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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

WASHINGTON, DC 20460

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OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

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OPP OFFICIAL RECORD
HEALTH EFFECTS DIVISION
SCIENTIFIC DATA REVIEWS
EPA SERIES 361

MEMORANDUM

SUBJECT: Chlorpyrifos (P.C. Code 059101) - Toxicology Data Review

FROM: Susan L. Makris, M.S. Susan L Makris 3/2/00
Toxicology Branch I
Health Effects Division (7509C)

TO: Deborah Smegal, Ph.D.
Reregistration Branch 3
Health Effects Division (7509C)

THRU: Alberto Protzel, Ph.D., Branch Senior Scientist Alberto Protzel 3/2/00
Toxicology Branch I
Health Effects Division (7509C)

TASK ID: DP Barcode: D254907
PC Code: 059101

Submission: S559875
Chemical: Chlorpyrifos

REGISTRANT: Dow AgroSciences LLC

Action requested: Review the following supplementary data:

Hoberman, A.M. (1999) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats; SAS Statistical Output. Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, Undated. MRID not assigned. Unpublished.

Hoberman, A.M. (1999) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats; Report Supplement 2 - Reanalysis of Morphometric Data. Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, March 19, 1999. MRID 44787301. Unpublished.

These data were submitted in supplement to the following study:

Hoberman, A. M. (1998) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats. Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, May 1, 1998. MRID 44556901. Unpublished.

Hoberman, A.M. (1998) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats; Report Supplement 1 (pathology data). Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, September 23, 1998. MRID 44661001. Unpublished.

Background Information

A developmental neurotoxicity study with chlorpyrifos (MRID 44556901), including a separate supplementary report addressing terminal pathology data (MRID 44661001), was submitted to the Agency by the Registrant (Dow AgroSciences). In the review of these data (HED Doc. No. 013081), it was noted that there were outstanding issues which had not been adequately addressed by the original study report documents. The study was graded **unacceptable**, due to an inadequate presentation of the statistical data analysis which resulted in the inability to determine the definitive developmental neurotoxicity NOAEL and LOAEL for the offspring.

One of the outstanding critical issues concerned review of the morphometric data for Subset 1 (PND 12) and Subset 4 (PND 66) males and females in the developmental neurotoxicity study with chlorpyrifos (HED Doc. No. 013081). These issues originated with the manner in which the statistical analyses of the brain measurements were presented and interpreted. The original report stated that the statistical tests were conducted at alpha = 0.02; it was also unclear exactly how PND 12 and PND 66 data were analyzed, and whether the analysis had included data from mid- and low-dose measurements.

Revised EXECUTIVE SUMMARY

In this developmental neurotoxicity study (MRID 44556901), 25 pregnant Sprague-Dawley rats/group were administered chlorpyrifos (99.8% a.i.) by gavage from gestation day 6 (GD 6) through lactation day 11 at 0, 0.3, 1, or 5 mg/kg/day. An additional 5 pregnant females/group were dosed at the same levels for the cholinesterase (ChE) phase of the study. Dams were examined for body weight, reproductive performance, number of viable pups, and postpartum behavior. During the dosing period, dams were observed daily for signs of autonomic function toxicity. Satellite dams were sacrificed four to five hours post-dosing on GD 20 for ChE analyses to be performed on brain, plasma, and erythrocytes. Offspring were examined for viability at birth, pup/litter survival, body weight, sex ratio, physical development landmarks (eye opening and pinna detachment), observed nursing behavior, and sexual maturation. F_1 generation litters were randomly standardized on lactation day 5 and assigned to 4 subsets for continued observation. On postnatal day (PND) 12, fixed brain weight measurements (10 pups/sex/dose) and neuropathological evaluations including morphometrics (6 pups/sex/dose) were performed on Subset 1 pups, with the remaining 10 pups/sex/dose necropsied for gross lesions. In Subset 2, 8 pups/sex/dose were selected for evaluation of learning and memory; evaluations were performed on PNDs 23-25 and 62-92. These Subset 2 animals were sacrificed on PNDs 97-101, following the last evaluation. The Subset 2 pups not selected for evaluation were necropsied for gross lesions on PND 22. The Subset 3 pups were tested for motor activity on PNDs 14, 18, 22, and 61 and auditory startle habituation on PNDs 23 and 62; all Subset 3

animals were sacrificed on PNDs 63 or 64 following the last evaluation. In Subset 4 pups, fixed brain weights were determined in 10 pups/sex/dose, neuropathological examinations were performed on 6 pups/sex/dose, and all remaining Subset 4 animals (10/sex/dose) were necropsied for gross lesions upon sacrifice on PND 66-77.

Maternal toxicity in the high-dose (5 mg/kg/day) animals was manifested as increased signs of autonomic function toxicity, apparent at the end of gestation as fasciculations (6/25 treated vs 0/25 controls), and during early lactation (days 1-5) as fasciculations (16/24 treated vs 0/25 controls), hyperpnea (8/24 treated vs 0/25 controls), and hyperreactivity (17/24 treated vs 2/25 controls). Dams with all pups dying were increased in the high-dose group (3/23 treated vs 0/25 controls). There were no significant effects on bodyweight, food consumption, or pregnancy parameters. There were no unscheduled deaths in the maternal animals.

Brain ChE activity was decreased in the high-dose (↓90%) and mid-dose (↓18%) dams as compared to control. Erythrocyte (↓41-99%) and plasma (↓43-92%) ChE activities were decreased in a dose-dependent manner in all treated groups.

