HED DOC. NO. 014088

DATE: April 6, 2000
MEMORANDUM

SUBJECT: ***CHLORPYRIFOS*** **- REEVALUATION BASED ON *PHASE 3* (PUBLIC COMMENTS) of the TRAC Process -** Report of the Hazard Identification Assessment Review Committee.

FROM: Deborah Smegal, Toxicologist and Risk Assessor
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Health Effects Division (7509C)

THROUGH: Jess Rowland, Co-Chairman
And
Elizabeth Doyle, Co-Chairman
Hazard Identification Assessment Review Committee
Health Effects Division (7509C)

TO: Steve Knizner, Branch Senior Scientist
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On **January 20, 2000**, the Health Effect Division's (HED) Hazard Identification Assessment Review Committee (HIARC) re-evaluated the toxicology endpoint selected for the chronic RfD, considered the impact of additional registrant data on the conclusions of the developmental neurotoxicity and companion studies, and evaluated recent scientific literature that primarily pertains to the increased susceptibility and sensitivity of infants and children.

On **February 2, 2000**, the HIARC reconvened to modify the definition of short-term duration from 1-7 days to 1-30 days because the exposure durations for the toxicity studies do not match the agricultural scenarios.

On **March 28, 2000** the HIARC met again to evaluate, specifically, the red blood cholinesterase data of the two year feeding study in dogs (MRID Nos. 00064933 and 00146519). The HIARC's review included: 1) the in-depth analysis of the data, 2) statistical analysis of the RBC ChE data conducted by Sielken/Holden on behalf of the registrant and submitted to OPP, and 3) the statistical analysis conducted by HED.

Presented in this report are the chronology of the various HIARC meetings on chlorpyrifos and a summary table of the doses and endpoints that are currently used for risk assessment of chlorpyrifos. In addition, HED has prepared responses to several of the Registrant's comments received during and subsequent to the Phase 3 of the Tolerance Reassessment Advisory Committee (TRAC) process.

Committee Members in Attendance: Bill Burnam, David Nixon, Elizabeth Doyle, Pam Hurley, Tina Levine, Elizabeth Mendez, Nicole Paquette, Jess Rowland, Vicki Dellarco, and Brenda Tarplee.

Other HED staff present at the meeting were Deborah Smegal and John Doherty, Re-Registration Branch 3, Susan Makris of Toxicology Branch 1, and Hans Allender of Chemistry and Exposure Branch 1.

Data Presentation:
and
Report Preparation

Deborah Smegal, MPH
Toxicologist

I. BACKGROUND

On **February 21, 1986**, the Health Effects Division's (HED) RfD/Peer Review Committee established the Reference Dose (RfD) of 0.003 mg/kg/day based on the NOAEL of 0.03 mg/kg/day in a study in humans and an Uncertainty Factor of 10 to account for intra-species variation. This RfD was re-affirmed at the subsequent meetings held on March 4, 1988, September 8, 1993, May 25, 1995 and November 23, 1995.

On **April 28, 1996**, HED's Toxicology Endpoint Selection Committee (TESC) selected the doses and endpoints for acute dietary as well as occupational and residential exposure risk assessments TES Document dated, August 15, 1994 **(HED Document No.** 013130).

On **December 11, 1997** , HED's Hazard Identification Assessment Review Committee (HIARC) re-assessed the RfD in response to a report (*Proposed Reference Dose (RfD) for Acute and Chronic Exposure to Chlorpyrifos Based on the Criteria Described by the Acute Cholinesterase Risk Assessment Task Force and the Available Animal and Human Data)* submitted by the Registrant (MRID No. 44271001). At this meeting, the HIARC also re-assessed the doses and endpoints selected for dietary and non-dietary exposure risk assessments by TESC, and discussed the critical scientific literature relevant to the potential risk to infants and children (as required by FQPA). The HIARC's conclusions are presented in the **HIARC report dated February 2, 1998 (HED Document No. 012471).**

On October 29, 1998 the HIARC evaluated the five additional studies listed below, the Registrant's rebuttal (of 8/4/98) and their impact on the RfD and FQPA assessment. Studies reviewed by HIARC were: Developmental Neurotoxicity (MRID No. 44556901 & 44661001); Cholinesterase and Metabolite Determination (MRID No. 44648102);Special Neurotoxic Esterase Assay (MRID No. 44273901); Cognitive Neurotoxicity (MRID No. 44020901) and Blood Time Course (Part A) (MRID No. 44648101). The HIARC's conclusions are presented in the **HIARC report dated December 7, 1998 (HED Document No. 013004).**

In **December 10-11, 1998**, the Science Advisory Board/Scientific Advisory Panel discussed both the ethical concerns and the scientific merit of using humans subjects for testing pesticides. The Agency is currently developing a policy for the use of human studies in risk assessment. In the interim, HED has taken the following course of action.

In **January, 1999**, the HIARC developed a specific outline of parameters and questions for the re-examination of human studies. Human studies were used in endpoint selection for risk assessment for eight organophosphates, including chlorpyrifos. These studies were re-evaluated according to the parameters and questions developed by the Committee. The HIARC then selected doses and endpoints from toxicity studies with animals for each of these eight organophosphates. The HIARC examined the human data in conjunction with the animal data to determine the appropriate inter-species uncertainty factor.

In the evaluation of the comparative toxicology data in laboratory animals and humans, when the data were suitable for comparison, the Committee relied mainly on the LOAEL for cholinesterase inhibition at comparable time points (duration). The comparative data were evaluated as follows:

> If the comparative data indicate (by the dose level and the magnitude of the effect) that humans are more sensitive than laboratory animals, there is no justification for reducing the 10x inter-species uncertainty factor.
>
> If the comparative data indicate (by the dose level and the magnitude of the effect) that humans and laboratory animals are equally sensitive or that humans are less sensitive than laboratory animals, consideration was given to reducing the inter-species uncertainty factor.

On **January 14, 1999,** the HIARC evaluated the oral (Coulston *et a*l., 1972; MRID No. 000112118) and the pharmacokinetic (Nolan *et al.*, 1982; 000249203) studies in humans with chlorpyrifos using the parameters developed for evaluation of the human studies. The HIARC classified these studies as *supplemental* because the results provided useful scientific information that can be used as supportive data along with the results from the animal studies, but the studies alone are not sufficient for endpoint selection or risk assessments due to technical limitations.

On **February 2, 1999,** the HIARC evaluated the doses and toxicology endpoints selected for chlorpyrifos based solely on animal toxicity studies. On **February 23, 1999,** the HIARC re-convened and determined the appropriate uncertainty factors and margins of exposures for dietary and non-dietary risk assessments. The HIARC's conclusions were presented in the March 4, 1999 (HED Doc No. 013249).

On **January 20, 2000,** the HIARC addressed issues raised in the Phase 3 Public comments and in the registrant's rebuttal. The committee re-evaluated the toxicology endpoint selected for the chronic RfD, considered the impact of additional registrant data on the conclusions of the developmental neurotoxicity and companion studies, and evaluated recent scientific literature that primarily pertains to the increased sensitivity and susceptibility of infants and children.

On **February 2, 2000,** the HIARC re-convened to modify the short-term duration from 1-7 days to 1-30 days because the exposure durations for the toxicity studies do not match the agricultural scenarios.

On **March 28, 2000** the HIARC met again to evaluate, specifically, the red blood cholinesterase data of the two year feeding study in dogs (MRID Nos. 00064933 and 00146519). The HIARC's review included: 1) the in-depth analysis of the data, 2) statistical analysis of the RBC ChE data conducted by Sielken/Holden on behalf of the registrant and submitted to OPP, and 3) the statistical analysis conducted by HED.

This report supersedes the previous HIARC documents with respect to toxicity endpoints and increased susceptibility issues as they relate to the FQPA safety factor. The reader is advised to refer to the HIARC reports listed below for details on dose and toxicology endpoint selections and FQPA assessments. The summary table in Section V presents the doses and endpoints based on animal studies that are currently used for the risk assessment of chlorpyrifos.

Date	HED Doc. No.	Report Title
February 2, 1998	012471	***CHLORPYRIFOS - FQPA REQUIREMENT*** - Report of the Hazard Identification Assessment Review Committee.
December 7, 1998	013004	***CHLORPYRIFOS - RE-EVALUATION*** - Report of the Hazard Identification Assessment Review Committee.
March 4, 1999	013249	***CHLORPYRIFOS - HAZARD IDENTIFICATION BASED ON ANIMAL STUDIES*** - Report of the Hazard Identification Assessment Review Committee.

II. HAZARD IDENTIFICATION

A. Acute Dietary (One-Day)

Study Selected: Concentration-Time Course of Chlorpyrifos and Chlorpyrifos-Oxon in Blood. [Non-Guideline]

MRID. Nos. 44648102 (Mendrala and Brzak 1998)

Executive Summary:

This study (MRID No.: 44648102) was conducted to help construct and validate a physiologically-based pharmacokinetic model for chlorpyrifos (Unlabeled - 99.8% a.i., Lot # MM930503-17; Labeled - 89.4% a.i., Lot # B930-51 [INV1134]) a weak inhibitor of acetyl-cholinesterase activity, and its metabolites, chlorpyrifos-oxon (OXON), a strong cholinesterase inhibitor and 3,5,6-trichloropyridinol. Groups of 24 Fischer 344 male rats were given a single gavage dose of 0.5, 1, 5, 10, 50, or 100 mg/kg chlorpyrifos in corn oil. Four rats from each group were killed 10 and 20 minutes and 1, 3, 6, and 12 hours after treatment. Cholinesterase (ChE) activity was measured in the brain and plasma at each time point, as well as the plasma concentration of the test material and its OXON metabolite. In a separate portion of the study, four male rats were given a single gavage dose of labeled chlorpyrifos at a concentration of 5 or 100 mg/kg and were sacrificed three hours later. Blood was collected from the animals at sacrifice and the concentration of the test material and its metabolites 3,5,6-trichloropyridinol (TCP) and OXON determined.

Plasma ChE activity decreased in a time- and dose-dependent manner. In rats, plasma ChE consists of predominantly the neuronal form, acetyl ChE (AChE), with an approximate ratio of 60% AChE to 40% butyryl ChE (BuChE). The plasma ChE activities of rats treated with 0.5, 1, 5 or 10 mg/kg were maximally decreased 3-6 hours after treatment, with both the decrease and recovery of activity being dose-dependent. Plasma ChE activity was not significantly inhibited in the 0.5 mg/kg group. In the 1 mg/kg dose group, plasma ChE activity was significantly inhibited approximately 28% and 40% relative to controls at 3 and 6 hours post exposure, respectively. By 12 hours post-exposure, plasma ChE activity was still significantly inhibited about 15%. The decrease in activity of rats treated with 50 or 100 mg/kg began within 10 minutes of treatment. By 12 hours after treatment, both groups were approximately 11% of the control group and had not shown signs of recovery.

Brain AChE activity was not affected as dramatically by test material treatment as plasma activity with only the 10, 50, and 100 mg/kg dose groups showing significant effects. The brain AChE activity of rats treated with 10 mg/kg test material began to decline within three hours of treatment and was significantly decreased by six hours after treatment. The brain AChE activity in the 50 or 100 mg/kg dose groups decreased significantly within one hour of treatments; and by 12 hours, it was approximately 30% and 20%, respectively, of control. In none of the affected groups did brain cholinesterase show signs of recovery.

Peak chlorpyrifos blood concentrations occurred within three hours of treatment in all but the lowest dose group. The area under the curve (AUC) was calculated as 0.4, 1.1, 5.0, and 12.5 μmole hr L^{-1} for the 5, 10, 50, and 100 mg/kg groups, respectively and yielded calculated blood half-lives of chlorpyrifos of 2.7, 1.5, 2.1, and 7.3 hours for the 5.0, 10.0, 50.0, and 100.0 mg/kg dose groups, respectively. Regardless of dose, the highest concentration of OXON detected was 2.5 ng/g found in the blood of rats treated with 50 mg/kg test material one hour post-treatment. Following treatment with 5 or 100 mg/kg labeled test material, ≥98% of the activity detected in the blood was identified as TCP metabolite with the remaining attributed to the parent compound. Since OXON is an intermediate in the formation of TCP and none of the metabolite was detected, these studies support that the half-life of the OXON metabolite is short (reportedly 10 seconds) and that *in vivo* metabolism of chlorpyrifos is rapid.

The LOAEL is 1 mg/kg/day based on 28 and 40% plasma ChE inhibition 3 and 6 hours, respectively following exposure. The NOAEL is 0.5 mg/kg/day.

This study is considered acceptable (nonguideline). This is a special study intended to investigate specific parameters and is acceptable for the purposes for which it was intended.

Dose and Endpoint for Establishing the acute RfD: NOAEL = 0.5 mg/kg/day based on significant 28-40% plasma ChE inhibition 3-6 hours after dosing in male rats (total of eight) with a single dose of 1 mg/kg. As noted previously, in the rat, approximately 60% of the plasma ChE is the neuronal form, AChE, while approximately 40% is present as BuChE. Although, the blood time course study did not measure red blood cell (RBC) ChE activity, it is likely that biologically significant RBC ChE inhibition would have been observed 3-6 hours post exposure at 1 mg/kg based on the results the Special Neurotoxic Esterase Study (Dittenber 1997, MRID 44273901). In addition, plasma and RBC ChE inhibition noted in the range of 0.75 and 1.5 mg/kg in adult male rats, respectively in the recent single dose study by Zheng et al. (2000) support the acute NOAEL of 0.5 mg/kg (see below discussion).

Comments about Study and Endpoint: The HIARC selected the dose and endpoint based on a weight of the evidence approach using the results of four single oral dose studies [Mendrala and Brzak 1998, Dittenber 1997 (44273901), Wilmer et al . 1992, (MRID 42669101 and 42943101), and Zheng et al. 2000]. Although the selected study focuses on the phamacokinetics of chlorpyrifos, the HIARC concluded that it provides valuable information and identifies a NOAEL and LOAEL of 0.5 and 1 mg/kg/day, respectively for plasma ChE activity. HIARC concludes that the NOAEL is appropriate for this exposure period of concern (i.e., after a single dose). This is the only registrant-submitted single dose animal study that measured cholinesterase activity at the peak time of inhibition of 3-6 hours post-exposure. The other two registrant-submitted studies either measured ChE activity 24 hours post-dosing (Dittenber 1997, MRID 44273901), or failed to measure ChE activity at all [i.e., acute neurotoxic study, Wilmer et al . 1992, (MRID 42669101 and 42943101)].

