from Table 25, pages 215-217. MRID 45912101.

M-Water Maze: Data are summarized in Table 13. There were no treatment-related differences for males or females at any dose level compared to controls with regard to trials-to criterion, time to escape, number of errors, or failure to meet criterion. The statistical increase ($p \leq 0.05$) in mean trial 1 duration for 1.0 ppm females is considered incidental to treatment.

TABLE 13. Water maze performance

Test day/parameter		Dose (ppm)			
		0	1.0	5.0	30
Males					
Session 1 (Learning)	Trials to criterion	8.1±2.4	7.1±2.4	7.4±2.9	8.3±3.0
	Trial 1 errors (mean ± SD)	1.1±1.3	0.7±1.4	0.9±1.1	1.1±1.5
	Trial 1 duration (sec) (mean ± SD)	21.6±17.7	18.0±16.9	22.4±17.6	17.8±14.3
	Trial 2 errors (mean ± SD)	0.9±1.2	0.4±0.6	0.8±1.1	0.6±1.1
	Trial 2 duration (sec) (mean ± SD)	19.6±15.7	12.5±10.9	15.8±15.8	14.1±11.9
	Failed to meet criterion	0/16 (0%)	0/15 (0%)	1/16 (6%)	2/16 (13%)
Session 2 (retention)	Trials to criterion	6.1±1.5	5.4±0.8	5.8±2.3	6.4±2.8
	Trial 1 errors (mean ± SD)	0.9±1.1	0.7±1.7	0.7±1.7	0.9±1.2
	Trial 1 duration (sec) (mean ± SD)	14.2±11.7	12.5±14.5	11.5±15.6	15.6±15.7
	Trial 2 errors (mean ± SD)	0.1±0.3	0.0±0.0	0.5±1.4	0.1±0.5
	Trial 2 duration (sec) (mean ± SD)	4.4±2.9	4.1±0.9	8.1±14.8	5.1±3.6
Females					
Session 1 (Learning)	Trials to criterion	6.4±2.0	8.2±2.3	8.5±4.2	6.6±1.5
	Trial 1 errors (mean ± SD)	0.6±0.7	1.1±1.0	0.4±0.6	0.7±0.8
	Trial 1 duration (sec) (mean ± SD)	12.9±8.3	23.1*±13.8	15.5±8.8	15.9±9.5
	Trial 2 errors (mean ± SD)	0.3±0.4	0.3±0.5	0.9±1.5	0.5±0.6
	Trial 2 duration (sec) (mean ± SD)	9.5±4.9	14.1±12.2	16.7±14.5	12.6±13.7
	Failed to meet criterion	0/16 (0%)	0/16 (0%)	3/16 (19%)	0/16 (0%)
Session 2 (retention)	Trials to criterion	7.0±2.6	5.9±1.9	6.9±3.1	6.6±2.1
	Trial 1 errors (mean ± SD)	0.2±0.4	0.1±0.5	0.5±0.9	0.1±0.3
	Trial 1 duration (sec) (mean ± SD)	7.3±4.7	7.2±5.7	9.7±8.0	5.5±3.3
	Trial 2 errors (mean ± SD)	0.3±0.7	0.2±0.5	0.4±0.8	0.2±0.5
	Trial 2 duration (sec) (mean ± SD)	4.6±2.8	5.0±5.1	5.8±4.5	4.9±3.9

[a]Data obtained from Table 26, pages 218-220, MRID 45912101. *p≤0.05.

e. **Ophthalmology:** There were no treatment-related ocular effects in any treated animals compared to controls.

5. **Postmortem results:**

a. **Brain weights:** No treatment-related effects were seen in absolute brain weight at any dose on any day measured. Mean brain weight data are presented in Table 14.