The maternal toxicity NOAEL was not observed.
The maternal LOAEL was < 0.3 mg/kg/day, based on plasma and RBC cholinesterase inhibition.

For the F_1 generation pups, the high-dose group bodyweights were significantly reduced (↓8-15%) at PND 1 and 5 (pre- and post-culling). Bodyweights were also reduced from birth to PND 22 in Subset 4 high-dose animals (↓5-19%); bodyweight gains were reduced in these animals during the same period (↓5-30%). Additionally, compared to the controls, reduced terminal body weights were observed in the Subset 1 (PND 12) high-dose animals (↓17-19%) and the Subset 4 (PND 66) high-dose males (↓10%). For the F_1 generation adults, body weights of the high-dose males were decreased at PND 22 through 66 (↓11-17% vs controls). High-dose F_1 adult females also weighed less than controls at PND 22 (↓17% vs controls), but were of similar weight at PND 66. Bodyweight gains were also decreased in the high-dose males for the PND 22-40 interval (↓13% vs controls) and PND 40-66 interval (↓7%). Food consumption was decreased immediately after weaning (PND 23-30) in high-dose males and females (↓13% vs controls).

Development as assessed by pinna unfolding was delayed (4.0 days in treated vs 3.5 days in controls) in the high-dose group. Sexual maturation was delayed as assessed by time to preputial separation (106% of controls) and vaginal patency (103% of controls).

Pup viability was reduced as assessed by the following parameters: surviving pups/ litter (↓27%) and live litter size at culling (↓16%), pup viability index (↓29%), and pups found dead or presumed cannibalized (day 1 - 7.2 % treated vs 0.0% controls; days 2 to 5 - 24.7% treated vs 1.3% controls).

There were no statistically significant differences between the groups in the average acquisition and delay training. Additionally, there were no differences among dose groups when comparing retention of information during PNDs 23-25 and 62-92. Motor activity was decreased in high

dose male and female pups on PND 14 (↓56% in males and ↓37% in females), and increased in high dose females on PNDs 18 and 22 (↑51% on both days). On PND 61, motor activity was increased for both sexes (↑16-17%). There was a statistically significant increase (↑16-25%) in the latency to peak response during the auditory startle habituation assessments on PND 23 in the high-dose animals compared to concurrent controls. At PND 62, the latency to peak response in the high-dose animals was 10-12% higher than in the controls. Additionally, the peak response amplitudes in the high-dose animals were decreased by 9 to 29% on PNDs 23 and 62 (not statistically significant) compared to the controls.

There were no gross or microscopic lesions of the nervous system in Subset 1 or 4 offspring. Subset 1 high-dose males at PND 12 had reduced absolute brain weights (↓9% vs controls), increased relative brain weights (↑13% vs controls), reduced anterior to posterior measurement of the cerebellum (↓24% vs controls), reduced height of the cerebellum (↓14% vs controls), decreased thickness of the parietal cortex (↓6% vs controls), and decreased thickness of the hippocampal gyrus (↓9% vs controls). High-dose female pups had reduced absolute brain weights (↓9% vs controls), increased relative brain weights (↑14% vs controls), decreased thickness of the parietal cortex (↓6% vs controls), decreased width of the caudate-putamen (↓10% vs controls), and decreased thickness of the hippocampal gyrus (↓12% vs controls). In Subset 4 F1 animals, killed on PND 66, morphometric analysis revealed significantly decreased parietal cortex measurements in high-dose (↓5%) and mid-dose (↓4%) females, as compared to controls. Decreases in the thickness of the hippocampal gyrus in high-dose females (↓7%) resulted in contradictory statistical results when compared to controls; decreases in mid-dose (↓4%) females as compared to control were not found to be statistically significant. There was no evaluation of the morphometric data for low dose females at PND 66. Brain weight in high dose females was similar to control brain weight at day 66 (↓0.3%).

It is not possible to definitively classify findings in the preweaning offspring as having originated with pre- or postnatal exposure, nor as resulting from developmental perturbation versus direct systemic- or neurotoxicity. However, adverse findings in the adult (~PND 66) offspring, i.e., alterations in motor activity, auditory startle response, and brain structure (decreased measurements of the parietal cortex and hippocampal gyrus, in the absence of brain weight deficits) can be interpreted to represent the long-term sequellae of developmental exposure to chlorpyrifos.

Adverse effects in the offspring have been identified at the MDT of 1.0 mg/kg/day; these include a significant treatment-related decrease in the measurement of the parietal cortex, supported by possible (although nonsignificant) alterations in the hippocampal gyrus, in the brain of female rats at postnatal day 66. However, due to the lack of morphometric data for low-dose (0.3 mg/kg/day) female rats at postnatal day 66, **the offspring NOAEL and LOAEL cannot be determined**.

Study Classification and Additional Information Required

While the offspring NOAEL and LOAEL have not yet been identified for this developmental neurotoxicity study, it is recognized that the study was well-conducted according to Agency guideline §83-6, and under GLP regulations. Remaining questions can be resolved with

additional information and statistical analysis, but there are no outstanding concerns regarding the quality of the animal data. It is recommended that the classification of the study be changed from **unacceptable** to **guideline-unacceptable** at this time, pending submission and review of additional morphometric data for PND 66 low-dose females (parietal cortex and hippocampus measurements).

cc: Kathleen Raffaele (7509C)
Mark Hartman (7508W)

EPA Reviewer: Susan L. Makris, M.S.
TOX 1 (7509C)
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Susan L Makris 3/2/00
Kathleen C. Raffaele 3/2/00

DATA EVALUATION RECORD

014014

STUDY TYPE: Developmental Neurotoxicity Study - Rat; Supplemental Analytical Data
OPPTS Number: 870.6300 OPP Guideline Number: §83-6

DP BARCODES: D251533, D254907 SUBMISSION CODES: S552530, S559875
P.C. CODE: 059101 TOX. CHEM. NO.: 219AA

TEST MATERIAL (PURITY): Chlorpyrifos (99.8% a.i.)