The acute RfD is also supported by a recent publication by Zheng et al. (2000) that evaluated single dose (gavage) exposure of 0, 0.15, 0.45, 0.75, 1.5, 4.5, 7.5 or 15 mg/kg to neonatal (7 day) and adult male rats (n=6-7/treatment/age/time point). Rats were sacrificed 4 hours after the acute dose. In this study, adult male rats exposed to a single dose of 1.5 mg/kg chlorpyrifos

exhibited mean 23.7% and 29.7% (statistically significant) inhibition of plasma and red blood cell ChE, respectively (the LOAEL). Exposure to 0.75 mg/kg was considered to be a single dose NOAEL for both plasma and RBC ChE inhibition for adult males. Neonatal rats exposed to 0.45 mg/kg exhibited statistically significant mean 27.1% plasma ChE inhibition (LOAEL), while higher exposure to 1.5 mg/kg resulted in mean 31.8% (statistically significant) and 20.6% RBC and brain ChE inhibition, respectively (LOAEL).

The Special Neurotoxic Esterase (NTE) Study (Dittenber 1997, MRID 44273901) also provides support for the acute RfD, and observed significant plasma ChE, RBC AChE and heart AChE inhibition of 45%, 17% and 19%, respectively 24 hours after dosing female F344 rats with 5 mg/kg/day, but no effects at 1 mg/kg/day. This study demonstrates that RBC and heart AChE inhibition are correlated with plasma ChE inhibition and would be expected to be similarly inhibited approximately 15-20% at 1 mg/kg/day during the peak period of inhibition, 3-6 hours following exposure. In addition, the female data from the NTE study support the findings of the male data from the blood time course study.

Based on current OPP policy, the human data were not considered in the development of the acute RfD, but are discussed for complete characterization of available information. The human data also provide support for the acute RfD based on animal data, but indicate that humans may be as sensitive and possibly even more sensitive than animals based on plasma butyryl ChE (BuChE) inhibition. No effects were observed in a human study of 4 male volunteers/dose after a single exposure to the highest dose of 0.1 mg/kg/day (plasma ChE activity ranged from 12% ↑ to 32% ↓relative to baseline measurements) (Coulston et al. 1972, MRID No.00095175). However, 6 adult males exposed to a single oral dose of 0.5 mg/kg/day exhibited peak plasma ChE inhibition of 64-88%,12 to 24 hours post-exposure (Nolan et al. 1982, MRID No. 00249203). In the latter study, there were no clinical signs of toxicity, although the plasma ChE activity did not return to pre-dose levels until 30 days post-exposure. HED notes that this long recovery is more characteristic of RBC acetyl ChE (AChE) inhibition, not plasma ChE inhibition, based on the 2 year dog data. It is interesting to note that peak RBC ChE inhibition of 11-52% occurred on post-exposure day 4. The registrant contends that the RBC ChE inhibition noted on day 4 is really an analytical artifact given what is known about the pharmacokinetics of chlorpyrifos. If this is the case, it raises concerns about the quality and reliability of the study data. A more recent single dose human study was submitted on April 27, 1999, but is an incomplete submission because two Appendices with critical data were omitted. HED will review this study once the submission is complete.

It is possible that the potential sensitivity in humans can be attributed to the species differences in composition of in plasma ChE between the rat and humans. For example, in rats, plasma ChE consists of approximately a 60:40 ratio of AChE and BuChE, while in humans, plasma ChE is predominantly as BuChE, which is more sensitive to inhibition than AChE.

Uncertainty Factor(s): 100 which includes inter-species (10X) extrapolation and intra-species (10X) variation.

$$\textbf{Acute RfD} = \frac{0.5 \text{ mg/kg/day}}{100} = \textbf{0.005 mg/kg/day}$$

This risk assessment is required.

B. <u>Chronic Dietary Risk Assessment</u> (Reference Dose)

On **February 2, 1999**, ***the HIARC, re-assessed the toxicology database for chlorpyrifos to select doses and endpoints based on animal toxicity studies for dietary and non-dietary exposure risk assessments. At this meeting the HIARC reviewed the results of 15 oral toxicity studies most of which were classified as acceptable/minimum fulfilling the Subdivision F Guideline requirements***. Those studies are as follows:

(1) 2-Year Feeding -Dog (McCollister et al. 1971 and Kociba et al. 1985);
(2) 2-Year Feeding - Rat (Crown et a. 1990);
(3) 2-Year Feeding - Rat (Young and Grandjean 1988)
(4) Two generation Reproduction - Rat (Breslin et al. 1991)
(5) Carcinogenicity - Mice (Gur 1992
(6) 90-day Capsule -Dog (Barker 1989);
(7) 90-day Feeding - Rat (Crown et al. 1985);
(8) 90 day Feeding - Rat (Szabo et al. 1988),
(9) Developmental Toxicity - Rat (Rubin et al. 1987a),
(10) Developmental Toxicity - Rat (Ouellette et al. 1983),
(11) Developmental Neurotoxicity - Rat (Hoberman 1998a,b)
(12) Developmental Toxicity - Rabbit (Rubin et al. 1987b),
(13) Developmental Toxicity - Mouse (Deacon et al. 1979),
(14) Companion study to the Developmental Neurotoxicity Study (Mattsson et al. 1998),
(15) 4- week cognitive study - Rat (Maurissen et al. 1996)

In addition to these animal studies, the HIARC also considered a study conducted in human volunteers (Coulston et al., 1972). Based on current OPP Policy, the human data <u>were not used</u> in the development of the chronic RfD, but are discussed for complete hazard characterization of the available data.

These studies demonstrated that chlorpyrifos, like other organophosphates, has anticholinesterase activity (ChEI) in several species including mice, rats, rabbits, and dogs. ChEI was seen in all three compartments [plasma, red blood cell (RBC) and/or brain] in all species tested, except rabbits for which no brain ChE data are available. In the evaluation of the study results, the HIARC considered the nature of the dose-response as well as consistencies in the level of inhibition seen in the three compartments in different studies. **Dogs appear to be the most sensitive species for cholinesterase inhibition (ChEI)** based on a comparison of effect levels.

HIARC used the weight of evidence (WOE) approach from the 15 studies for selecting the dose and endpoint for deriving the chronic RfD. The first step in the WOE approach was to identify the critical species and study/studies. Of the 15 oral animal toxicity studies considered, the HIARC determined 5 guideline studies to be critical. The NOAELs and LOAELs in these studies are presented in Table 1. Next, the 5 studies were examined with respect to dose, effect, and consistency in results. The dose and effect information from these critical studies taken together show significant plasma and RBC cholinesterase inhibition at lower doses (0.03 to 0.3 mg/kg/day)

when compared to the occurrence of ChEI at higher doses (0.9 to 3 mg/kg/day) in the remaining studies.

Following the selection of the 5 critical studies, the HIARC determined that the remaining 10 studies are not appropriate for the weight of evidence considerations. The reasons for not selecting these studies are provided in Table 2. The dose and effect information from these studies showed the presence of plasma and RBC ChEI at higher doses (0.9 to 3 mg/kg/day). In 7 studies, plasma ChEI was not seen until 1 mg/kg/day. One study did not meet the guideline requirement. RBC ChEI data were either not available or not reliable in 3 studies, while 2 studies failed to test doses low enough to identify a NOAEL. One of the ten studies (companion study to DNT) actually provides results identical to the developmental neurotoxicity and could be used as yet another critical study in the WOE considerations. Executive summaries for the five critical studies are presented in Appendix A.

Table 1. Critical Studies in the Weight of Evidence Considerations

Study	NOAEL (mg/kg/day)		LOAEL (mg/kg/day)	
	Plasma	**RBC**	**Plasma**	**RBC**
2-Year Feeding -Dog (McCollister et al. 1971/ Kociba et al. 1985)	0.01	0.03	0.03	Not established
90-Day -Capsule - Dog (Barker 1989)	0.01	0.01	0.22	0.22
90-Day Feeding - Rat (Crown et al. 1985)	Not Identified	Not Measured	0.025 (LDT)	Not Measured
2-Year Feeding -Rat (Crown et al., 1990)	0.013	0.013	0.33	0.33
Developmental Neurotoxicity - Rat (Hoberman et al. 1998a,b), verified by Mattsson et al. 1998 in DNT companion study	Not Identified		0.3 (LDT)	0.3 (LDT)

LDT = lowest dose tested

The critical studies demonstrate that:

- **Plasma ChEI**: **NOAEL** consistent at 0.01 mg/kg/day in 3 studies and was not identified in the other 2 studies because plasma ChEI was seen at the lowest dose tested .

- **RBC ChEI: NOAEL** 0.01 mg/kg/day in 2 studies; 0.03 mg/kg/day in 1 study; not

measured in 1 study; and not identified in 1 study because RBC ChEI seen at the lowest dose tested.

- **Plasma ChEI**: **LOAEL** was 0.03 mg/kg/day in 2 studies, 0.22 mg/kg/day in 1 study, and 0.3 mg/kg/day in 2 studies.

- **RBC ChEI: LOAEL** was 0.22, 0.3, and 0.33 mg/kg/day in 3 studies, could not be established in 1 study due to data quality issues, and was not tested in the remaining study.

Table 2. Studies Not Used in Weight of Evidence Considerations.

Study	Reasons for Not Considering in Weight of Evidence Considerations.
2-Year Rat Feeding (Young and Grandjean 1988)	Results inconsistent with the results of the other chronic study in rats. The LOAEL (1 mg/kg/day) for plasma and RBC ChEI was **3 times higher** than the LOAEL (0.33 mg/kg/day) established in the *Crown et al., 1990* chronic rat study.
Two- Generation Reproduction - Rat (Breslin et al. 1991)	Following 13 weeks of exposure, the LOAEL (1 mg/kg/day) for plasma ChEI is **3 times higher** than the LOAEL (0.33 mg/kg/day) in the same species (rats) after similar exposure period (i.e., 90 days) in the *Crown et al 1985* subchronic study. A comparison of the LOAELs for RBC ChEI could not be made since this was not measured in the Crown et al study.
90-Day Rat (Szabo et al., 1988)	Results inconsistent with the other 90-day study in the same species (i.e., *Crown et al 1985*). The LOAEL (1 mg/kg/day) for plasma ChEI in the *Szabo et al* study is **40x higher** than the LOAEL (0.025 mg/kg/day) established in the *Crown et al* study. A comparison of the LOAELs for RBC ChEI could not be made since this was not measured in the Crown et al study.
Carcinogenicity - Mice (Gur 1992)	RBC ChEI results not used because of concern for the validity of these data. The LOAEL of 0.9 mg/kg/day for plasma ChEI is higher than the LOAELs for rats and dogs.
Developmental Toxicity - Rat (Rubin et al. 1987a)	Not appropriate for use because RBC ChEI was not measured and a NOAEL was not established for plasma ChEI (plasma ChEI LOAEL=0.5 mg/kg/day)
Developmental Toxicity - Rat (Ouellette et al 1983)	Plasma ChEI was inconsistent with the Rubin study in the same species and comparable exposure duration. The LOAEL of 3 mg/kg/day is higher compared to the LOAEL of 0.5 mg/kg/day seen in the Rubin study and the LOAEL of 0.3 mg/kg/day in the DNT (used in WOE).

Table 2. Studies Not Used in Weight of Evidence Considerations.	
Study	**Reasons for Not Considering in Weight of Evidence Considerations.**
Developmental Toxicity - Rabbit (Rubin et al., 1987b)	Not appropriate because RBC ChEI was not measured and a NOAEL for plasma ChEI was not established (plasma ChEI LOAEL =1 mg/kg/day)
Developmental Toxicity Mouse (Deacon et al, 1979)	Study unacceptable
Cognitive Study -Rat (Maurissen et al. 1996)	LOAEL (1 mg/kg/day) for plasma and RBC ChEI was higher than those seen in this species in other studies.
DNT Companion Study - Rat (Mattsson et al, 1998)	Same results as that of the DNT which was selected as one of the 5 critical studies.

Based on the results of the plasma and RBC ChEI in these 5 critical studies, the HIARC selected the 0.03 mg/kg/day as the NOAEL for derivation of the chronic RfD. The rationale for selection of this dose as appropriate for risk assessment is based on the following factors:

1) 0.01 mg/kg/day was the NOAEL for plasma ChEI in 3 studies (2-year dog, 2 year rat, 90-day dog).
2) 0.01 mg/kg/day was the NOAEL for RBC ChEI in 2 studies (2-year rat and 90-day dog).
3) 0.03 mg/kg/day was the NOAEL for RBC ChEI in 1 study (2-year dog).
4) 0.03 mg/kg/day was the LOAEL for plasma ChEI in 2 studies (2-year dog and 90-day rat). This dose as a LOAEL is not appropriate for use in a risk assessment, because:
    - the magnitude of inhibition was marginal and variable in the 2-year dog study;
    - it was not always statistically or biologically significant at all intervals in the 1-2 year dog study,
    - not supported by RBC ChEI in male dogs;
    - in the 90-day rat study, plasma inhibition was marginal and occurred only in males at 0.025 mg/kg/day.
5) In addition, the results of the animal studies were corroborated by the results of the study conducted in human volunteers (Coulston et al., 1972). In this study, no effects on ChE were seen in four adult males given 0.03 mg/kg/day for 21 days.

Thus, HIARC concluded that, although the lowest NOAEL observed in some studies is 0.01 mg/kg/day, taken *in toto*, 0.03 mg/kg/day shows a consistent pattern of no adverse effect of cholinesterase inhibition. Although the Agency's interim policy does not allow the use of human studies in regulatory risk assessments, findings in the 1972 human study were consistent with

those of the animal studies and therefore played a part in the weight of evidence approach.

The HIARC determined that the dose of 0.1 mg/kg/day is not a NOAEL based on the following factors:

1) 0.22 mg/kg/day was the LOAEL for RBC ChEI in one study (90-day dog)
2) 0.33 mg/kg/day was the LOAEL for plasma & RBC ChEI in one study (2 year rat).
3) 0.3 mg/kg/day was the LOAEL for plasma & RBC ChEI in one study (DNT-Rat).
4) In addition, the results of the animal studies were corroborated by the results of the study conducted in human volunteers (Coulston et al., 1972). In this study, 36 to 82% plasma ChEI were observed in 4 adult males given 0.1 mg/kg/day for 9 days. In addition, one individual developed possible clinical signs (blurred vision, feeling of faintness and runny nose) related to treatment. These data suggest that humans may be more sensitive than animals based on the extent of ChEI (i.e., up to 80% ChEI at 0.1 mg/kg/day but only up to 43% ChEI at 0.3 mg/kg/day in animals), and possible clinical signs.

Uncertainty Factor (UF): 100 which includes inter-species (10X) extrapolation and intra-species (10X) variation.

$$\textbf{Chronic RfD} = \frac{\text{0.03 mg/kg/day (NOAEL)}}{\text{100 (UF)}} = \textbf{0.0003 mg/kg/day}$$

This risk assessment is required.

C. Occupational/Residential Exposure Risk Assessments

1. Dermal Absorption

Study selected :- None.

MRID No.: None.