22

TABLE 14. Mean (±SD) Brain Weight Data in Offspring [a]				
Parameter	Dose (ppm)			
	0	1.0	5.0	30
Males				
Day 21				
Terminal body weight (g)	45.9±4.5	47.0±4.1	47.4±3.9	46.7±4.4
Absolute brain weight (g)	1.413±0.079	1.412±0.038	1.420±0.062	1.403±0.068
Termination				
Terminal body weight (g)	318.3±15.2	320.1±20.7	301.8±29.8	*±*
Absolute brain weight (g)	1.836±0.081	1.823±0.084	1.845±0.080	1.830±0.080
Females				
Day 21				
Terminal body weight (g)	45.5±1.7	43.3±5.1	43.3±5.2	45.9±3.6
Absolute brain weight (g)	1.342±0.047	1.383±0.059	1.390±0.066	1.364±0.037
Termination				
Terminal body weight (g)	195.8±15.7	194.5±9.2	200.00±11.7	195.5±12.9
Absolute brain weight (g)	1.743±0.062	1.685±0.064	1.758±0.070	1.733±0.061

[a] Data obtained from pages 884-889, MRID 45912101 *The Table entry on page 888 reads "21539.8±67167.2". This is assumed to be an error
N = 9-10/sex/dose

b. **Cholinesterase activity**: Results of cholinesterase activity assessment are presented in Table 15. For the day 4 offspring, inhibited cholinesterase activity was not affected at any dose level. For the day 21 offspring, all three cholinesterase activities were affected in high-dose animals. Only plasma cholinesterase activity was inhibited significantly ($p \leq 0.05$, 30% inhibition) in high-dose males; whereas, plasma, erythrocyte, and brain cholinesterase activities were inhibited ($p \leq 0.05$) 27%, 33%, and 8%, respectively, in high-dose females. Effects in the dams were briefly discussed above (Section II.A.4).

TABLE 15. Cholinesterase activity in dams and offspring

Cholinesterase [mean ± SD (% inhibition relative to control)]	Dose (ppm)			
	0	1.0	5.0	30
Lactation day 21 dams				
Plasma (IU/mL)	0.62 ± 0.15	0.58 ± 0.08 (-6)	0.49* ± 0.09 (-21)	0.20* ± 0.05 (-68)
RBC (IU/mL)	1.25 ± 0.30	1.05 ± 0.27 (-16)	0.27* ± 0.08 (-78)	0.19* ± 0.20 (-85)
Brain (IU/g)	13.0 ± 0.6	12.5 ± 0.7 (-4)	12.2 ± 0.3 (-6)	8.3* ± 1.3 (-36)
Day 4 male & female offspring combined				
Plasma (IU/mL)	0.59 ± 0.07	0.62 ± 0.08 (+5)	0.57 ± 0.07 (-3)	0.59 ± 0.06 (0)
RBC (IU/mL)	1.40 ± 0.33	1.36 ± 0.36 (-3)	1.35 ± 0.35 (-4)	1.30 ± 0.30 (-7)
Brain (IU/g)	4.2 ± 0.4	4.2 ± 0.4 (0)	4.2± 0.2 (0)	4.3 ± 0.8 (+2)
Day 21 male offspring				
Plasma (IU/mL)	0.67 ± 0.09	0.63 ± 0.07 (-6)	0.61 ± 0.08 (-9)	0.47* ± 0.08 (-30)
RBC (IU/mL)	1.40 ± 0.34	1.35 ± 0.34 (-4)	1.36 ± 0.36 (-3)	1.14 ± 0.23 (-19)
Brain (IU/g)	11.2 ± 0.5	11.4 ± 0.4 (+2)	11.1 ± 0.4 (-1)	10.8 ± 0.5 (-4)
Day 21 female offspring				
Plasma (IU/mL)	0.62 ± 0.05	0.62 ± 0.06 (0)	0.57 ± 0.13 (-8)	0.45* ± 0.10 (-27)
RBC (IU/mL)	1.25 ± 0.18	1.47 ± 0.38 (+18)	1.33 ± 0.22 (+6)	0.84* ± 0.32 (-33)
Brain (IU/g)	11.6 ± 0.5	11.8 ± 0.4 (+2)	11.4 ± 0.5 (-2)	10.7* ± 1.0 (-8)

Data obtained from Appendix Tables, pages 849-855, MRID 45912101. Percentage inhibition from p. 49, MRID 45912101.
*$p \leq 0.05$.