SYNONYMS: Dursban

CITATION: Hoberman, A.M. (1999) Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats; SAS Statistical Output. Argus Research Laboratories, Inc., Horsham, Pennsylvania, Laboratory study No. 304-001, Sponsor study No. K-044793-109, Undated. MRID not assigned. Unpublished.

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Supplemental to:

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SPONSOR: Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268

I. Background Information

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II. Additional Data Submitted by Registrant

The Registrant submitted Report Supplement 2 - Reanalysis of Morphometric Data (MRID 44787301), to address the concerns of the Agency reviewers, and to present their views on the interpretation of the study data. Previously (December 4, 1998), the Registrant had also provided a volume of information entitled Copy *of Statistical Output from SAS File* to Agency reviewers (no MRID assigned), to the Health Effects Division (HED). A review of the information contained within these submissions follows.

A. Statistical Output from SAS File

The SAS output file submitted to the agency confirmed the original statistical findings, and included sufficient information to enable reviewers to determine how the original statistical analysis of the morphometric data was performed. The output file also included the statistics for some of the statistical interactions not included in the original report. Contrary to what was reported in the original review (HED document No. 013081, based on the original study report), the procedures used in the original statistical analysis appear to have been as follows:

1) The original Analysis of Variance (ANOVA) included data from the high-dose and control groups only (both sexes and time points); data from the mid- and low-dose groups (both sexes and time points at PND 12, mid-dose females at PND 66) were not included.

2) Detailed results of statistical findings were reported in the original review, and will not be repeated here, except to note that the results were not entirely in agreement with those of the submitted re-analysis. In particular, findings in the original analysis with respect to

hippocampal gyrus and parietal cortex measurements were: a) statistically significant decreases in hippocampal gyrus measurements, when high dose and control data for both sexes were compared across time points (main effect of Group, p=0.001); differences were consistent across sex and across time (interactions were not statistically significant: Group X Time and Group X Sex interactions both had p-values greater than 0.5); b) statistically significant decreases in parietal cortex measurements, when high dose and control data were compared across time points (main effect of Group, p=0.02); differences were again consistent across time points and sexes (interactions were not statistically significant: Group X Time, p=0.25; Group X Sex, p=0.098), although the consistency of effect across sexes was less compelling.

B. Report Supplement 2 - Reanalysis of Morphometric Data (MRID 44787301)

Supplement 2 consists of two parts: a statistical reanalysis of the brain morphometric data; and arguments supporting the Registrant's interpretation of the morphometric findings. Copies of three cited references were also included in the submission.

1. Brain Weight and Morphometric Measurements

Statistical analysis of morphometric data

In this submission, brain morphometric data were reanalyzed using statistical procedures different from those used in the original report. In the current reanalysis, separate ANOVAs were conducted for each time point; data for males and females were also analyzed separately. In addition, all available treatment groups were included in each analysis (for day 12, all doses were evaluated for both sexes; for day 66, control and high dose males, control, mid, and high dose females). If the ANOVA was statistically significant at an alpha level of $p < 0.05$, the data from each dose level were compared to control by Dunnett's test conducted at alpha = 0.05.

The results of the reanalysis provided in the supplement are summarized below in Table 1. The individual and summary morphometric data for PND 66 animals are presented in Attachment 1. The data for high-dose PND 12 male pups demonstrate significant decreases in the measurements of anterior to posterior cerebellum and cerebellum height. For high-dose PND 12 females, the caudate/putamen measurement was significantly decreased. At the mid-dose, only the cerebellum height was significantly decreased for PND 12 females. For PND 66 adult offspring, significant decreases in the measurement of the parietal cortex were noted for mid- and high-dose females. The decrease in the measurement of the hippocampal gyrus was not statistically different from control for either sex or time point. No significant differences were observed for any brain measurement for males at PND 66. The study pathologist dismissed the significant decreases in the parietal cortex of mid- and high-dose PND 66 females as being related to variability in the data; historical morphometric data were not provided to support this position.

As noted above, the current findings are not in complete agreement with those reported

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previously. Note that (for example), in contrast with the results of the earlier analysis, the differences between high dose and control for the hippocampal gyrus are no longer statistically significantly different for either sex or time point. Findings for parietal cortex remain significant only for high dose females at day 66; the difference between controls and mid-dose females (not included in the previous analysis) was found to be statistically significant.