Because the registrant has not submitted a study that quantifies dermal absorption of chlorpyrifos in animals, the HIARC estimated a dermal absorption factor of 3% by comparing the dermal LOAEL of 10 mg/kg/day from the 21 day rat dermal study (Calhoun and Johnson 1988, MRID No.40972801) to the oral LOAEL of 0.3 mg/kg/day in the rat developmental neurotoxicity study (Hoberman 1998a,b, MRID Nos. 44556901, 44661001). Both LOAELs are based on cholinesterase inhibition. The resulting estimated dermal absorption is 3% (oral LOAEL of 0.3 ÷ dermal LOAEL of 10 x 100 = 3%). This absorption factor is comparable to the dermal absorption estimated from human data of 1-3% (Nolan et al. 1982, MRID No. 00249203).

Dermal Absorption Factor: **3%** (extrapolated)

On **February 03, 2000**, ***the HIARC revised the short-term duration from 1-7 days to 1-30 days to be more consistent with the exposure durations for the agricultural scenarios. HIARC determined that the 21-day dermal study is appropriate to assess exposures up to 30 days.***

2. Short-Term Dermal - (1-30 days)

Study Selected: 4-Day Dermal Probe and 21-Day Dermal Toxicity Study in Rats (Calhoun and Johnson 1988)

MRID No. 40972801

Executive Summary:

In a 21-day dermal toxicity study (MRID 40972801), 5 Fischer 344 rats/sex/dose were dermally exposed to 0, 0.1, 0.5 , 1 or 5 mg/kg/day chlorpyrifos (100% a.i.) in corn oil on a 12 cm^2 area of the back of each animal once per day, 6 hours/application, 5 days/week for a total of 15 applications in 21 days. In a 4-day dermal probe study used to select the doses, 4 female Fischer 344 rats/dose were treated via dermal application at dose levels of 0, 1, 10, 100 or 500 mg/kg/day chlorpyrifos in corn oil for four consecutive days.

In the 21-day study, there were no signs of treatment-related systemic or dermal toxicity at doses up to 5 mg/kg/day, including effects on cholinesterase inhibition, body weight, food consumption, ophthalmological examination, hematology, or clinical chemistry. In the 4-day probe study, 2 of 4 females in the 1 and 10 mg/kg/day groups developed slight erythema. Dose-related plasma (45, 92 and 98%↓) and red blood cell (16, 49 and 75%↓) cholinesterase inhibition were observed in the 10, 100 and 500 mg/kg/day groups relative to controls. However, statistical analyses were not conducted. The cholinesterase activities of the 1 mg/kg/day females were slightly decreased, but within the historical control range. No other treatment-related effects were noted in the dermal probe study.

The NOAEL and LOAEL for plasma and red blood cell cholinesterase inhibition are 5 and 10 mg/kg/day, respectively, based on the results of both the 21-day and 4-day dermal probe studies.

The combination of the 4-day probe study and the 21-day dermal study are classified as ACCEPTABLE-GUIDELINE and satisfy the guideline requirement (82-2), but not guideline 870.3200, which requires 10 animals/sex/dose for dermal toxicity testing. However, these studies were determined to be useful for risk assessment.

Dose and Endpoint for Risk Assessment: NOAEL =5 mg/kg/day based on plasma and red blood cell ChE inhibition of 45 and 16%, respectively in rats dermally exposed to 10 mg/kg/day for 4 days, but no ChE inhibition in rats exposed to 5 mg/kg/day for a total of 15 applications over a 21 day period. Because the NOAEL is from an animal study, a MOE of 100 should be used in the risk assessment.

For comparison to biomonitoring data in the risk assessment, which evaluates total exposure from oral, dermal and inhalation routes, HIARC recommends using the 21-day dermal study with an adjustment for 3% dermal absorption. Therefore, the dermal absorbed NOAEL = 0.15 mg/kg/day

(i.e., 5 mg/kg/day * 0.03). The corresponding dermal absorbed LOAEL = 0.3 mg/kg/day (i.e., 10 mg/kg/day * 0.03). This dermal absorption adjustment is appropriate because there is a good understanding of the metabolic profile of chlorpyrifos in humans based on the Nolan et al. (1982, 1984) studies, and both the animal and human data support a 3% dermal absorption factor. In addition, the absorbed dermal LOAEL is identical to the oral LOAEL of 0.3 mg/kg/day identified from the developmental neurotoxicity study (Hoberman et al. 1998a,b), and the 2 year rat study (Crown et al. 1990), and similar to the oral LOAEL of 0.22 mg/kg/day identified from the 90 day dog study (Barker 1989) for plasma and RBC ChE inhibition.

Comments about Study and Endpoint: This study is appropriate for the route (dermal) and duration (1-30 days) of exposure concern. Both studies were used in the determination of the dose for risk assessment. In a human pharmacokinetic study, 5 white adult males dermally exposed to 5 mg/kg/day for a single dose had peak plasma ChE inhibition of 27-45% on day 3 and mean RBC ChE inhibition of 8.6% on day 4 (Nolan et al. 1982). On day 9 post dosing, plasma ChE activity was inhibited 13-53% (mean 14%) from baseline levels. These data indicate that humans may be more sensitive to the dermal toxicity of chlorpyrifos than animals. The registrant contends that the plasma and RBC ChE inhibition noted on days 3 and 4 are really an analytical artifact given what is know about the pharmacokinetics of chlorpyrifos. If this is the case, then this raises concern about the data quality of the study. The registrant contends that the kinetic data suggest that in humans, dermal absorption is complete within 24 hours and that plasma esterase returned to baseline within 24-48 hours post dosing.

This risk assessment is required.

3. Intermediate-Term and Long-Term Dermal (30 Days to Several Months; and Several Months to Lifetime, respectively)

Studies Selected: The HIARC selected the same 5 critical studies (based on a weight of evidence approach) used for deriving the chronic RfD for this exposure scenario.

MRID Nos.
(1) 2-Year Dog Feeding Study (00064933, and 00146519)
(2) 90-day Dog Study (42172801);
(3) 2-Year Rat Feeding Study (42172802);
(4) 90-day Rat Study (40436406); and
(5) Rat Developmental Neurotoxicity Study (44556901)

Executive Summary: See Chronic Dietary Discussion and Appendix A.

Dose and Endpoint for Risk Assessment: NOAEL = 0.03 mg/kg/day based on weight of the evidence for plasma and red blood cell ChE inhibition in dogs and rats. See discussion under chronic RfD. Because the NOAEL is from animal studies, a MOE of 100 should be used in the risk assessment.

Comments about Study and Endpoint: The HIARC determined that the 21 day dermal toxicity study is of insufficient duration for this (30-to several months) risk assessment because no 21-day LOAEL was established, and effects were seen in the 4-day probe study (with only 4 females) after only 4 doses. In addition, while the combination of the 4-day probe study and the 21-day dermal study satisfy the older 82-2 guideline requirement, they do not meet the current 870.3200 guidelines, which requires 10 animals/sex/dose for dermal toxicity testing. HIARC selected a dose (NOAEL) based on ChE inhibition observed at 0.22 to 0.3 mg/kg/day after 2 weeks to 2 years in the 5 studies evaluated as part of a weight of evidence. Since an oral dose was identified, a dermal absorption rate of 3% should be used for dermal risk assessments. Because the NOAEL is from an animal study, a MOE of 100 should be used in the risk assessment.

This risk assessment is required.

4. Short- and Intermediate-Term Inhalation Exposure (1 Day to Several Months)

Studies Selected: Two 90-day Inhalation Toxicity Studies - Rat (vapor exposure) (Newton 1988, and Corley et al. 1996a,b)

MRID No(s). 40013901, 40166501, 40908401

Executive Summaries: In the first study (MRID Nos.40013901 & 40166501), Fischer 344 rats (10/sex/concentration) were exposed nose only to Chlorpyrifos at vapor concentrations of 0, 5.2, 10.3, or 20.6 ppb (0, 72, 143 or 287 µg/m^3, respectively) 6 hours/day, 5 days/week for 13 weeks. Cholinesterase activity was measured at study termination. The maximum dose to rats in the 20.6 ppb group was estimated to be 0.044-0.082 mg/kg/day based on average study specific body weights of 0.15 and 0.282 kg for female and male control animals, respectively and the EPA default rat ventilation rate of 0.00715 m^3/hr (average for males and females).

There were no treatment-related effects on body weight, clinical signs, urinalysis, hematology, clinical chemistry, organ weights, gross pathologic or histopathologic evaluations, or plasma, red blood cell or brain cholinesterase activities. Although female rats of all treatment groups had a slight (<4%) but significant decrease in red blood cell count, and males of all treatment groups had slightly elevated (approximately 13%) serum urea nitrogen, these observations were not considered treatment-related due to a lack of dose-response, and all values were within the historical control range.

A LOAEL was NOT identified in this study. Therefore, the NOAEL for systemic toxicity and cholinesterase inhibition exceeds 20 ppb or 0.082 mg/kg/day the highest dose tested.

In the second study (MRID No. 40908401), Fischer 344 rats (10/ sex/ concentration) were exposed nose only to Chlorpyrifos (95% a.i.) at vapor concentrations of 0, 5, 10, or 20.6 ppb (0, 72, 143 or 287 µg/m^3, respectively) 6 hours/day, 5 days/week for 13 weeks. These concentrations resulted in estimated maximum exposures of 0, 0.024, 0.048 and 0.097 mg/kg/day, respectively

based on the EPA default ventilation rate of 0.00715 m^3/hr for rats (average of males and females), and average study specific body weights of 0.189 and 0.127 kg for male and female controls, respectively. The study author stated that the saturation or near saturation level was 20 ppb.

There were no treatment-related effects on mortality, body weight, clinical signs, ophthalmoscopy, hematology, gross pathology or histopathology. In females, food consumption was slightly depressed throughout the study in all dose groups without correlation to the dose level, although this observation was not considered of toxicological significance due to only slight decreases in corresponding body weights. There were some sporadic differences in clinical chemistry parameters, although these were not considered treatment-related due to a lack of dose-response and inconsistency between interim and terminal values. Sporadic differences in organ weights also were not considered treatment-related and appeared to be attributed to the increase mean body weights.

Significant plasma cholinesterase (ChE) inhibition was observed in the high dose males (23%) and females (25%) at the terminal sacrifice. Significant plasma ChE inhibition was also noted in females of the 5 and 10 ppb groups (26 and 40%, respectively), although a dose-response relationship was not apparent. Interim (8 week) measurements were similar or slightly greater than controls. Red blood cell (RBC) (interim and terminal) and brain (terminal) ChE activities were not significantly inhibited at any dose level. It should be noted that the chlorpyrifos concentrations in the exposure chambers at 13 weeks were approximately 12, 16 and 24 ppb, which exceeds the 5, 10 and 20 ppb average exposure levels and this may partially explain the terminal results, while the 8 week concentrations were closer to the average levels. The plasma ChE inhibition was not considered of toxicological significance because of the minimal inhibition (23-25%) at the high dose, lack of dose-response, and an absence of inhibition in the 8 week interval.

A LOAEL was NOT identified in this study. Therefore, the NOAEL for systemic effects and plasma cholinesterase inhibition exceeds 20 ppb or 0.097 mg/kg/day the highest dose tested.

Dose/Endpoint for Risk Assessment: NOAEL = 0.1 mg/kg/day based on no effects on plasma or RBC ChE inhibition in two rat inhalation studies at the highest dose of 20.6 ppm (0.082-0.097 mg/kg/day) (rounded to 0.1 mg/kg/day, the saturation level). The inhalation data are supported by the developmental neurotoxicity study that observed biologically significant decreases in plasma and red blood cell ChE of 43% and 41%, respectively relative to controls in rats orally exposed to 0.3 mg/kg/day (the lowest dose tested) (LOAEL) from gestation day 6 (GD 6) through gestation day 20 (approximately 2 weeks) (Hoberman 1998a,b MRID Nos. 44556901, 44661001). Because the NOAEL is from an animal study, a MOE of 100 should be used in the risk assessment.

Comments about Study and Endpoint: Animal inhalation data were selected with support from the oral data in animals. There are no human inhalation data to indicate whether humans are more sensitive than animals following inhalation exposure. The HIARC concluded that the weight of

evidence indicates that an inhalation hazard from technical Chlorpyrifos is unlikely based on the following factors: 1) the low vapor pressure of Chlorpyrifos, which is 1.87×10^{-5} mmHg at 25°C (Merck Index, 11th Edition); 2) the maximum attainable vapor concentration of Chlorpyrifos is 25 ppb at 25°C; and 3) the two 90 day inhalation studies (nose only) that yielded no adverse effects at concentrations up to 20 ppb or 287 $\mu g/m^3$ (0.097 mg/kg/day). However, low airborne concentrations (< 1 $\mu g/m^3$) persist in homes at least one year, and possibly up to 8 years (Wright et al. 1994) following termite treatments, and at least 10 days in homes following crack and crevice treatment with chlorpyrifos, and homeowners could be exposed to air concentrations as high as 20 $\mu g/m^3$ following lawn treatment with chlorpyrifos. In addition, agricultural workers are exposed via inhalation during application to crops.

Although HIARC recognizes that human exposure to chlorpyrifos occurs through inhalation of both vapor and aerosol, a waiver for an aerosol study was previously granted in 1989. HIARC selected an inhalation NOAEL based on two vapor studies. It is possible this NOAEL may overestimate the toxicity associated with aerosol inhalation exposures to an unknown degree.

This risk assessment is required.

5. Long-Term Inhalation Exposure (Several Months to Life-Time)

The HIARC selected the same 5 critical studies (based on a weight of evidence approach) used for deriving the chronic RfD for this exposure scenario.

MRID Nos.
(1) 2-Year Dog Feeding Study (00064933, and 00146519)
(2) 90-day Dog Study (42172801);
(3) 2-Year Rat Feeding Study (42172802);
(4) 90-day Rat Study (40436406); and
(5) Rat Developmental Neurotoxicity Study (44556901)

Executive Summary: See Chronic Dietary Discussion and Appendix A

Dose/Endpoint for establishing the RfD: NOAEL = 0.03 mg/kg/day based on weight of the evidence for plasma and red blood cell ChE inhibition in dogs and rats. See discussion under chronic RfD. Because the NOAEL is from a animal studies, a MOE of 100 should be used in the risk assessment.

Comments about Study and Endpoint: Oral animal data were selected because the 90 day inhalation studies are of insufficient duration to evaluate long-term exposures, especially given that low airborne concentrations (< 1 $\mu g/m^3$) persist in homes at least one year, and possibly up to 8 years (Wright et al. 1994) following termite treatments. Since an oral dose was identified, the inhalation absorption rate should be assumed to be equivalent to the oral absorption rate (i.e., use a default factor of 100%) for long-term inhalation risk assessments.

Although HIARC recognizes that human exposure to chlorpyrifos occurs through inhalation of

both vapor and aerosol, a waiver for an aerosol study was previously granted in 1989. HIARC selected an inhalation NOAEL based on two vapor studies. This NOAEL is likely to overestimate the toxicity associated with aerosol inhalation exposures to an unknown degree.

This risk assessment is required.