C. Neuropathology

1. **Macroscopic examination:** No treatment-related effects were reported for male or female offspring at postnatal day 21 or study termination.

2. **Microscopic examination:** No significant treatment-related effects were noted on postnatal day 21 or study termination.

3. **Brain Morphometry:** . Data are summarized in Table 16. On PND 21, male pups at the high dose exhibited statistically significant decreases in the parietal cortex thickness (4%, $p<0.05$) and the cerebellum height measurement (7%, $p<0.01$) when compared with control measurements. No treatment-related morphomeric effects were observed any animals at study termination. Data for the low and mid dose groups were not reported.

TABLE 16. Mean (±variance or SD) morphometric data in offspring [a]

Parameter	Dose (ppm)			
	0	1.0	5.0	30
Males [Day 21]				
Frontal cortex	1.8371±0.0018	NA	NA	1.833±0.005
Parietal cortex thickness	1.9159±0.0078	NA	NA	1.8309±0.003*
Caudate putamen	3.1721±0.01	NA	NA	3.0593±0.03
Corpus callosum	0.5179±0.02	NA	NA	0.4860±0.01
Hippocampal gyrus	1.6269±0.015	NA	NA	1.5772±0.006
Cerebellum height	4.306±0.0294	NA	NA	4.0106±0.036**
Anterior to posterior cerebrum length (mm)	13.46±0.34	13.42±0.29	13.60±0.38	13.72±0.30
Anterior to posterior cerebellum length (mm)	7.12±0.26	7.16±0.29	7.05±0.24	7.07±0.50
Males [Termination]				
Frontal cortex	1.7880±0.008	NA	NA	1.7337±0.004
Parietal cortex thickness	1.8437±0.002	NA	NA	1.8064±0.007
Caudate putamen	3.4774±0.02	NA	NA	3.4681±0.04
Corpus callosum	0.6002±0.05	NA	NA	0.4975±0.005
Hippocampal gyrus	1.7023±0.019	NA	NA	1.6252±0.02
Cerebellum height	4.4344±0.07	NA	NA	4.2668±0.11
Anterior to posterior cerebrum length (mm)	14.76±0.22	14.79±0.38	14.74±0.32	14.79±0.38
Anterior to posterior cerebellum length (mm)	7.73±0.32	7.76±0.33	7.81±0.34	7.72±0.39
Females [Day 21]				
Frontal cortex	1.8426±0.011	NA	NA	1.8195±0.008
Parietal cortex thickness	1.9047±0.0033	NA	NA	1.8905±0.0069
Caudate putamen	3.1222±0.01	NA	NA	3.1±0.03
Corpus callosum	0.5547±0.009	NA	NA	0.5149±0.004
Hippocampal gyrus	1.6269±0.02	NA	NA	1.5772±0.006
Cerebellum height	4.2821±0.0254	NA	NA	4.3381±0.0233
Anterior to posterior cerebrum length (mm)	13.27±0.23	13.27±0.21	13.42±0.51	13.40±0.31
Anterior to posterior cerebellum length (mm)	6.99±0.33	7.16±0.24	7.15±0.36	6.96±0.24
Females [Termination]				
Frontal cortex	1.7197±0.01	NA	NA	1.6835±0.006
Parietal cortex thickness	1.7571±0.005	NA	NA	1.7790±0.01
Caudate putamen	3.3990±0.02	NA	NA	3.3637±0.01
Corpus callosum	0.5195±0.003	NA	NA	0.4821±0.007
Hippocampal gyrus	1.6468±0.015	NA	NA	1.6945±0.02
Cerebellum height	4.2433±0.04	NA	NA	4.2885±0.11
Anterior to posterior cerebrum length (mm)	14.10±0.27	14.07±0.23	14.33±0.21	14.35±0.44
Anterior to posterior cerebellum length (mm)	7.83±0.41	7.81±0.28	7.93±0.33	7.71±0.41

a Data obtained from pages 884-889. MRID 45912101. Parietal cortex thickness and cerebellum height are on page 894 and 895 of the report. * Significant at <0.05 ** Significant at p<0.01.
N = 10/sex/dose