Table 1: Brain weight and linear measurements expressed as percent of control

Variable	PND 12						PND 66		
	Males			Females			Males	Females	
	Low	Mid	High	Low	Mid	High	High	Mid	High
Brain weight	110.6	105.5	88.5*	98.9	97.6	91.3*	100.0	101.1	97.4
AP cerebrum	107.1	104.7	93.8	102.4	103.2	98.0	101.8	100.1	99.4
AP cerebellum	105.6	102.0	75.5*	95.3	103.7	94.3	99.7	99.7	97.6
Frontal cortex	100.9	100.3	94.4	100.9	98.5	99.4	98.7	100.2	98.9
Parietal cortex	108.4	108.4	94.0	99.7	99.1	94.5	102.1	95.8*	94.9*
Caudate/putamen	100.0	103.2	99.3	93.3	96.0	90.3*	98.0	99.1	105.0
Corpus callosum	103.4	99.0	100.0	93.2	99.0	89.1	92.8	105.2	95.4
Hippocampal gyrus	111.1	107.5	91.2	97.4	99.6	88.5	98.3	96.3	93.2
Cerebellum height	98.6	97.5	85.8*	90.4	88.8*	91.3	99.1	97.4	99.0
Ext germinal layer	103.1	107.6	101.3	94.0	106.5	105.6	-----	-----	-----

Statistical analyses were performed before conversion to percent of control.
* $p<0.05$, Dunnett's.
Data extracted from MRID 4478301, page 12.
Note that findings for the ANOVAs were also significant for AP cerebellum, parietal cortex, and hippocampal gyrus for day 12 males, in the absence of significant effects for individual comparisons.

Variability in morphometric measurements

The Registrant argues that the limited sample size (n=6/sex) used for morphometric measurements (as specified by EPA testing guideline §83-6) resulted in increased variability and is a confounding factor in the interpretation of the morphometric data in this developmental neurotoxicity study with chlorpyrifos. Additionally, it was suggested that technical aspects related to histological processing of brain tissues, such as variation in brain size, difficulty in

standardizing brain sections, degree of tissue dehydration during processing, and differences in section level that result from "facing in" procedures used in preparing slides from tissue blocks, may have contributed to any variability observed in the PND 12 morphometric measurements in this study. EPA reviewers note, however, that high variability in the data would be more likely to contribute to a lack of statistical significance in the face of true differences than to lead to spurious findings of statistical significance in the absence of true differences. More importantly, an examination of the individual morphometric data from this study reveals an absence of excessive overall variance in both the PND 12 and 66 measurements; the average coefficient of variation for the data at each time point are below 8% (analysis by Dr. Kevin Crofton, USEPA, National Health and Evironmental Effects Laboratory) .

Agency reviewers considered significant differences noted in the morphometric data to be treatment-related. The morphometric data for PND 12 pups demonstrate significant decreases in various parameters for males and females; these data are consistent with the significantly reduced brain weight data for both sexes at this age. In contrast to the Registrant's interpretation, it could be argued that the small sample size contributes to the inability to detect statistically significant differences from control for endpoints other than those currently identified for PND 12. For example, examination of the individual data indicates that the statistically significant decreases in the hippocampal measurements identified in the original analysis, but not in the current re-analysis, may also be treatment-related at the high-dose for both sexes at PND 12 and for females at PND 66; there were also some indications of a possible effect for the mid-dose females at PND 66.

In addition to the apparent decrease in the hippocampal gyrus measurements on PND 66, the morphometric findings in the parietal cortex of female offspring indicate a treatment-related effect at the mid- and high-dose levels (1 and 5 mg/kg/day). The significant reduction in the parietal cortex measurement occurs only in females on PND 66 and was not observed in either sex at PND 12 in the current reanalysis (although the original statistical analysis did identify statistically significant differences at the high dose, which were consistent across time and sex). However, the decrease cannot be attributed to individual outliers at either the mid- or high-dose (see Attachment 1). Further analysis, conducted by Dr. Karl Jensen (USEPA, National Health and Evironmental Effects Laboratory), demonstrates the following:

a) The coefficient of variation for the PND 66 parietal cortex measurements is 3%, indicating a lack of excessive variance from any source, biological or technical.
b) These data are within the historical range for adult female parietal cortex measurements, established by an examination of the individual morphometric data from several developmental neurotoxicity studies submitted to the Agency in support of pesticide registration.
c) Examination of the individual parietal cortex measurements in the chlorpyrifos developmental neurotoxicity study reveals that the minimal difference between values is 24 μm, and that all values are divisible by 24. Consequently it is reasonable to assume that for parietal cortex, this is a raw unit of measurement; the minimal raw unit of measurement may

be considered a reasonable threshold for detection of differences. The differences between the mid- or high-dose group means are between 3 to 4 raw units, and thus at least 3 times the value of a minimal threshold for detection.

d) As indicated in the study report, an ANOVA indicates that the effect of dose is significant, and a Dunnett's test indicates that the high- and mid-dose values are different from control. An analysis with Scheffe's test confirms this finding and further indicates that the high- and mid-dose values are not different from one another. Therefore, the effect is a result of treatment, but the magnitude of the effect is not dependant on the dosage.

In conclusion, the morphological alterations in the parietal cortex of female offspring at PND 66 are both statistically and biologically significant at the mid- and high-dose levels and are a clear indication that the structure of the brain has been altered by treatment. As a result, evaluation of the brain measurements for the low-dose PND 66 females becomes critical to establish the offspring NOAEL for this study. As previously noted in the Agency review of MRID 44556901, no brain measurements were presented in the data for low- and mid-dose males or for low-dose females at PND 66; it is presumed that these were not measured. At a minimum, the parietal cortex and hippocampal gyrus measurements from the low-dose females should be provided to the Agency to further examine this issue and establish a NOAEL for this endpoint.

<u>The interrelationship of pup body weight, brain weight, and morphometric measurements</u>

There is no disagreement between the Agency reviewers and the Registrant regarding the characterization of effects seen on pup body and brain weight in this study. High dose pups were observed to weigh less than control throughout lactation and had significantly reduced absolute brain weights at PND 12 necropsy, although relative brain weights were increased. No effects attributable to treatment were noted in the pup body and brain weight data for the low- or mid-dose groups.