D. Margins of Exposure for Occupational/Residential Exposures:

A MOE of 100 should be used for all of the occupational risk assessment scenarios because these endpoints were based on NOAELs from animal studies. The Committee concluded that an MOE of 100 is appropriate because the effect level in the single dose dermal study in humans (5.0 mg/kg) is the same as the NOAEL in the 21-day dermal toxicity study in rats which was used for dermal risk assessments. The MOEs for residential exposure risk assessment scenarios will be determined during risk characterization by the FQPA Safety Factor Committee.

E. Recommendations for Aggregate Exposure Risk Assessments

For **acute** aggregate exposure risk assessment, combine the high end exposure values from food + water and compare it to the acute RfD.

For **Short-Term** aggregate exposure risk assessment, route specific data are available for the oral, dermal and inhalation exposures with a common endpoint (ChEI); therefore, the following method should be used:

$$MOE_{Total} \quad \frac{1}{\frac{1}{MOE_{(Oral)}} + \frac{1}{MOE_{(Dermal)}} + \frac{1}{MOE_{(Inhalation)}}}$$

For **Intermediate-Term** aggregate exposure risk assessment route specific data were available only for the inhalation exposure whereas an oral dose was selected for dermal exposure. The dermal exposure should be converted to an oral equivalent dose (using 3% dermal absorption) and compared to the oral NOAEL to calculate the MOE. Therefore, the following method should be used:

$$MOE_{Total} \quad \frac{1}{\frac{1}{MOE_{(Oral+dermal\ \ oral\ equivalent)}} + \frac{1}{MOE_{(Inhalation)}}}$$

For **Long-Term** aggregate exposure risk assessment, the oral, dermal exposure converted to an oral equivalent dose (using 3% dermal absorption) and the inhalation exposure converted to an oral equivalent dose (assuming inhalation absorption is 100% of oral absorption) should be combined and compared to the oral NOAEL .

IV. FQPA CONSIDERATIONS

1. Neurotoxicity Data

This issue was previously addressed in the February 2, 1998 and December 7, 1998 HIARC reports (HED Document Nos. 012471 and 013004, respectively).

2. Determination of Susceptibility

The HIARC discussed a number of new scientific literature studies pertaining to the potential sensitivity and susceptibility of children following chlorpyrifos exposure. **A detailed analysis of the relevant studies prepared by K. Baetcke, V. Dellarco, S. Makris and D. Smegal is attached as Appendix B.**

V. SUMMARY OF TOXICOLOGY ENDPOINTS SELECTION

The doses and toxicological endpoints selected for various exposure scenarios are summarized below.

Summary of Toxicological Endpoints for Risk Assessment			
EXPOSURE SCENARIO	**DOSE (mg/kg/day)**	**ENDPOINT**	**STUDY**
Acute Dietary	NOAEL=0.5 UF = 100	40% plasma cholinesterase inhibition at peak time of inhibition (6 hours post exposure) at 1 mg/kg. Significant RBC ChE inhibition at 1.5 mg/kg. 4 hours post exposure	Blood Time Course Study (Mendrala and Brzak 1998) with support from Zheng et al. (2000)
	Revised Acute RfD =0.005 mg/kg/day		
Chronic Dietary	NOAEL= 0.03 UF= 100	Plasma and RBC cholinesterase inhibition	Weight of Evidence from 5 studies: 2 year dog; 90 day dog; 2 yr rat; 90 day rat; developmental neurotoxicity
	Revised Chronic RfD =0.0003 mg/kg/day		
Short-Term **(Dermal)**	Dermal NOAEL =5 Absorbed Dermal NOAEL = 0.15 (for biomonitoring) (a)	Plasma and RBC cholinesterase inhibition of 45 and 16%, respectively at 10 mg/kg/day following 4 days. (Dermal absorption factor not necessary for administered dermal NOAEL)	21-day dermal rat
Intermediate- and Long-Term **(Dermal)**	Oral NOAEL =0.03	Plasma and RBC cholinesterase inhibition. 3% dermal absorption factor is required due to the use of an oral NOAEL.	Weight of Evidence from 5 studies: 2 year dog; 90 day dog; 2 yr rat; 90 day rat; developmental neurotoxicity
Short-,and Intermediate-Term (Inhalation)	Inhalation NOAEL= 0.1	Lack of effects in 2 rat inhalation studies at the highest dose tested.	Two 90 day rat inhalation studies
Long-Term (Inhalation)	Oral NOAEL= 0.03 100% absorption	Plasma and RBC cholinesterase inhibition	Weight of Evidence from 5 studies: 2 year dog; 90 day dog; 2 yr rat; 90 day rat; developmental neurotoxicity

UF = Uncertainty Factor

(a) Use absorbed dermal NOAEL of 0.15 mg/kg/day (5 mg/kg/day * 0.03 dermal absorption factor) for comparison with absorbed biomonitoring exposure.

IV RESPONSE TO REGISTRANT'S COMMENTS RELEVANT TO HIARC DURING PHASE 3 OF THE TRACK PROCESS.

A. Response to Registrants Comments on Age-Related Sensitivity And the Chronic RfD.

Comment: Because cholinesterase inhibition is the endpoint upon which the RfD is established for chlorpyrifos, the Registrant has made the assumption that all sensitivity considerations should be based upon the comparison of this specific toxicological response to chlorpyrifos administration at a range of doses in adult and perinatal animals (rats).

Response: While the registrant comment is not consistent with current OPP policy on the influence of age-related sensitivity on the determination of the FQPA safety factor (USEPA, 1999), the comparison of cholinesterase inhibition in adult and juvenile animals is important to the weight-of-evidence discussions on this issue.

The study by Mattsson et al. (MRID 44648102) evaluated cholinesterase inhibition and determined levels of chlorpyrifos and its principle metabolites in maternal rats and their offspring. While cholinesterase inhibition was observed in dams at doses of 0.3, 1, and 5 mg/kg/day, it was observed in pups only at 5 mg/kg/day. There are, however, some important considerations that must be examined in order to determine the appropriateness of using these data, in combination with blood cholinesterase levels, in a direct comparison to determine age-related sensitivity. Maternal and perinatal cholinesterase levels were determined either on gestation day 20 or on lactation/postnatal days 1, 5, and 11. For all analyses, samples were taken from dams and fetuses 4 hours postdosing, and from pups 2 hours postdosing of the dams. In a study by Mendrala and Brzak (MRID 44648101), the concentration-time course of chlorpyrifos and chlorpyrifos-oxon in blood was examined in male Fischer rats. The time to peak concentration of chlorpyrifos and chlorpyrifos oxon in the blood following oral administration at 1, 5, 10, 50, and 100 mg/kg was found to be 3 hours. Maximum inhibition of plasma cholinesterase was observed at 3 hours after oral doses of 0.5 mg/kg and at 6 hours after doses of 1, 5, and 10 mg/kg. Similar time-course information was not available for lactating dams or for pups of any age; however, if it is assumed that there are some similarities in the time course data for adult rats, it is very unlikely that blood samples collected from pups 2 hours after their dams were dosed were representative of peak blood chlorpyrifos and maximum cholinesterase inhibition levels related to that particular daily dose. On page 39 of MRID 44648102, it states that "most of the exposure from milk would likely have occurred between 3 and 6 hours post-maternal dosing (based on peak blood levels Fig.7) with a further delay of a few hours due to time necessary to digest the milk (based on Byczkowski et al., 1994)." *[Note: Figure 7 graphically represents the time-course data from the results of the Mendrala and Brzak study.]*

Based upon this information, it is concluded that the blood chlorpyrifos levels and cholinesterase inhibition levels in dams and pups, at the maternal posttreatment times that they were sampled, were not biologically comparable. Therefore, to use numerical comparisons of these data to support any conclusions regarding comparative sensitivity of adult and neonatal rats would not be valid. Specifically, the Registrant's comparison of maternal and pup cholinesterase inhibition at empirically-determined chlorpyrifos blood levels, in order to demonstrate a lack of differential

sensitivity, is invalid, since it is based upon incorrect assumptions regarding the dose to the offspring. Likewise, it is not appropriate to correlate the incidence of pup deaths with maternal and offspring blood chlorpyrifos levels and cholinesterase inhibition, based upon the fact that samples were not collected from pups at the time of peak effect.

A review of the Mattsson study data, submitted to the chlorpyrifos docket by M. Dourson and J. Zhao of Toxicology Excellence for Risk Assessment (TERA), concluded that a comparison of chlorpyrifos tissue dose with the level of cholinesterase inhibition in five maternal and offspring tissues indicates that "neonatal and young animals are equally or perhaps less sensitive than adults to the cholinesterase inhibition on a tissue dose and tissue response specific basis." It is noted, however, that all samples were collected after multiple exposures to dams and offspring, and therefore did not assess sensitivity after acute exposures to chlorpyrifos. The Agency is in agreement that little evidence of sensitivity is observed in rat fetuses at gestation day 20, or after repeated postnatal exposures to neonatal or juvenile rats. This response may be due to increased new synthesis or more rapid turnover of inhibited molecules of cholinesterase in the fetal or neonatal rat as compared to the adult. Nevertheless, sufficient evidence exists in the peer-reviewed literature that an age-related sensitivity can be demonstrated in the toxicological response (as measured by cholinesterase inhibition and/or clinical signs) of young rodents to acute doses of chlorpyrifos.

While a number of studies have reported age-related sensitivity in the rat, these studies were often conducted at near-lethal doses or by routes of administration that were not comparable to oral exposure (i.e., subcutaneous or intra peritoneal injection). A study by Moser and Padilla (1998) examined the comparative doses at which cholinesterase inhibition was observed in adult rats versus juvenile rats and identified a 5-fold age-related sensitivity in this endpoint, although at acute oral doses that were relatively high, in comparison to the dose levels examined in the developmental neurotoxicity study with chlorpyrifos. The doses tested were 15 mg/kg/day in postnatal day 17 pups and 75 mg/kg/day in adults (approximately postnatal day 70). In a follow-up study (Moser, et al., 1998), oral doses examined were 5 or 20 mg/kg/day at postnatal day 17, 20 or 50 mg/kg/day on postnatal day 27, or 20 or 80 mg/kg/day for adults. A 2- to 5-fold sensitivity was identified in preweaning rats, but again these doses were relatively high. However, a more recent study (Zheng et al., 2000 [in press]), was conducted at doses that were more comparable to those used in the developmental neurotoxicity study; dose levels were 0.15 to 7.5 mg/kg for pups (postnatal day 7) and adults. In this study, 3- and 5-fold age-related susceptibility was observed in plasma and brain cholinesterase inhibition for juvenile rats as compared to adults when chlorpyrifos was administered by acute oral dosing. Probable explanations for the difference in conclusions of this study and of the studies submitted by the registrant include the fact that chlorpyrifos was administered by direct oral dosing to pups in the Zheng et al. study, versus the indirect exposure via the maternal animal that was used in the developmental neurotoxicity study and the companion study (MRIDs 44556901 and 44648102) .

Second, the blood levels in pups in the Zheng et al. study were measured at 4 hours posttreatment, a time period that is more likely to be comparable to time of maximum blood levels of chlorpyrifos and time of peak cholinesterase inhibition, as estimated by the data generated in adult animals (MRID 44648101). Therefore, the adult and postnatal cholinesterase levels in this study are more biologically relevant and comparable than the levels generated in the Mattsson study (MRID 44648102), and there is greater confidence in the results and conclusions of the Zheng et al. study.

Additionally, it cannot be assumed that any adverse effects in offspring that were demonstrated in the developmental neurotoxicity study (MRID 44556901), are only the result either of postnatal inhibition of cholinesterase in the pups or of maternal toxicity. Adverse effects in the offspring could be related to a treatment-induced alteration to development of the nervous system that occurs either pre- or postnatally. The developmental neurotoxicity study protocol is not designed to distinguish the source or timing of the disruption, nor to identify the precise mechanism by which it occurs. Nevertheless, information in the peer reviewed literature and results of the guideline developmental neurotoxicity study (MRID 44556901) are supportive of the possibility that chlorpyrifos exposure may affect early nervous system development via mechanisms which may be independent of cholinesterase inhibition (e.g., neuronal destruction, interruption of DNA synthesis, and disruption of the structural architecture of the brain), and which may result in irreversible alterations of nervous system structure and/or function. In the developmental neurotoxicity study, neuropathological evaluation revealed statistically significant treatment-related decreases in measurements of the parietal cortex in female offspring at postnatal day 66 (following chlorpyrifos exposure from gestation day 6 through postnatal day 10) at maternal dose levels of 1 and 5 mg/kg/day. Cholinesterase inhibition was not observed in the offspring at 1 mg/kg/day or less, and the resulting morphometric alterations were observed long after the acute affects of cholinesterase inhibition would have been present, even at the high dose level. There are a number of studies in the peer reviewed literature that support the plausibility of this finding. Slotkin (1999) summarized a large body of evidence suggesting that chlorpyrifos elicits damage by both noncholinergic and cholinergic mechanisms extending from early stages of neural cell proliferation through late stages of axonogenesis and terminal differentiation, suggesting that the window of developmental vulnerability is likely to extend from the embryonic period into postnatal life. Brimijoin and Koenigsberger (1999), in a similar survey of recent research, concluded that it is possible that anticholinesterase agents can adversely affect the process of neural development itself, leading to permanent deficits in the architecture of the central and peripheral nervous systems. Eskenazi et al. (1999) also provided a summary of studies to demonstrate the multiple mechanisms of developmental disruption that have been identified in recent research on chlorpyrifos. Studies that support the hypothesis that chlorpyrifos can elicit morphogenic alterations on development, include the following:

- In a study by Whitney et al. (1995), one-day old rats showed significant inhibition of DNA synthesis in all brain regions within 4 hours of treatment with subtoxic doses (2 mg/kg) of chlorpyrifos administered by subcutaneous injection. At 8 days of age, inhibition of DNA synthesis was also seen regionally in the brain. It was determined that the effects of chlorpyrifos on DNA and protein synthesis were not secondary to generalized cell damage

or suppression of cell metabolism. The study authors concluded that low doses of chlorpyrifos target the developing brain during the critical period in which cell division is occurring, effects which may produce eventual cellular, synaptic, and behavioral aberrations after repeated or prolonged subtoxic exposures.

S Johnson et al. (1997) treated neonatal rats with subtoxic doses of chlorpyrifos (1 or 5 mg/kg/day, injected subcutaneously) on postnatal day 1-4 or 11-14, respectively. Significant alterations in cellular RNA concentration and content were observed in the brainstem and the forebrain, two brain regions that are targeted for delayed neurotoxicity. The study authors concluded that these results suggest that chlorpyrifos can elicit delayed neurotoxicity by targeting the pivotal macromolecules that control cell differentiation in a critical postmitotic period, and that the developing brain is a selective target for chlorpyrifos.