III. DISCUSSION AND CONCLUSIONS:

A. **INVESTIGATORS' CONCLUSIONS:** The investigators concluded that the overall NOAEL is 1.0 ppm for dams based on slight inhibition of erythrocyte and plasma cholinesterase activity at 5.0 ppm on lactation day 21. The investigators also concluded that the overall NOAEL for offspring is 5.0 ppm based on decreased cholinesterase activity in males and females in the 30 ppm group on PND 21.

B. **REVIEWER COMMENTS**

In dams, no treatment-related effects on mortality, clinical signs, body weight, body weight gain, food consumption, or FOB parameters were noted.

In offspring, there were no treatment-related deaths or clinical signs or effects on birth weight, body weight or body weight gain pre- or post-weaning, food consumption, developmental landmarks, pupil constriction, FOB parameters, motor or locomotor activity, acoustic startle, passive avoidance, learning and memory, and opthalmological parameters. At necropsy, there were no treatment-related gross or micropathological effects and no effect on absolute or relative brain weight or brain morphometry. Cholinesterase activity was inhibited ($p \leq 0.05$) in maternal animals at 5.0 and 30 ppm. Inhibitions were 21% and 78% at 5.0 ppm, for plasma and erythrocyte cholinesterase activities, respectively, and 68%, 85%, and 36% at 30 ppm, for plasma, erythrocyte and brain cholinesterase activities, respectively.

In the offspring, cholinesterase activity was not affected at any dose level in offspring on PND 4. For the day 21 offspring, all three cholinesterase activities were affected in high-dose animals. Only plasma cholinesterase activity was inhibited significantly ($p \leq 0.05$, 30% inhibition) in high-dose males; whereas, plasma, erythroctye, and brain cholinesterase activities were inhibited ($p \leq 0.05$) 27%, 33%, and 8%, respectively, in high-dose females. Brain morphometric analysis showed statistically significant decreases in the parietal cortex thickness (7%,) and the cerebellum height measurement (4%) when compared with control measurements in male offspring on PND 21. No treatment-related morphometric effects were observed in any animals at study termination. Data for the low and mid dose groups were not reported. However, it was determined that these data are not necessary because the magnitude of the changes seen at the highest dose (30 ppm) would not be expected to occur at a dose (1 ppm) which is 30-times lower.

For maternal systemic toxicity, the NOAEL is 30 ppm (2.77 mg/kg/day), the highest dose tested. A LOAEL was not established.

For maternal cholinesterase inhibition the NOAEL is 1.0 ppm (0.09 mg/kg/day). The LOAEL is 5.0 ppm (0.47 mg/kg/day) based on inhibition of plasma and erythrocyte cholinesterase activities.

For offspring systemic toxicity, the NOAEL is 5.0 ppm (0.47 mg/kg/day). The LOAEL is 30 ppm (2.77 mg/kg/day) based on morphometric changes in the brain of PND21 males.

For offspring cholinesterase inhibition, the NOAEL is 5.0 ppm (0.47 mg/kg/day). The LOAEL is 30 ppm (2.77 mg/kg/day) based on inhibition of plasma, erythrocyte and brain cholinesterase activities.

This study is classified **Acceptable** and may be used for regulatory purposes, however it does not satisfy the guideline requirement for a developmental neurotoxicity study in rats (OPPTS 870.6300, §83-6); OECD 426 (draft) at this time pending a comprehensive review of all available positive control data.

C. **STUDY DEFICIENCIES:** None