The Registrant attributes these alterations in pup body weight, brain weight, and morphometrics to postnatal undernutrition of the pups, citing a study by Peeling and Smart (1994) which concluded that body weight, brain weight, and brain morphometrics are related. In attempting to demonstrate the correlation between brain weight and morphometrics in this study, the Registrant averaged the relative values (% control) for the nine distinct morphometric measurements by sex for the high dose group, and compared these values and average high-dose brain weight values against controls. Specifically, for males the average morphometric value was 92.8% of control and the average brain weight was 88.5% of control; for females, the average morphometric value was 94.5% of control and the average brain weight was 91.3% of control (MRID 44787301, page 12). These comparisons, however, are an inappropriate and inconclusive manipulation of the data, since a numerical value derived from averaging the relative values for all external and internal morphometric measurements is not meaningful. Such a derived number would not evaluate the differences between alterations in growth patterns or disruptions in discreet areas of the brain, which could be differentially altered as an adverse consequence of treatment.

While the Agency reviewers agree with the Registrant that body weight, brain weight, and brain

morphometrics can be related in preweaning rat pups, high-dose observations on pups in this study cannot be dismissed on that basis. At a dose which is unequivocally toxic to adult animals, and in the presence of information from a companion study (MRID 44648102) that fetuses and pups were exposed to chlorpyrifos *in utero* and in maternal milk, adverse effects observed in the offspring at PND 12 are interpreted by Agency reviewers as being related to treatment. It is not possible to separate the origin or interrelationship of these specific effects, and classify findings in the preweaning offspring as having originated with pre- or postnatal exposure, nor as resulting from developmental perturbation versus direct systemic- or neurotoxicity. However, findings observed in the adult (~PND 66) offspring, long after exposure *via* the dam has ceased, namely alterations in motor activity, auditory startle response, and brain structure (decreased measurements of the parietal cortex and hippocampal gyrus, in the absence of brain weight deficits) can be interpreted to represent the long-term sequellae of developmental exposure.

2. Pup Undernutrition and Maternal Neglect

The Registrant has proposed that pup body and brain weight decrements during lactation were the result of undernutrition which was attributable to maternal toxicity or to maternal neglect. The inference made is that any treatment-related alterations in structure or function in the offspring were not the result of a direct effect of exposure to chlorpyrifos during development.

As support for this hypothesis, the work of Peeling and Smart (1994) and Carney et al. (1998) are cited. These two studies demonstrate 1) that undernutrition during early development causes slower body and organ growth, although brain tissue is "spared," and 2) that pups of feed-restricted dams grew more slowly than pups of dams that were not feed-restricted. In the developmental neurotoxicity study with chlorpyrifos, food consumption of the dams was measured during gestation and lactation. High-dose values were statistically similar to control values, although a 5-15% decrease in food consumption (g/day) was observed during early lactation, and a 5-12% decrease in relative food consumption (g/kg/day) was observed during the treatment period. These small decreases in food consumption could not be termed "undernutrition" or "feed restriction," and it is very unlikely that they would have resulted in the alterations to offspring development as described in Peeling and Smart and in Carney et al.

An attempt was also made to link the preweaning motor activity deficits in the chlorpyrifos rats with maternal undernourishment. Gramsbergen and Westerga (1991) demonstrated a transient delay in locomotor development of pups from dams that were 40% feed-restricted throughout gestation and lactation. In the chlorpyrifos study, the decreased motor activity at the high dose was observed on PND 14, 18, and 22 for males and on PND 14 only for females. Increased motor activity, which was observed in females on PND 18 and 22, was also observed in both sexes at PND 61. The non-transient nature of the alterations to motor activity in the chlorpyrifos study, following only non-significant maternal food consumption deficits during the first few days after delivery, is an indication that the insult is probably not comparable to that induced

experimentally in the Gramsbergen and Westerga study.

Furthermore, there is no support for the supposition (as proposed by the Registrant) that offspring findings at the high dose (5 mg/kg/day) should be attributed solely to maternal neglect. At that dose level, maternal brain cholinesterase was significantly inhibited (to 10% of control), cholinergic clinical signs (fasciculations, hyperpnea, and/or hyperactivity) of unknown duration and severity were observed in most dams during lactation following chlorpyrifos administration on an average of 2 separate dosing days (only during lactation days 1-6, although dams were dosed daily through lactation day 10), and maternal weight gain was decreased in late gestation (days 17-20) and early lactation (postnatal days 0-3). While the type of pup weight deficits and decreased survival observed at 5 mg/kg/day during the early postnatal period are not inconsistent with lack of maternal care, there is no evidence in the reported results that the effects on the offspring in this study were, in fact, due solely to maternal neglect. For example, the maternal clinical observation data (MRID 44556901, Table B15, p 138) did not indicate that the dams were not gathering the pups into the nest, remaining with their litters in the nest, or grooming the pups. There was no indication anywhere in the reported data that there was an alteration in high dose maternal lactation and nursing behavior. The clinical observations on live pups (report Table 24, p 205) did not describe the absence of visible milk in the stomach (often described as a "milk spot"). The gross necropsy data (report Table B28, p 220) indicated a lack of milk in the stomach of 12 pups of one litter that had died on PND 1, and in 2 pups from separate litters that had died on lactation days 2 and 5, although a total of 48 liveborn pups (from 13 litters) died and were necropsied between birth and postnatal day 5. Therefore, the statement that "neonatal deaths often were associated with a lack of milk in their stomachs" (page 8 of MRID 44787301) is not supported by the data. Nevertheless, even had there been overwhelming evidence of a lack of nourishment to the offspring, it would be impossible to discern whether nursing was interrupted or altered by treatment-related toxicity to the dams or to the pups, especially since there is evidence in the data of a companion study (MRID 44648102) that chlorpyrifos was available to the pups in maternal milk. An additional important consideration is that the observed treatment-related effects on the offspring at the high dose level also included continued postweaning body weight deficits, increased motor activity on PNDs 18 and 22 (females) and PND 61 (both sexes), decreased peak response and increased latency to peak response in auditory startle habituation tests on PND 23 and 62, decreased PND 12 brain weight, and alterations in internal brain measurements at PND 12 in both sexes and PND 66 in females. Many of these findings continued past the time of weaning. Full recovery of body weight, motor activity, auditory startle, and morphometric alterations did not occur during the posttreatment period (of approximately 55 days), even though the rate of posttreatment weight gain appeared comparable among control and treated groups.