S In a study by Campbell et al. (1997), chlorpyrifos was administered to young rats by subcutaneous injection. Neurocellular growth and brain development were gauged by macromolecular (DNA and protein) synthesis. At a dose of 5 mg/kg/day on postnatal days 1-4, there was significant mortality and the survivors exhibited severe cell loss in the brainstem; brainstem growth was maintained by enlargement of the remaining cells. At a dose of 1 mg/kg/day on postnatal days 11-14, the major target for cell loss shifted from the brainstem to the forebrain and was expressed between postnatal days 15 and 20 rather than during the duration of chlorpyrifos treatment (delayed cell death). The study authors concluded that the results of this study indicated that even when growth or survival are unaffected, chlorpyrifos produces cellular deficits in the developing brain that could contribute to behavioral abnormalities.

S In a study by Song et al. (1997), neonatal rats were exposed to doses of chlorpyrifos that produced no weight loss or mortality, either on postnatal day 1-4 or 11-14. Effects on the components of the adenylyl cyclase cascade were evaluated in the forebrain, cerebellum, and heart. In all three areas, chlorpyrifos evoked deficits in multiple components of the adenylyl cyclase cascade: expression and activity of adenylyl cyclase, functioning of G-proteins that link neurotransmitter and hormone receptors to cyclase activity, and expression of neurotransmitter receptors that act through this cascade. Adverse effects appeared after a delay of several days. It was concluded that the results suggested that chlorpyrifos can affect cell development by altering the activity and reactivity of the adenylyl cyclase signaling cascade, a major control point for trophic regulation of cell differentiation.

S In a study by Song et al. (1998), inhibition of DNA synthesis by chlorpyrifos was demonstrated in undifferentiated PC12 cells (rat pheochromocytoma cells, a clone cell line that initially resembles sympathetic neuronal precursor cells, but that differentiates to resemble sympathetic neurons morphologically, physiologically, and biochemically).

S Das and Barone (1998) used pheochromocytoma (PC12) cells to examine the effects of

cholinesterase-inhibiting pesticides on neurite outgrowth. Chlorpyrifos and its metabolites (chlorpyrifos-oxon and TCP) inhibited neurite outgrowth. Since this could occur in the absence of cholinesterase inhibition, the study authors suggested that an alternate mechanism could be involved in the inhibition of differentiation.

- In a study by Roy et al. (1998), *in vitro* studies in rat embryo cultures, using chlorpyrifos concentrations that showed no evidence of growth reduction or dysmorphogenesis, demonstrated abnormalities of mitosis in the developing brain at the neural tube stage. Embryos were incubated with chlorpyrifos for 48 hours beginning at embryonic day 9.5. Examination of the forebrain and hindbrain regions revealed reduced and altered mitotic figures with dispersion and disorientation of the mitotic layer. Additionally, cytotoxicity was evidenced by cytoplasmic vacuolation, enlargement of intercellular spaces, and the presence of a significant number of apoptotic figures.

- Bigbee, et al. (1999) utilized DRG neuronal cultures prepared from E-15 rat embryos, which were maintained for 14 days and then examined to determine the extent and pattern of outgrowth, ultrastructural changes, and the distribution of neurofilaments in the presence of a acetylcholinesterase inhibitor. It was concluded that acetylcholinesterase has an extrasynaptic, noncholinergic role during neural development.

- In a study by Dam et al. (1999) neonatal rats were treated with 1 mg/kg/day of chlorpyrifos on postnatal days 1-4 and 5 mg/kg/day on postnatal days 11-14. Widespread disruption of cholinergic and catecholinergic pathways resulted. The study authors concluded that the results support the view that chlorpyrifos affects neonatal brain development through multiple mechanisms, some directed toward the intracellular processes controlling cell replication and differentiation, whereas others target the development of specific neural pathways, compromising cholinergic synaptogenesis in the forebrain, reducing cholinergic synaptic activity, and enhancing catecholaminergic activity.

B. Response to Registrant Comments on Developmental Neurotoxicity Study

Comment: DAS contends that effects in offspring at high dose of developmental neurotoxicity (DNT) study were due to maternal neglect.

Response: There is no support for the supposition that offspring findings at the high dose (5 mg/kg/day) are attributable 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 (report 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. Nevertheless, even if there had 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 in males, 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.

C. Response to Registrants Comments on the RBC Inhibition in the Two-Year Feeding Study in Dogs (McCollister et al., 1971 / Kociba, 1985):

At the February 2, 1999 meeting the HIARC concluded that for RBC cholinesterase activity, the LOAEL was 0.1 mg/kg/day based on the inhibition of plasma and RBC cholinesterase inhibition observed in both sexes of dogs at this dose; the NOAEL was 0.03 mg/kg/day based

On December 27, 1999, the Registrant during the Phase 3 public comment period contends that in the above referenced two year feeding study the RBC ChE NOAEL should be 0.1 mg/kg/day, and submitted data to support this conclusion.

In response to this issue, the toxicologists in Reregistration Branch 3 conducted a detailed reanalysis of the RBC ChE, and Dr. Hans Allender conducted independent statistical analysis of the data. Both Phase A and Phase B of the dog study were reassessed for plasma and RBC ChE activity using separate comparisons with the predose data and the control group. Results of this analyses is presented in the revised Data Evaluation dated April 6, 2000, D264801.

In general, both Phase A and Phase B data have large standard deviations. Phase A plasma and RBC ChE activity were fairly consistent over time. There was however, significant variability in three predose RBC ChE activity measurements for individual dogs in Phase A as shown on Table 9 (i.e., predose activity varied an average of 25 and 28% for controls, 29 and 36% for 0.03 mg/kg/day and 19 and 36% for 0.1 mg/kg/day for males and females, respectively), which confounds the data interpretation. Phase B ChE activity varied considerably (i.e., at 730 day RBC ChE activity was 175 and 149% of predose activity for males and females, respectively). In addition, the fluctuation in control activity appeared to coincide with fluctuations in exposure group activity indicating that analytical irregularities (i.e., equipment calibration, assay preparation, and individual technique) could be a contributing factor in the data variation. Consequently, HED believes that it is more appropriate to compare phase B data results with the controls values, rather than predose values to account for this variation. This is consistent with the recommendation of the World Health Organization (1990) as follows: "A biologically significant reduction in erythrocyte cholinesterase is normally considered to be a reduction of >20% of pretest levels in the same animals in short-duration studies *or in concurrent controls in longer studies*."

To address the registrants' comments, in addition to using unadjusted ChE inhibition data presented, HED also estimated ChE inhibition results versus control activity based on a consideration of the differences in predose activity (i.e., if the exposure group predose activity was 80% of controls, a mean 50% inhibition was adjusted to only 30% inhibition to account for the predose activity differences).

1. Plasma Cholinesterase

The NOAEL and LOAEL of 0.01 and 0.03 mg/kg/day, respectively were reaffirmed by the reanalysis. In phase A, mean and 351 day plasma ChE inhibition were 13 and 32% for females and

18 and 34% for males at 0.03 mg/kg/day versus both controls and predose activity even after adjustment for differences in predose activity. In phase B, minimal inhibition was noted in females (0-8%) versus controls after adjustment for predose activity, while mean and 730 day plasma ChE activity ranged from 18% decrease to 51% increase for males at 0.03 mg/kg/day versus predose and control activity. It should be noted that the 51% increase in activity on day 730 in Phase B males was 33% less than the increase in controls (i.e., control activity was 184% relative to predose activity at day 730), and therefore was considered of significance. As noted previously, this increase was attributed to possible differences in analytical variation. While HED identified several data quality issues (similar to the RBC ChE data), overall the data for plasma ChE inhibition were still considered consistent between Phase A and B, and between sexes. There was a clear dose response at all levels showing inhibition, and statistically significant inhibition was consistently noted at 0.03 mg/kg/day in both sexes of Phase A and B.

2. Red Blood Cell Cholinesterase

In general, the RBC ChE data were inconsistent between Phase A and Phase B and between comparisons versus predose and controls. There was significant variability in predose RBC ChE activity measurements in individual dogs of Phase A (i.e., predose activity varied an average of 25 and 28% for controls, 29 and 36% for 0.03 mg/kg/day and 19 and 36% for 0.1 mg/kg/day for males and females, respectively), which confounds the data interpretation. In addition, RBC activity in Phase B was variable over the course of the study indicating that analytical irregularities could be contributing to the variation in data results. Because of these inconsistencies, the HED determined that it is inappropriate to combine the results of Phase A and Phase B. Although there was possible inhibition in the Phase A females at 0.03 mg/kg/day compared to predose activity (mean of 25%, and 34% on day 351), this inhibition was not considered biologically significant because there was no dose-response (i.e., there was similar inhibition at 0.1 mg/kg/day), adjusted inhibition compared to controls was 13-19%, and similar inhibition was not seen in Phase B.

At 0.1 mg/kg/day there was statistically significant RBC ChE inhibition in Phase A and B females and Phase B males versus controls based on a comparison of all time interval data combined (student's t-test at $p<0.05$). In addition, significant RBC ChE inhibition was noted in females of both Phase A and B versus controls for the terminal study measurements (i.e., days 351 and 730). Red blood cell ChE of the 0.1 mg/kg/day group appeared to return to predose after 44 days post exposure, while near predose activity was achieved at 92 days post exposure for the 1 and 3 mg/kg/day groups. In some cases, RBC ChE inhibition at 0.1 mg/kg/day still demonstrated a concern even after adjusting for differences in predose activity where adjusted inhibition was up to 26% for males (phase B males at day 730) and 20% for females (phase B at day 730) versus controls. In other cases, (i.e., Phase A males), there were no indications of inhibition at 0.1 mg/kg/day relative to controls. Overall, the data were considered too variable and inconsistent to conclude that 0.1 mg/kg/day is a NOAEL or a LOAEL. Exposure to 1 mg/kg/day and 3 mg/kg/day resulted in clear inhibition of RBC ChE despite the data limitations.

In the original study submission, the study authors reported statistical significance for RBC ChE

inhibition in Phase B females at 0.1 mg/kg/day only for the 365 day (25% decrease or an adjusted 4% decrease) and 730 day (41% decrease or an adjusted 20% decrease) measurements relative to controls, and in day 465 measurement (12% decrease) relative to predose levels (31% decrease versus controls). At 365, 465 and 730 days, RBC activity was increased 28%, decreased 12% and increased 7%, respectively relative to predose measurements. However, it should be noted that the control activity on days 365 and 730 was 134% and 142% above predose control values suggesting that the increase in RBC ChE activity is due to differences in analytical irregularities at these time intervals. Therefore, the 28% and 7% increases actually reflect 6% and 35% decreases relative to the control activity increases. On day 465 for 0.1 mg/kg/day, activity was 12% below predose values and 10% (adjusted) below control values.

HED notes that in the 90 day dog study (Barker 1989, MRID 42172801), statistically significant RBC ChE inhibition was noted in both males (32-46%) and females (24-38%) following exposure for 6 and 12 weeks at 0.22 mg/kg/day. This dose level was identified as a LOAEL, with the next lower dose level of 0.01 mg/kg/day established as a NOAEL. It is very reasonable to predict that since 24% to 46% inhibition is seen at 0.22 mg/kg/day, that there would be significant inhibition at 0.1 mg/kg/day as the dose response is not that steep.

3. HIARC' Review of the Reanalysis

On January 20, 2000, the HIARC evaluated the registrants contention that 0.1 mg/kg/day is the NOAEL for RBC ChEI. The HIARC concluded that additional analysis of the RBC ChE data conducted by Re-Registration Branch 3 scientists showed that both Phase A and B have procedural problems that result in large standard deviations and/or unexplained large increases in activity over time that confound the interpretation of the data.

On March 8, 2000, the registrant submitted statistical analysis conducted by Sielken/Holden to support their determination of 0.1 mg/kg/day as the NOAEL for RBC ChEI in the dog study. This submission evaluated the dog data using in their opinion a "statistical repeated measures analysis of variance to reflect a lot more of the information about the changes in cholinesterase levels across the different dose levels". This submission contends that this statistical approach is more powerful than the two-sample and paired t-test.

On March 28, 2000, the HIARC reviewed the additional in-depth analysis of the RBC ChE data conducted by Reregistration Branch 3 scientists, independent statistical analyses conducted by Dr. Hans Allender, in addition to the Sielken/Holden submission. HIARC concluded that the statistical analyses approach used in the Sielken/Holden submission is not clear and that further explanation is needed on the graphical analysis presented. For example, on the first page, the report expresses that the graphical analysis is more powerful than the standard two-sample and paired t-test, however the report fails to clarify this point or cite references that sustain that point. Figures 1 to 6 of the report present a segment that contains a 5% level, however no portion of the report indicates what was done to calculate the length of this segment. The submission also contends that the

graphical analyses of the dose-response pattern and the statistical repeated measures analyses of variance is more powerful than other "less powerful analyses (such as two-sample and paired t-tests)". However, no rationale was given for rejecting the t-test, which is the standard statistical method used for data analyses. Most importantly, the HIARC concluded that the RBC ChE data are not sufficiently robust for statistical analyses given the sample size, internal inconsistencies between Phase A and Phase B and between comparisons versus predose and controls and the significant variability in predose RBC ChE activity in Phase A which confounded the data interpretation.

Even though there is low confidence in these data (poor quality) they elicit concerns about possible RBC ChE inhibition at 0.1 mg/kg/day based on the following factors:

- There was apparent inhibition at 0.1 mg/kg/day depending upon whether the data were assessed relative to the predosing mean for each animal or relative to the control group at each interval. There were inconsistencies in both Phase A and Phase B and the method of comparison.
- Decreases in Phase A females both when results are compared to predose values (22 to 30%) and adjusted control values (10 to 14%)
- Decreases in Phase B males both when results are compared to predose values (21 to 46%) and adjusted control values (14 to 26%)
- Decreases in Phase B females both when results are compared to predose values (9 to 35%) and adjusted control values (6 to 20%)
- Dose dependent decreases in both sexes in both phases at the next two higher dose levels (1 and 3 mg/kg/day)
- Statistical analysis (two-tailed students t-test) showed predose activity of the 0.1 mg/kg/day group is significantly lower than controls for Phase A and B females, and is significantly increased relative to controls for Phase A males.
- Statistical analysis showed significant inhibition for females of both Phase A and B and males of Phase B based on comparison of all time intervals. In addition, there is significant inhibition in females of both Phase A and B at the terminal measurement.

Based on these considerations, the HIARC concluded that the dose of 0.1 mg/kg/day can not be assigned as the NOAEL for RBC ChE inhibition as contended by the registrant. Also, because of the variability and internal inconsistencies that confound the interpretation of the data, the HIARC did not identify a NOAEL or a LOAEL for RBC ChEI in this dog study.

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APPENDIX A: EXECUTIVE SUMMARIES FOR CHRONIC RFD BASIS

(1) 2-Year Dog Feeding Study (McCollister et al. 1971, Kociba et al. 1985)

Executive Summary:

The chronic toxicity study (MRIDs 00064933, 00146519) in dogs consisted of two phases. In Phase A, chlorpyrifos (97.2-98.8% a.i) as Dowco® 179 was administered to 3 beagle dogs/sex/dose in diet at dose levels of 0, 0.01, 0.03, 0.1, 1 or 3 mg/kg/day for one year (Phase A). One dog/group was sacrificed at one year, and the remaining 2 dogs/group were sacrificed after a 3 month recovery period. In Phase B, chlorpyrifos was administered to 4 beagle dogs/sex/dose at the same dose levels for a total of two years (Phase B), at which time all dogs were sacrificed.