III. Conclusions

Several issues had remained outstanding following the review of the developmental

neurotoxicity study with chlorpyrifos (MRID 44556901). These have been resolved in part by Supplement 2. Questions regarding the statistical treatment of the behavioral data, which were raised in HED Doc. No. 013081, were not addressed. These included the lack of some parameters (i.e., Group X Sex X Time or Group X Sex X Time X Block) in the analysis of variance performed on replicate data such as motor activity or auditory startle, and the absence of an explanation for the use of a significance level (alpha) of 0.02. However, the issues surrounding the statistical analyses of these data were not judged by Agency reviewers to be critical to the overall interpretation of the study or the determination of the lowest adverse effect level for the offspring. The issue of greater concern and impact was the statistical analysis and interpretation of morphometric data for the offspring. Sufficient data were provided in the SAS Output file and in Supplement 2 (information on the statistical methodology, and the results of statistical analyses of the mid-dose group morphometric data) to enable the Agency reviewers to arrive at an independent conclusion.

The maternal findings and effect levels were not reexamined and remain as indicated below:

> **The maternal toxicity NOAEL was not observed.**
> **The maternal LOAEL was < 0.3 mg/kg/day, based on plasma and RBC cholinesterase inhibition.**

However, based upon the discussions above, the offspring conclusions have been revised:

> Adverse effects in the offspring have been identified at the MDT of 1.0 mg/kg/day; these include a significant treatment-related decrease in the measurement of the parietal cortex, supported by possible (although nonsignificant) alterations in the hippocampal gyrus, in the brain of female rats at postnatal day 66. However, due to the lack of morphometric data for low-dose (0.3 mg/kg/day) female rats at postnatal day 66, **the offspring NOAEL and LOAEL cannot be determined.**

C. Study Classification

While the offspring NOAEL and LOAEL have not yet been identified for this developmental neurotoxicity study, it is recognized that the study was well-conducted according to Agency guideline §83-6, and under GLP regulations. Remaining questions can be resolved with additional information and statistical analysis, but there are no outstanding concerns regarding the quality of the animal data. It is recommended that the classification of the study be changed from **unacceptable** to **guideline-unacceptable** at this time, pending submission and review of additional morphometric data for PND 66 low-dose females (parietal cortex and hippocampus measurements).

D. Revised EXECUTIVE SUMMARY:

In this developmental neurotoxicity study (MRID 44556901), 25 pregnant Sprague-Dawley rats/group were administered chlorpyrifos (99.8% a.i.) by gavage from gestation day 6 (GD 6)

through lactation day 11 at 0, 0.3, 1, or 5 mg/kg/day. An additional 5 pregnant females/group were dosed at the same levels for the cholinesterase (ChE) phase of the study. Dams were examined for body weight, reproductive performance, number of viable pups, and postpartum behavior. During the dosing period, dams were observed daily for signs of autonomic function toxicity. Satellite dams were sacrificed four to five hours post-dosing on GD 20 for ChE analyses to be performed on brain, plasma, and erythrocytes. Offspring were examined for viability at birth, pup/litter survival, body weight, sex ratio, physical development landmarks (eye opening and pinna detachment), observed nursing behavior, and sexual maturation. F_1 generation litters were randomly standardized on lactation day 5 and assigned to 4 subsets for continued observation. On postnatal day (PND) 12, fixed brain weight measurements (10 pups/sex/dose) and neuropathological evaluations including morphometrics (6 pups/sex/dose) were performed on Subset 1 pups, with the remaining 10 pups/sex/dose necropsied for gross lesions. In Subset 2, 8 pups/sex/dose were selected for evaluation of learning and memory; evaluations were performed on PNDs 23-25 and 62-92. These Subset 2 animals were sacrificed on PNDs 97-101, following the last evaluation. The Subset 2 pups not selected for evaluation were necropsied for gross lesions on PND 22. The Subset 3 pups were tested for motor activity on PNDs 14, 18, 22, and 61 and auditory startle habituation on PNDs 23 and 62; all Subset 3 animals were sacrificed on PNDs 63 or 64 following the last evaluation. In Subset 4 pups, fixed brain weights were determined in 10 pups/sex/dose, neuropathological examinations were performed on 6 pups/sex/dose, and all remaining Subset 4 animals (10/sex/dose) were necropsied for gross lesions upon sacrifice on PND 66-77.

Maternal toxicity in the high-dose (5 mg/kg/day) animals was manifested as increased signs of autonomic function toxicity, apparent at the end of gestation as fasciculations (6/25 treated vs 0/25 controls), and during early lactation (days 1-5) as fasciculations (16/24 treated vs 0/25 controls), hyperpnea (8/24 treated vs 0/25 controls), and hyperreactivity (17/24 treated vs 2/25 controls). Dams with all pups dying were increased in the high-dose group (3/23 treated vs 0/25 controls). There were no significant effects on bodyweight, food consumption, or pregnancy parameters. There were no unscheduled deaths in the maternal animals.

Brain ChE activity was decreased in the high-dose (↓90%) and mid-dose (↓18%) dams as compared to control. Erythrocyte (↓41-99%) and plasma (↓43-92%) ChE activities were decreased in a dose-dependent manner in all treated groups.

The maternal toxicity NOAEL was not observed.
The maternal LOAEL was < 0.3 mg/kg/day, based on plasma and RBC cholinesterase inhibition.

For the F_1 generation pups, the high-dose group bodyweights were significantly reduced (↓8-15%) at PND 1 and 5 (pre- and post-culling). Bodyweights were also reduced from birth to PND 22 in Subset 4 high-dose animals (↓5-19%); bodyweight gains were reduced in these animals during the same period (↓5-30%). Additionally, compared to the controls, reduced terminal body weights were observed in the Subset 1 (PND 12) high-dose animals (↓17-19%) and the Subset 4 (PND 66) high-dose males (↓10%). For the F_1 generation adults, body weights of the

high-dose males were decreased at PND 22 through 66 (↓11-17% vs controls). High-dose F_1 adult females also weighed less than controls at PND 22 (↓17% vs controls), but were of similar weight at PND 66. Bodyweight gains were also decreased in the high-dose males for the PND 22-40 interval (↓13% vs controls) and PND 40-66 interval (↓7%). Food consumption was decreased immediately after weaning (PND 23-30) in high-dose males and females (↓13% vs controls).

Development as assessed by pinna unfolding was delayed (4.0 days in treated vs 3.5 days in controls) in the high-dose group. Sexual maturation was delayed as assessed by time to preputial separation (106% of controls) and vaginal patency (103% of controls).

Pup viability was reduced as assessed by the following parameters: surviving pups/ litter (↓27%) and live litter size at culling (↓16%), pup viability index (↓29%), and pups found dead or presumed cannibalized (day 1 - 7.2 % treated vs 0.0% controls; days 2 to 5 - 24.7% treated vs 1.3% controls).

There were no statistically significant differences between the groups in the average acquisition and delay training. Additionally, there were no differences among dose groups when comparing retention of information during PNDs 23-25 and 62-92. Motor activity was decreased in high dose male and female pups on PND 14 (↓56% in males and ↓37% in females), and increased in high dose females on PNDs 18 and 22 (↑51% on both days). On PND 61, motor activity was increased for both sexes (↑16-17%). There was a statistically significant increase (↑16-25%) in the latency to peak response during the auditory startle habituation assessments on PND 23 in the high-dose animals compared to concurrent controls. At PND 62, the latency to peak response in the high-dose animals was 10-12% higher than in the controls. Additionally, the peak response amplitudes in the high-dose animals were decreased by 9 to 29% on PNDs 23 and 62 (not statistically significant) compared to the controls.

There were no gross or microscopic lesions of the nervous system in Subset 1 or 4 offspring. Subset 1 high-dose males at PND 12 had reduced absolute brain weights (↓9% vs controls), increased relative brain weights (↑13% vs controls), reduced anterior to posterior measurement of the cerebellum (↓24% vs controls), reduced height of the cerebellum (↓14% vs controls), decreased thickness of the parietal cortex (↓6% vs controls), and decreased thickness of the hippocampal gyrus (↓9% vs controls). High-dose female pups had reduced absolute brain weights (↓9% vs controls), increased relative brain weights (↑14% vs controls), decreased thickness of the parietal cortex (↓6% vs controls), decreased width of the caudate-putamen (↓10% vs controls), and decreased thickness of the hippocampal gyrus (↓12% vs controls). In Subset 4 F1 animals, killed on PND 66, morphometric analysis revealed significantly decreased parietal cortex measurements in high-dose (↓5%) and mid-dose (↓4%) females, as compared to controls. Decreases in the thickness of the hippocampal gyrus in high-dose females (↓7%) resulted in contradictory statistical results when compared to controls; decreases in mid-dose (↓4%) females as compared to control were not found to be statistically significant. There was no evaluation of the morphometric data for low dose females at PND 66. Brain weight in high dose females was similar to control brain weight at day 66 (↓0.3%).

It is not possible to definitively classify findings in the preweaning offspring as having originated with pre- or postnatal exposure, nor as resulting from developmental perturbation versus direct systemic- or neurotoxicity. However, adverse findings in the adult (~PND 66) offspring, i.e., alterations in motor activity, auditory startle response, and brain structure (decreased measurements of the parietal cortex and hippocampal gyrus, in the absence of brain weight deficits) can be interpreted to represent the long-term sequellae of developmental exposure to chlorpyrifos.

Adverse effects in the offspring have been identified at the MDT of 1.0 mg/kg/day; these include a significant treatment-related decrease in the measurement of the parietal cortex, supported by possible (although nonsignificant) alterations in the hippocampal gyrus, in the brain of female rats at postnatal day 66. However, due to the lack of morphometric data for low-dose (0.3 mg/kg/day) female rats at postnatal day 66, **the offspring NOAEL and LOAEL cannot be determined.**

REFERENCES (cited by the Registrant):

Carney, E., B. Scortichini and J. Crissman (1998) Feed restriction during in utero and neonatal life: effects on reproductive and developmental end points in the CD rat. *Toxicologist* 42, Suppl. 1(abstract 506):102-103.