There was a significant increase in the absolute and relative liver weights in the high dose males, although no concurrent histolopathological changes were observed. The male liver/body weight ratios were 2.6 for the control group and 3.5 for the highest dose group. There were no treatment related effects on body weight, mortality, clinical signs, clinical chemistry, food consumption, hematology, urinalysis, or gross pathology.

Plasma cholinesterase (ChE) activity in the Phase A study was significantly and dose-dependently decreased in all male and female dogs except the 0.01 mg/kg/day group when compared with the control group at each time interval. Inhibition was apparent from 7 days on-ward. Mean plasma ChE inhibition for all intervals for the 0.01, 0.03, 0.1, 1 and 3 mg/kg/day dose groups relative to controls for males was 15%, 29%, 48%, 71%, and 75%, respectively and for females was 6%, 23%, 39%, 58% and 72%, respectively. At 0.03 mg/kg/day, maximum plasma ChE inhibition was 42% in males (day 93, phase B) and 42% in females (day 352, phase A) versus controls. ChE returned to predose levels 14 days after treatment cessation at 0.03, 0.1, 1 and 3 mg/kg/day. Plasma ChE activity in Phase B was similar to Phase A. Mean plasma ChE inhibition for 0.01, 0.03, 0.1, 1 and 3 mg/kg/day groups was 4%, 24%, 49%, 59% and 75%, respectively for males and +14% (increase), -10%, 35%, 52% and 65%, respectively for females. Inhibition was similar or less based on a comparison of predose activity levels. Overall statistical significance for plasma ChE inhibition was reached at 0.03 mg/kg/day for both Phase A and B in both males and females.

HED identified a number of data quality issues for RBC ChE that confound the data interpretation. The RBC ChE data were inconsistent between Phase A and Phase B and between comparisons versus predose and controls. There was significant variability in the three predose RBC ChE activity measurements in individual dogs in Phase A (i.e., predose activity varied an average of 25 and 28% for controls, 29 and 36% for 0.03 mg/kg/day and 19 and 36% for 0.1 mg/kg/day for males and females, respectively) which confounds the data interpretation. In addition, RBC activity in Phase B was unacceptably variable over time in both controls and exposure groups probability due to analytical problems. For example, at day 730 male RBC ChE activity was 175%, 153%, 159% and 129%, while female RBC ChE activity was 142%, 145%, 149% and 107% for controls, 0.01, 0.03 and 0.1 mg/kg/day groups, respectively relative to the mean predose activity. At 0.1 mg/kg/day there was statistically significant RBC ChE inhibition in Phase A and B females and Phase B males versus controls based on a comparison of all time interval data combined (student's t-test at $p<0.05$). In addition, significant RBC ChE inhibition was noted at 0.1 mg/kg/day in females of both Phase A and B versus controls for the terminal study measurements (i.e., days 352 and 730). During Phase A, red blood cell ChE in the 0.1 mg/kg/day group appeared to return to

predose after 44 days post exposure, while for the 1 and 3 mg/kg/day groups, predose activity was achieved (or nearly achieved) after 92 days post-exposure. In some cases, RBC ChE data at 0.1 mg/kg/day still demonstrated a concern for inhibition even after adjusting for differences in predose activity where adjusted inhibition was up to 26% for males (phase B males at day 730) and 20% for females (phase B at day 730) versus controls. In other cases, (i.e., Phase A males), there were no indications of inhibition at 0.1 mg/kg/day. Overall, the data were considered too variable and inconsistent to conclude that 0.1 mg/kg/day is a NOAEL or a LOAEL.

Brain ChE activity was not markedly different from controls in the one-year study, although only one dog/sex/dose was evaluated. No significant brain ChE inhibition was noted in the two-year study, although mean inhibition in males relative to controls was 2%, 7%, 8%, 7% and 21% for the 0.01. 0.03, 0.1, 1 and 3 mg/kg/day groups, respectively. In females, the brain ChE activities relative to controls were +7%, +3%, +1%, +6% and -19%, respectively. The brain ChE inhibition in the high dose group is considered toxicologically significant.

The NOAEL and LOAEL for plasma ChE inhibition are 0.01 and 0.03 mg/kg/day based on consistent mean inhibition of 10% to 29% at 0.03 mg/kg/day compared to controls for both males and females in Phases A and B. HED did not identify a NOAEL and LOAEL for RBC ChE inhibition due to inconsistencies in the data and the large standard deviations that confounded the interpretation of the data at lower dose levels. The NOAEL and LOAEL for brain ChE were 1 and 3 mg/kg/day. The systemic NOAEL and LOAEL are 1 and 3 mg/kg/day based on liver weight effects.

CLASSIFICATION:

The chronic toxicity study in dogs in conjunction with the addendum that contains supplemental information are ACCEPTABLE-GUIDELINE and satisfy the guideline requirement (83-1b).

(2) 90-Day Dog (Barker 1989)

Executive Summary:

In a subchronic oral toxicity study in dogs (MRID 42172801), chlorpyrifos (95.8% a.i.) was administered by gelatin capsule to 4 beagle dogs/sex/dose at dose levels of 0, 0.01, 0.22, or 5 mg/kg/day each day for 13 weeks.

There were no treatment related effects on mortality, clinical signs, body weight, food consumption, ophthalmological examination, urinalysis, or organ weights. Although some statistically significant differences were noted in some hematological parameters, these findings were not considered biologically significant, or treatment related. No biologically significant differences were noted for clinical chemistry parameters, with the exception of cholinesterase (ChE) inhibition. Significant and dose-related plasma and red blood cell ChE inhibition were observed in both sexes throughout the study. At 0.01 mg/kg/day plasma ChE inhibition was noted in females at week 6 (20-24%, $p<0.05$) and week 12 (24%, not significant). In males there was 15% plasma ChE inhibition at week 13 that was not significant. Plasma ChE was significantly inhibited in males (33-63%) and females (42-67%) exposed to 0.22 mg/kg/day and in males (69-85%) and females (64-87%) exposed to 5 mg/kg/day. Only the inhibition noted at 0.22 mg/kg/day was considered to be of sufficient magnitude and consistency to be biologically and toxicologically meaningful. Red blood cell ChE was also significantly inhibited in males (32-46%) and females (24-38%) exposed to 0.22 mg/kg/day during weeks 6 and 12 and in males (38-85%) and females (29-86%) exposed to 5 mg/kg/day during weeks 1, 6 and 12. Brain ChE activity was significantly reduced 46% at 5 mg/kg/day in both males and females. Although possible treatment-related gross and microscopic pathology changes were observed in the high dose animals, these findings were not observed in the 2-year dog study, and only occurred in one male and one female. These include the a thickened muscular wall of the duodenum and an area of papillomatous hyperplasia (pyloric).

The NOAEL and LOAEL for plasma and red blood cell cholinesterase inhibition are 0.01 and 0.22 mg/kg/day, respectively.

This subchronic toxicity in dogs is classified as ACCEPTABLE-GUIDELINE and satisfies the guideline requirement for a subchronic oral study (82-1) in dogs.

(3) 2-year Rat Study (Crown et al. 1990).

Executive Summary:

In a carcinogenicity toxicity study (MRID 42172802), chlorpyrifos (96.1% a.i) was administered to 55 Fisher F344 rats/sex/dose in diet at dose levels of 0, 0.2, 5 or 100 ppm (equivalent to approximately 0, 0.0132, 0.33, or 6.99 mg/kg/day for males and 0, 0.0146, 0.365 or 7.78 mg/kg/day for females, respectively) for 104 weeks. Plasma cholinesterase (ChE) activity (10/animals/sex/group) was measured on weeks 14, 32, 45, 78 and 104, while red blood cell (RBC) ChE activity (10/animals/sex/group) was measured at weeks 45, 78 and 104. Plasma, RBC and brain ChE activities were measured on 5 animals/sex/group at week 50 and in 10 animals/sex/group at terminal sacrifice.

Rats in the 100 ppm group exhibited significantly decreased body weights in both sexes, and a significant increased incidence of non-neoplastic lesions (cataracts and diffuse retinal atrophy) in females. Plasma ChE activity was significantly inhibited at 5 and 100 ppm in both sexes. Significant plasma cholinesterase inhibition in the 5 ppm group ranged from 15 to 51% throughout the study in both sexes. In females exposed to 0.2 ppm, red blood cell ChE was also significantly inhibited 42% at the 50 week sacrifice, but was elevated 14% at the terminal sacrifice. Red blood cell ChE was also significantly inhibited in the 50 week sacrifice for the 5 and 100 ppm females (39 and 45% ↓, respectively), but inhibition was less pronounced at the terminal sacrifice where inhibition was 11 and 18%, respectively. At the week 50 measurements, the decrease in RBC ChE activity in the treated groups appeared to be seriously influenced by the high control value (3891 U/g tissue) compared to the other control values which ranged from 2092 to 2586 U/g tissue. Therefore, the RBC ChE inhibition in females at 50 weeks is discounted because of the unusually high control value. Brain ChE was significantly reduced in both high dose males and females at the 50 week and terminal sacrifices (57-80% ↓), but was not significantly decreased at the other doses. At terminal sacrifice, males in the high dose group had significantly lower absolute liver and kidney weights that were not significant after correction for body weight, and therefore were not considered treatment-related. There were no treatment related effects in mortality, clinical signs, food consumption, or hematology.

At the doses tested, there was no treatment related increase in tumor incidence when compared to controls. Dosing was considered adequate based on decreased body weights and the increased incidence of non-neoplastic lesions.

The LOAEL and NOAEL for plasma inhibition are 5 and 0.2 ppm, respectively (0.33 and 0.0132 mg/kg/day, respectively). The LOAEL and NOAEL for systemic effects of decreased body weights in both sexes, and increased incidence of cataracts and diffuse retinal atrophy in females are 100 and 5 ppm, respectively (6.99 and 0.33 mg/kg/day, respectively).

This carcinogenicity study in rats is classified as ACCEPTABLE-GUIDELINE and satisfies the guideline requirement for a carcinogenicity study (83-2a).

(4) 90-Day Rat Study (Crown et al. 1985)

Executive Summary:

In a subchronic oral toxicity study in rats (MRID 40436406), chlorpyrifos (95.5% a.i.) was fed to 20 rats/sex/dose at dose levels of 0, 0.5, 10 or 200 ppm (equivalent to 0, 0.025, 0.5 or 10 mg/kg/day) for 13 weeks.

There were no treatment related effects on mortality, clinical signs, histopathology or organ weights. A significant decrease in body weight gain was observed in high dose males during the first half of the study, and in high dose females during the first three weeks. However, body weight in exposed animals was similar to controls by week 13. Food consumption in the high-dose animals was also significantly increased during the time of increase body weight gain. Hematological effects were observed in both high-dose males and females, characterized by significantly reduced packed cell volume (PCV), hemoglobin (HB) and erythrocyte (RBC) group means relative to controls, which is suggestive of anemia. However, these parameters were within the normal range. Urinalysis revealed that males in the high dose group had a significantly reduced urine volume, increased urine pH, a higher specific gravity and a higher protein grading, which appear to be treatment-related.

No biologically or significant or treatment-related differences were noted for clinical chemistry parameters, with the exception of cholinesterase (ChE) inhibition. Significant and dose-related plasma ChE inhibition of 22, 37 and 72% was observed in the 0.5, 10 and 200 ppm male groups, respectively. In females, plasma cholinesterase was also significantly inhibited at 91 and 57% for the 10 and 200 ppm groups, respectively, but was not inhibited in the low dose group (10% increase). However, the registrant acknowledged the possibility that the cholinesterase data for the 10 and 200 ppm female groups were accidently switched. Red blood cell and brain cholinesterase activity were not evaluated in this study.

The LOAEL for plasma cholinesterase inhibition is 0.5 ppm (0.025 mg/kg/day) for males, which is the lowest dose tested. No NOAEL was observed for cholinesterase inhibition. The systemic NOAEL and LOAEL are 10 and 200 ppm, respectively (0.5 and 10 mg/kg/day, respectively) based on decreased body weight gains and possible anemia.

This subchronic toxicity study in rats, in conjunction with the registrant's discussion of the female cholinesterase values, is classified as ACCEPTABLE-GUIDELINE and satisfies the guideline requirement for a subchronic oral study (82-1a) in rats.

(5) Developmental Neurotoxicity Study in Rats (Hoberman et al. 1998a,b)

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 body weight, 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%, statistically significant) 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 body weights were significantly reduced (↓8-15%) at PND 1 and 5 (pre- and post-culling). Body weights were also reduced from birth to PND 22 in Subset 4 high-dose animals (↓5-19%); body weight 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. Body weight 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

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).

APPENDIX B: Chlorpyrifos Children's Hazard: Sensitivity and Susceptibility

Chlorpyrifos
Children's Hazard: Sensitivity and Susceptibility

March 28, 2000

Office of Pesticide Programs
Health Effects Division
U.S. Environmental Protection Agency
Washington, D.C.

Prepared by: Karl Baetcke, Vicki Dellarco, Susan Makris, Deborah Smegal

HED Hazard Identification Assessment Review Committee Concurrence

Chlorpyrifos: Children's Hazard
March 30, 2000

HIARC Members	Signature
William Burnam	
Vicki Dellarco	
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Preface

There has been increasing concern about the potential effects on fetuses, infants, and children posed by exposure to chlorpyrifos. In recent years there has been an emergence of new information from laboratory studies which expands our knowledge and adds to the concern for increased sensitivity and susceptibility for the young. The purpose of this document is to assess and characterize this new information in an integrative manner. It is not a comprehensive review of all the literature and studies. It should be noted that the older literature is consistent with the findings of the newer studies. Furthermore, this document does not deal with endpoint selection of NOAELs/LOAELS for RFD derivation. Instead it is intended as a science analysis of the potential for increased sensitivity and susceptibility of pre- and postnatal animals to adverse effects that might result from exposure to chlorpyrifos.

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Summary

Increased concerns regarding the potential hazard to children associated with both pre- and postnatal exposure to chlorpyrifos centers around consideration of data showing both increased sensitivity *(i.e.,* the young will respond more than the adult when given the same dose) and susceptibility (*i.e.*, there is a unique biological target such as the developing brain that predisposes the individual). A number of gestational or postnatal dosing studies in rats conducted with chlorpyrifos (and its metabolites) were designed to evaluate age-related differences in cholinesterase inhibition, behavioral responses, the metabolism of the agent, and the occurrence of biochemical and morphologic effects in the developing brain.

Multiple gavage studies in rats using adult, perinatal and postnatal chlorpyrifos treatment demonstrate differential responses and an increased sensitivity to cholinesterase inhibition, body weight changes, acute lethality and behavioral effects in young animals. The differential between young and adult animals is dependent on dose and duration of treatment as well as the age of the young animal. For example, an increased sensitivity to cholinesterase inhibition (2- to ~5-fold) can be seen at relatively low doses after a single treatment. Following repeated dosing of 7-day old rats to chlorpyrifos, only a small differential in sensitivity (~1.5-fold difference) is seen for brain cholinesterase inhibition compared to adult animals. An increased sensitivity is seen for other effects including, but not limited to, behavioral effects and body weight changes. Data indicate that if the dam is treated with chlorpyrifos, cholinesterase inhibition is found in fetal tissues (brain and blood). Additionally, there are data that show age-related differences in detoxification enzyme activities that may result in less protection of the fetus and neonate, if exposed to chlorpyrifos.

The available data not only demonstrate an increased sensitivity of the young but also an unique susceptibility of the developing brain. A recent rat developmental neurotoxicity (DNT) study found biologically and statistically significant changes in brain structure following pre-and postnatal gavage dosing with chlorpyrifos. Other recent peer reviewed literature show potential perturbation of key neurodevelopmental processes following treatment of neonatal rats or by treatment of neural cell cultures. At relatively low subcutaneous doses, chlorpyrifos can alter macromolecular synthesis, neurotransmitter levels, and cell signaling in the developing rat brain. These literature studies did not include measurements of functional or morphological adverse outcomes. Nevertheless, the findings from the biomarkers evaluated raise concern for the potential hazard for chlorpyrifos to damage the developing brain, and thus provide evidence of the unique susceptibility of the neonatal animal, and possibly the fetus given the results of the DNT.

The weight of evidence conclusion based on the most sensitive endpoint (*i.e.,* cholinesterase inhibition), demonstrates that there is a clear differential response (2- to ~5-fold) in the young compared to the adult animal after an acute treatment with a relatively low dose of chlorpyrifos. There is also increased sensitivity at higher doses (LD_{10} and MTD) found after acute dosing (up to 9-fold). Moreover, recent data have shown effects on the developing rat brain (*e.g.*, structural defects and changes in macromolecular synthesis, neurotransmitter levels and cell signaling). It is these neurodevelopmental effects that raised a high degree of concern for the potential unique susceptibility of the young to chlorpyrifos. The susceptibility parameters evaluated can not be directly linked, however, to cholinesterase inhibition or functional outcomes. Furthermore, whereas data are available to evaluate quantitative differences in the sensitivity of the young animal compared to the adult, there are insufficient data to establish the quantitative dose-response for the susceptibility indicators. Nevertheless, special attention should be given to children because of evidence showing the potential for a disproportionate hazard to chlorpyrifos compared to adults

(*i.e.*, the qualitative evidence showing unique susceptibility of the developing brain along with the evidence of increased sensitivity).

The key lines of evidence are discussed in detail in Sections 1 and 2. Section 3 provides a weight of evidence characterization of the strength and weaknesses of the data, as well as an integrative interpretation of the available studies.

1. Evaluation of Differential Sensitivities Among Fetal, Neonatal, Adult Female and Male Rats to Chlorpyrifos Treatment

Tables 1 and 2 summarize results of studies showing differences in toxicological responses of adult male and female, prenatal, neonatal, and young rats to chlorpyrifos treatment. Toxicological endpoints related to the comparative sensitivities of rats of different ages are presented below. Where possible, comparisons of relative potency between young and adult animals are based on the effective dose that results in 50% cholinesterase inhibition (*i.e.*, ED_{50}). Evaluating the differential sensitivity between young and adult animals for inhibition of cholinesterase activity at lower doses is viewed as less reliable because of the inherent variability in measures of cholinesterase inhibition as the response approaches background levels..

1.1 Cholinesterase inhibition (ChEI)

The magnitude of the difference in sensitivity to chlorpyrifos treatment of different age groups and sexes of rats is influenced both by the dose levels administered and the duration of treatment. Comparisons of the responses observed after acute and repeated treatments with chlorpyrifos are discussed below.

ChEI Following Acute Dosing

Postnatal day 17 (PND 17) juvenile rats are about 5-fold more sensitive than adult male or female rats to an acute chlorpyrifos dose that produces 70-90% ChEI (brain or blood). The dose level for this response is 15 mg/kg or 80 mg/kg, juvenile and adult, respectively (Moser and Padilla, 1998). The magnitude of ChEI has been shown to be inversely related to age. At a single dose of 20 mg/kg chlorpyrifos, brain ChEI was reported to be 39% (male adult rats), 66% (female adult rats), 76% (PND 27 rats), and 87% (PND 17 rats). In the same study, a dose of 5 mg/kg produced ~50% brain ChEI in PND 17 animals, suggesting ~ 4-fold greater sensitivity of PND 17 animals compared with adult animals. In addition, adult non-pregnant females are more sensitive to ChEI than are males (Moser *et al.,* 1998).

In a study with adult males and PND 7 offspring, an increase in sensitivity of young animals relative to adults was observed for acute doses that resulted in 50% RBC and plasma ChEI (*i.e.*, neonates were ~2 to3-fold more sensitive than adults) (Zheng *et al.*, 2000). In this study, no statistically significant effects were seen in brain ChEI in adult males up to a dose of 15 mg/kg. With respect to brain ChEI, neonatal rats are >5-fold more sensitive when compared to adult males. This comparison is based on an estimated ED_{50} (a dose resulting in 50% ChEI) of 2.9 mg/kg in young rats and no effect in adults to brain ChEI at 15 mg/kg. The data from the studies on the response of rats of different ages to ChEI (discussed above) support the assumption that the neonatal animal (7 day old) is $\geq$ 5-fold more sensitive than the adult animal to chlorpyrifos-induced brain ChEI, as suggested by data from the Zheng *et al*. study. In addition, adult female rats appear to be somewhat more sensitive (<2-fold) than adult male rats to ChEI after an acute treatment with chlorpyrifos (Moser *et al.,* 1998).

ChEI Following Repeat Dosing

In 14 day repeated oral dose studies, plasma ChE was observed to be inhibited by 33-43% in rat dams at 0.3 mg/kg/day (dosing from GD 6-GD 20) and by 24% at 0.45 mg/kg/day in male rats (Hoberman, 1998; Mattsson *et al.*, 1998; Zheng *et al.*, 2000). Corresponding RBC ChEI was 26-41% and 39% for dams and male rats, respectively. Brain (cortex) ChEI was reported to be 87-90% at 5 mg/kg/day in the dams and 81% at 7.5 mg/kg/day (whole brain) in male rats. These data suggest that pregnant female rats are more sensitive than male rats to chlorpyrifos treatment; but no

firm conclusions should be made regarding relative sensitivities of pregnant female and male rats based on these data alone because these were not direct comparisons in the same laboratory.

Repeated (14 day) chlorpyrifos treatment of adult male rats or neonatal (PND 7) Sprague-Dawley (SD) rats of both sexes did not result in pronounced differences in sensitivity to ChEI between adult and neonatal rats (Zheng *et al.,* 2000). In the Zheng *et al*. study, based on a comparison of ED_{50}'s, there is some indication of an increase (1.5-fold) in sensitivity of PND 7 neonate to brain ChEI, but RBC and plasma ChEI were found to be equal or greater in the adult male rat compared to the PND 7 neonate.

Chlorpyrifos was shown to inhibit ChE to a higher degree in maternal brain tissue than in fetal brain tissue when dams were treated during gestation days 14-18 (Lassiter *et al*., 1998; Hunter *et al.,* 1999). Similarly, treatment of dams during GD 6 - GD 20 led to more brain ChEI in dams than the fetus at a dose of 5 mg/kg/day (Mattsson *et al.,* 1998). At a dose of 1 mg/kg/day in the same study, there was 61%-82% plasma and RBC ChEI in dams, but no ChEI was observed in these tissues in the fetus.

Overall, the data on ChEI from neonatal and young rats treated with repeated doses of chlorpyrifos do not indicate an increased sensitivity of neonatal or young animals compared with adult animals. Additionally, the fetus has less ChEI than the dam when the dam is treated with chlorpyrifos during gestation. Nevertheless, it is apparent that cholinesterase activity is inhibited in the fetus if the dam is treated with a chlorpyrifos dose which can be absorbed by the fetus.

 # Recovery

Young rats (PND 17) appear to recover more rapidly than adults from ChEI after acute chlorpyrifos treatment. At one week postdosing, PND 17 rats treated with 15 mg/kg chlorpyrifos were observed to return to control levels of ChE activity, whereas adult female rats treated with 80 mg/kg had not recovered by 2 weeks (Moser and Padilla, 1998). These were equipotent dosages with regard to initial ChEI.

Brain ChE was inhibited by 29% one day following the last dose after gavage administration of chlorpyrifos to rats every other day at a low dose of 3 mg/kg from PND 1 through PND 21 (Tang *et al*., 1999). In mid-dose animals (3 doses of 3 mg/kg and 8 doses of 6 mg/kg), brain cholinesterase was inhibited 57% at the same sacrifice time. Brain ChEI was not detected after a 9 day recovery period in the low dose group but persisted after 19 days recovery in the mid- and high-dose groups (Tang *et al.,* 1999). Thus, although juvenile rats appear to return to control levels of cholinesterase activity within a relatively short period of time after an acute treatment with chlorpyrifos, repeated treatments can lead to prolonged ChEI.

The lack of an apparent increased sensitivity of the fetus or neonate versus the maternal animal to ChEI following repeated treatment of dams with chlorpyrifos may be due to the increased new synthesis or more rapid turnover of inhibited molecules of cholinesterases in the fetal brain than in the adult (Lassiter *et al*., 1998; Mortensen *et al*., 1998). This more rapid recovery of ChEI has also been demonstrated following dosing of neonates (Pope and Liu, 1997).

1.2 Body Weight Changes

At a chlorpyrifos dose of 5 mg/kg/day administered to dams (GD 6 - LD 11), there were no significant effects on body weight, food consumption, or pregnancy parameters. However, body weights, body weight gains, and food consumption were reduced in offspring that received less than

5 mg/kg/day via the dam's milk, indicating that offspring are more sensitive to body weight changes (Hoberman, 1998). Body weights were significantly reduced at 12 days during repeated treatments with 4.5 mg/kg/day in 7 day neonates versus 15 mg/kg/day at day 10 for adults (~3.3-fold increased sensitivity of 7 day neonates) (Zheng *et al.*, 2000).

1.3 Maximum Tolerated Doses (MTD)

At 10 days of age in rats, there is a 7-fold increase (adult MTD ÷ neonatal MTD) in sensitivity of the neonate compared to adult animals to an acute oral dose that results in approximately10% mortality (Moser and Padilla, 1998).

1.4 Acute Lethality

The LD_{10} for chlorpyrifos is 15 mg/kg and 136 mg/kg for the 7-day neonate and adult, respectively, ~9-fold difference in sensitivity (Zheng *et al.*, 2000).

1.5 Muscarinic Receptor Down-Regulation

Juvenile rats (PND 17) treated with a single dose of 15 mg/kg seem to have more extensive muscarinic down-regulation in brain tissues than adult rats treated by gavage with a single dose of 80 mg/kg (Moser and Padilla, 1998). In a separate study, decreases in brain muscarinic acetylcholine receptor density were reported for pups treated by gavage with 3 mg/kg every other day from PND 1 to PND 21 (Tang *et al.*, 1999). Transient decreases in muscarinic acetylcholine receptor density (^{3}H-methylscopolamine binding) were noted as early as PND 6, or following 3 treatments with the 3 mg/kg dose. Differences in ^{3}H-quinuclidinyl benzilate binding were not observed in this dose group. In the medium dose group of pups (3 treatments every other day with 3 mg/kg, then 8 treatments every other day with 6 mg/kg), binding for both ligands was reduced at PND 22, after ChEI had reached a constant level of 54-57%.

1.6 Behavioral Effects

Behavioral effects appear to occur at a lower dose in juvenile (PND 17) rats than adult rats. A single dose of 20 mg/kg led to clinical/behavioral signs of neurotoxicity in 17 day juvenile rats whereas adult behavioral neurotoxicity was observed at 50 mg/kg, about a 3-fold difference (Moser *et al*., 1998). PND 17 juvenile rats also showed behavioral effects at a five-fold lower acute dose (15 mg/kg) than an acute dose that resulted in similar effects in adult female rats (80 mg/kg) (Moser and Padilla, 1998). After repeated exposures (14 days), functional signs of toxicity were reported in 7 day old neonates administered chlorpyrifos at 7.5 mg/kg/day versus 15 mg/kg/day in adults, a two-fold difference in sensitivity (Zheng *et al*., 2000).

1.7 Distribution

TCP (the major metabolite of chlorpyrifos) concentration is 2-fold to 4-fold higher in the fetal brain than in the maternal brain following chlorpyrifos treatment of dams with 3 or 7 mg/kg/day during GD 14-18, suggesting that the fetal nervous system may be exposed to a higher concentration of chlorpyrifos during gestation than revealed by brain ChEI alone (Hunter *et al.*, 1999). The differences in TCP concentration between adult and fetal tissues could not be attributed to differences in accumulation, as the half-life of TCP was found to be identical in all maternal and fetal tissues examined (*i.e.*, liver, brain, and blood).

1.8 Detoxification Enzyme Activities

Carboxylesterase and chlorpyrifos-oxonase activities are less in fetal and young rats than adult rats (Lassiter *et al.*, 1998). Activities of these enzymes, which are involved in the detoxification of chlorpyrifos and its metabolites, are low at birth (<10% of adult activity) and rise

gradually to adult levels. By 20 days of age, liver and plasma chlorpyrifos-oxonase activities approach adult levels. Carboxylesterase activities in liver and plasma of 20-day old rats are 40-50% of adult activities in the same tissues. It has been postulated that the age-related differences in sensitivities to ChEI in rats treated with chlorpyrifos can be explained, in part, by the lower levels of these detoxification enzymes in the young animal (Moser *et al.*, 1998).

2. Evaluation of Susceptibility following Gestational and Postnatal Dosing in Rats

There are a number of studies from the peer reviewed literature concerning the susceptibility of the developing brain to chlorpyrifos. In addition, there is a rat developmental neurotoxicity study that also provides some information (*e.g.*, data on behavioral and structural evaluations) regarding the susceptibility of the fetus or young.