Gramsbergen, A. and J. Westerga. (1991) Locomotor development in undernourished rats. *Behavioral Brain Research* 48:57-64.

Peeling, A.N. and J.L. Smart. (1994) Review of literature showing that undernutrition affects the growth rate of all processes in the brain to the same extent. *Metabolic Brain Disease* 9(1):33-42.

Attachment 1

Individual Morphometric Data - Adult (PND 66) F1 Generation Male Rats									
Rat No.	Brain Wt.	Ant/Post Cerebrum	Ant/Post Cerebellum	Frontal Cortex	Parietal Cortex	Caudate Putamen	Corpus Callosum	Hippocampal Gyrus	Cerebellum
0 mg/kg/day									
405	2.221	15.6	5.5	1920	1656	2928	317	1488	5040
414	2.249	16.2	5.9	1872	1800	2976	269	1680	4944
416	2.264	15.6	5.4	1656	1728	2784	269	1656	5376
418	2.371	15.5	5.6	1680	1680	2544	230	1752	5280
463	2.394	16.5	5.7	1824	1824	2640	250	1584	4896
478	2.300	16.0	6.0	1800	1848	2928	259	1680	5376
Mean	2.300	15.900	5.683	1792.000	1756.000	2800.000	265.667	1640.000	5152.000
S.D.	0.069	0.400	0.232	104.797	79.478	176.145	29.049	91.913	218.211
5 mg/kg/day									
403	2.266	15.9	6.0	1728	1728	2784	259	1536	5040
443	2.298	16.0	5.8	1704	1824	2592	250	1608	5376
444	2.310	16.2	5.4	1872	1872	2736	250	1488	5040
451	2.318	16.0	5.6	1824	1824	2880	211	1680	5376
454	2.282	16.5	5.7	1800	1728	2784	259	1608	5328
466	2.317	16.5	5.5	1680	1776	2688	250	1752	4464
Mean	2.299	16.183	5.667	1768.000	1792.000	2744.000	246.500	1612.000	5104.000
S.D.	0.021	0.264	0.216	75.387	58.131	97.980	17.942	95.297	350.980

Data extracted from MRID 44787301, page 13. Brain weight are in grams. Anterior to posterior measurements of the cerebrum and cerebellum are in millimeters. All other parameters are linear measurements in micrometers.

Individual Morphometric Data - Adult (PND 66) F1 Generation Female Rats									
Rat No.	Brain Wt.	Ant/Post Cerebrum	Ant/Post Cerebellum	Frontal Cortex	Parietal Cortex	Caudate Putamen	Corpus Callosum	Hippocampal Gyrus	Cerebellum
0 mg/kg/day									
806	2.092	16.0	5.3	1704	1824	2784	211	1728	4896
807	2.060	15.3	5.6	1776	1776	2496	259	1728	5184
825	2.155	15.5	5.5	1704	1800	2400	221	1728	5136
835	2.125	15.5	5.7	1680	1824	2640	278	1680	4896
846	2.191	16.0	5.8	1776	1800	2592	250	1776	4992
870	1.993	15.4	5.2	1824	1728	2544	250	1608	4992
Mean	2.103	15.617	5.517	1744.000	1792.000	2576.000	244.833	1708.000	5016.000
S.D.	0.071	0.306	0.232	56.114	36.133	131.161	24.766	57.633	120.479
1 mg/kg/day									
809	2.005	15.5	5.5	1680	1680	2400	240	1632	4992
828	2.201	15.8	5.4	1824	1728	2400	288	1776	4704
839	2.055	15.7	5.3	1680	1680	2592	259	1416	4704
841	2.142	15.3	5.3	1680	1728	2736	259	1608	4992
853	2.187	16.2	6.0	1824	1704	2784	259	1752	5040
862	2.172	15.3	5.5	1800	1776	2400	240	1680	4896
Mean	2.127	15.633	5.500	1748.000	1716.000	2552.000	257.500	1644.000	4888.000
S.D.	0.079	0.344	0.261	75.004	36.398	178.097	17.604	129.467	150.008

Data extracted from MRID 44787301, pages 14. Brain weight are in grams. Anterior to posterior measurements of the cerebrum and cerebellum are in millimeters. All other parameters are linear measurements in micrometers.

Individual Morphometric Data - Adult F1 (PND 66) Generation Female Rats (continued)									
Rat No.	Brain Wt.	Ant/Post Cerebrum	Ant/Post Cerebellum	Frontal Cortex	Parietal Cortex	Caudate Putamen	Corpus Callosum	Hippocampal Gyrus	Cerebellum
5 mg/kg/day									
843	2.129	15.5	5.3	1752	1632	2832	221	1632	5088
848	1.993	15.6	5.3	1728	1728	2784	250	1560	4944
849	2.022	15.1	5.3	1776	1632	2640	211	1584	5232
858	2.054	15.8	5.5	1824	1752	2784	259	1440	4992
859	2.012	15.4	5.4	1608	1704	2640	221	1680	4608
874	2.079	15.7	5.5	1656	1752	2544	240	1656	4944
Mean	2.048	15.517	5.383	1724.000	1700.000	2704.000	233.667	1592.000	4968.000
S.D.	0.050	0.248	0.098	79.478	55.599	112.228	18.886	86.755	207.569

Data extracted from MRID 44787301, page 14. Brain weight are in grams. Anterior to posterior measurements of the cerebrum and cerebellum are in millimeters. All other parameters are linear measurements in micrometers.