2.1 Developmental Neurotoxicity Study

In a guideline developmental neurotoxicity study performed with Sprague-Dawley rats , pregnant/nursing females were administered chlorpyrifos by gavage (GD 6-LD 11) at doses of 0, 0.3, 1, or 5 mg/kg/day (Hoberman, 1998; MRID 44556901). The results of this study are summarized in Table 3. Maternal toxicity was observed as signs of fasciculations, hyperpnea, and hyperactivity at the high-dose level (5 mg/kg/day), and dose-dependent decreases in cholinesterase activity (plasma, RBC, and/or brain) at all dose levels. In the high-dose offspring, early postnatal viability and survival were decreased, and growth was impaired during lactation and following weaning (as manifested by decreased body weight and body weight gain, delayed pinna unfolding, and delayed sexual maturation in both sexes). Also at 5 mg/kg/day, behavioral evaluations demonstrated decreased motor activity for both sexes on PND 14, increased motor activity in females on PNDs 18, 22, and PND 61. Auditory startle habituation assessments identified increased latency to peak response and decreased peak response amplitudes on PNDs 23 and 62 in the high-dose offspring. Postmortem evaluation of high-dose pups on PND 12 revealed reduced absolute brain weights, increased relative (to body weight) brain weights, and decreased brain measurements in one or both sexes (anterior to posterior cerebellum, cerebellum height, caudate-putamen, parietal cortex, and hippocampal gyrus). On PND 66, morphometric evaluation of the brain revealed significant decreases in the thickness of the parietal cortex and nonsignificant decreases in the hippocampal gyrus of female offspring at the mid- and high-dose levels. Since morphometric data were not provided for PND 66 female offspring at the 0.3 mg/kg/day dose level, no conclusions can be drawn regarding the potential effects on neurological development at that dose; this establishes an issue of uncertainty in the risk assessments for infants and children.

As described above, adverse effects in the offspring are observed only at dose levels which are unequivocally toxic to maternal animals. However, an examination of the findings indicates that the offspring observations include apparent alterations in brain development, expressed as decreased thickness in specific morphological regions (*i.e.*, parietal cortex and hippocampal gyrus), while maternal toxicity is comprised of transient cholinesterase inhibition and cholinergic clinical signs. This difference in the qualitative severity of the findings seen in adult and neonatal animals is indicative of susceptibility of the developing brain. Additionally, a number of the treatment-related findings in the offspring appear to be the delayed expression of perturbations in earlier neurological development, since functional and morphological changes are observed at study termination (~PND 61-66), approximately 50-55 days after cessation of maternal dosing. These findings included increased motor activity in both sexes at PND 61, alterations in auditory startle measurements (increased latency to peak response and decreased peak response amplitudes) at PND 62, and morphometric alterations in the parietal cortex and hippocampal gyrus on PND 66.

The effects on the thickness of the parietal cortex and hippocampal gyrus in PND 66 female offspring was minimal (4-6%). Nevertheless, they can not be dismissed as evidence that chlorpyrifos treatment leads to effects in brain morphology. They are substantiated by the treatment-related effects shown in other brain regions at an earlier time point (PND 12) at the

highest dose tested (5 mg/kg/day), in spite of the fact that interpretation of the PND 12 data are complicated by the presence of maternal and offspring toxicity (*e.g.*, delayed pup growth, significant reductions in absolute but not relative pup brain weight). The reductions observed in the brains of female offspring at PND 66 are not accompanied by decreased absolute brain weight. Further, based upon comparisons with historical data as well as a low coefficient of variance (3%), and using a number of statistical tests, the effect on parietal thickness has been shown to be statistically significant at both the mid- and high-doses. Therefore, it is likely that effects on brain structure are treatment related.

Results of the developmental neurotoxicity study, when considered in conjunction with other evidence that the brain of neonates is vulnerable to the effects of chlorpyrifos treatment, raise concerns for the susceptibility of the fetus or neonate. Although functional outcomes of morphometric changes are unknown, the effects on brain development could potentially lead to adverse consequences. The nature (*i.e.*, persistence to adulthood) of behavioral findings in this study are also indicative of potential long-term consequences sequella.

2.2 Effects on Neurodevelopmental Processes

There is a fine genetic control that leads to a coordinated cascade of events during critical windows of normal brain development. So, there must be gene expression, cell-to-cell communication, cell migration, proliferation and differentiation occurring at the right times in the right regions of the developing brain. Because the developing brain is highly interactive, a perturbation during one of the developmental windows may result in a "derailment" with little opportunity to repair the mistake. There may be permanent effects which may not be expressed until later in life.

Chlorpyrifos has been shown in recent studies to alter macromolecular synthesis and function of cell signaling cascades in the brain during early and late postnatal development (see Table 4). Although direct measures of functional or morphological outcomes were not made, such perturbations may elicit abnormalities in neuronal cell proliferation and differentiation, leading to shortfalls in the number of cells as well as altered synaptic function and axonogenesis.

This work with chlorpyrifos has predominantly originated in one academic laboratory and is summarized in a recent review by Slotkin (1999). These studies generally followed a protocol that involved chlorpyrifos treatment of neonatal rats (usually Sprague-Dawley) at 1 mg/kg during postnatal days (PND) 1-4 or at 5 mg/kg on PND 11-14. Three brain regions which have different maturation profiles and cholinergic innervation (*i.e.*, brainstem, forebrain, and cerebellum) were evaluated. These *in vivo* studies administered chlorpyrifos in dimethyl sulfoxide (DMSO) by subcutaneous (SC) injection. The issue of the DMSO/SC dosing regime is addressed later. Results of key studies are presented below.

2.2.1 Macromolecular Synthesis

Several studies have shown that brain DNA synthesis is inhibited when rats are exposed to chlorpyrifos during the postnatal period (Dam *et al.*, 1998; Song *et al.*, 1998; Whitney *et al.*, 1995). Dam *et al. (*1998) used the incorporation of [^{3}H]thymidine into DNA as a measure of DNA synthesis. Effects on DNA synthesis were evaluated 4 or 24 hours after the last dose. The authors found that DNA synthesis was inhibited, with the earliest effect occurring in the brainstem 4 hours after the last dose in rats administered 1 mg/kg chlorpyrifos during PND 1-4. The inhibitory effects of chlorpyrifos on DNA synthesis were most evident (*i.e.*, ~20% inhibition) 24 hours after the last dose (PND 5) in both the brainstem and forebrain, which are cholinergically enriched. The

cerebellum, which is less cholinergically innervated and which develops later, was less affected. Thus, the degree and time of DNA synthesis inhibition appeared to parallel the maturation profile of the different brain regions evaluated. No significant effects on DNA synthesis were found in the heart, which is fully developed at birth and cholinergically innervated (Dam *et al.,* 1998). These findings suggest that the effects of chlorpyrifos were targeted to the developing postnatal brain, and raise the question of whether direct cholinergic hyperstimulation accounts for the observed effects. Inhibition of DNA synthesis was found after intracisternal administration (an equivalent dose of ~2 mg/kg body weight), thus leading the authors to propose that the effects of chlorpyrifos may be unrelated to cholinesterase inhibition (Whitney *et al.,* 1995). A shortcoming of the Whitney *et al. (*1995) study, however, is the lack of accompanying cholinesterase activity measures in either blood or brain.

Whitney *et al. (*1995) also showed that when 1-day old rats were administered a higher dose of chlorpyrifos (2 mg/kg), both DNA ([^{3}H]thymidine) and protein ([^{3}H]leucine) synthesis were inhibited within 4 hours of a single treatment. Mortality was reported as "insignificant" (*i.e.*, ~2%) at the 2 mg/kg dose of chlorpyrifos. Campbell *et al.* (1997) using different biomarkers (*i.e.*, measures of DNA and protein content) also found effects on brain macromolecules. Changes in DNA and protein content were more evident at 5 mg/kg chlorpyrifos administered during PND11-14 compared to the 1 mg/kg treatment during PND 1-4. The total amount of DNA declined between postnatal days 15 and 20 after the last dose.

Chlorpyrifos treatment resulted in decreased RNA concentration (total RNA per gram of tissue) and content (total RNA per tissue) in brainstem or forebrain with early (1mg/kg, PND 1-4) and late postnatal (5 mg/kg, PND 11-14) dosing (Johnson *et al.*, 1998). Further, because only total RNA is measured, it is unclear what type of RNA chlorpyrifos treatment affects (*i.e.*, mRNA, tRNA, rRNA). A further limitation of this study is presented by the fact that no specific precautions (*e.g.,* RNase inhibitor treatment) were taken to prevent RNA degradation which could be considerable. While the techniques used to measure RNA levels are not precise, the extent of changes seen are significant, and thus raise concern about potential damage to the developing brain. These effects were found shortly after the cessation of treatment and were more prominent in the brainstem, which develops earlier than the forebrain. Using a different biomarker, Dam *et al.* (1998) found an effect on DNA synthesis, but very little effect on RNA synthesis ([^{3}H]uridine incorporation) and protein synthesis ([^{3}H]leucine incorporation) after 1 mg/kg chlorpyrifos treatment during PND 1-4.

Chlorpyrifos was also evaluated for its effects on macromolecular synthesis in rat pheochromocytoma (PC12) cells, which express cholinergic receptors (Song *et al.*, 1998). In undifferentiated cells, chlorpyrifos treatment (0.5 - 1.5 ug/ml) inhibited DNA synthesis in a concentration-dependent manner. RNA and protein synthesis was less affected under similar conditions. The effects of chlorpyrifos on DNA synthesis could not be blocked by cholinergic receptor antagonists, thus suggesting that these effects may not be mediated through receptor activation. As nerve growth factor (NGF)-induced differentiation proceeded in these cells in the presence of chlorpyrifos, the inhibition of DNA synthesis continued and the inhibition of RNA and protein synthesis was greater initially but later disappeared. Therefore, the effects of chlorpyrifos on macromolecular synthesis following *in vitro* exposure generally mimic what was observed in the *in vivo* postnatal rat studies discussed above.

In summary, chlorpyrifos treatment of postnatal rats and *in vitro* neuronal cultures suggest dose-related changes in brain macromolecular synthesis (DNA, RNA, protein). Normal

macromolecular synthesis is critical to proliferation in the developing nervous system. These effects are unlikely to be due to nonspecific toxicity given the regional and temporal pattern of the responses. Further, the low dose treatment regime used in the above studies did not affect body weight or result in mortality. It is important to point out that none of the above studies included direct measures of cell proliferation or cell number (*e.g.*, morphometric analysis of brain regions). Nonetheless, the biomarkers evaluated (*i.e.*, effects on DNA synthesis, DNA or protein content) raise the concern for the potential of chlorpyrifos to damage the developing brain by interfering with cell replication and growth during critical periods of development. These effects of chlorpyrifos on cell proliferation in the brains of neonates have also been extended to effects on fetal brain in experiments with whole embryo cultures (Roy *et al.,* 1998). Near mid-gestation, embryos exposed to as low as 0.5 µg/ml chlorpyrifos for 2 days displayed evidence of altered proliferation, increased number of apoptotic cells, and alterations in cell polarity.

2.2.2 Differentiation and Synaptogenesis

Neurite outgrowth and branching (morphological markers of differentiation) are reduced in NGF-induced PC12 cells treated with noncytotoxic concentrations of chlorpyrifos or its metabolites, chlorpyrifos-oxon and TCP (Das and Barone, 1999). The inhibitory effects of an *in vitro* subacute treatment of chlorpyrifos or its metabolites were not observed during early neuronal differentiation but during the later elaboration phase in the differentiation process (at 168 hours). In more differentiated cells, the lowest acute concentrati ons that inhibited neurite outgrowth after 24 hours of incubation were 1 µg/ml for chlorpyrifos or TCP and 1 ng/ml for chlorpyrifos-oxon. At these concentrations, there was no inhibition of ChE activity found for chlorpyrifos while 90% ChE inhibition is caused by the chlorpyrifos-oxon. No anticholinesterase activity is found for TCP (at any concentration tested). Chlorpyrifos and TCP may affect differentiation (*i.e.,* neurite outgrowth) through a mechanism(s) other than inhibition of ChE. At the low concentrations used, chlorpyrifos and TCP continued to inhibit neurite outgrowth 24 hours after treatment.

In vivo exposure to chlorpyrifos during either PND 1-4 (1 mg/kg/d) or PND 11-14 (5 mg/kg/d) had significant effects on markers of synaptogenesis and synaptic activity (Dam *et al.,* 1999). Chlorpyrifos affected biomarkers for both the development of cholinergic synapses and nerve impulse activity. Choline acetyltransferase activity (ChAT), which is a marker for cholinergic synaptic outgrowth, was decreased in the forebrain following early postnatal treatment (1 mg/kg, PND 1-4). This effect on ChAT persisted through PND 10. From an earlier study, restoration of ChE activity was nearly complete at PND 10 following the 1 mg/kg chlorpyrifos treatment during PND 1-4 (Song *et al.,* 1997). Hemicholinium-3 binding (HC-3), a marker for neuronal impulse activity was only decreased with late postnatal chlorpyrifos treatment (5 mg/kg, PND 11-14). In the brainstem, both cholinergic synaptic development and activity were affected (*i.e.,* decreases in ChAT activity and HC-3 binding) but only after late postnatal treatment. These effects on synaptogenesis were observed in both cholinergic and catecholaminergic neurotransmitter systems in different brain regions. The effects on the developing catecholaminergic systems may be unrelated to cholinergic hyperstimulation based upon their occurrence in a region with very low intrinsic cholinergic activity, the cerebellum, and the temporal nature of the findings, which apparent later than the initial episode of ChE inhibition.

Follow up *in vitro* studies examined the nature of the acute effects of the parent compound chlorpyrifos had on synaptic function using rat brain synaptosomes pre-loaded with [^{3}H]norepinephrine. These studies found that catecholaminergic synaptic neuronal activity was altered after *ex vivo* exposure to chlorpyrifos (50 µg/ml)(Dam *et al.,* 1999b). In this *ex vivo* examination of chlorpyrifos effects on synaptosomes, it appeared that neonatal synaptosomes were

affected to a lesser extent compared to adult.

2.2.4 Synaptic Activity

Synaptic activity during critical windows of development is believed to have qualitatively different effects on the patterning of the developing nervous system than acute changes in synaptic activity in adult. This novel role of neurotransmitters as trophic molecules is reviewed in several recent publications (Buznikov *et al.,* 1996; Buznikov *et al.,* 1999; Lauder and Schambra, 1999). Dam *et al.* (1999b) provides evidence that chlorpyrifos may alter cell-cell communication or cell signaling during axonogenesis and synaptogenesis. This paper reports highly significant effects on both cholinergic and catecholaminergic pathways following postnatal chlorpyrifos treatment of rats. As discussed earlier, cholinergic synaptic activity was affected (as measured by decreased HC-3 binding) following early or late postnatal treatment. The effects of chlorpyrifos on catecholamine systems was different than the chlorpyrifos effects on cholinergic activity. Instead of suppression, chlorpyrifos treatment of 1 mg/kg during PND 1-4 or 5 mg/kg during PND 11-14 in rats increased catecholaminergic activity as determined by increased neurotransmitter (norepinephrine and dopamine) levels and turnover. Compared to brainstem and forebrain, the cerebellum was most affected with respect to chlorpyrifos effects on catecholaminergic markers. For example, cerebellum norepinephrine turnover (which is indicative of increased neuronal activity) increased approximately 70 - 80% from controls. The effects of chlorpyrifos on cholinergic and catecholaminergic pathways tended to disappear by 20 days of age.

2.2.5 Function of Cell Signaling Cascades

Chlorpyrifos has been reported to affect cell signaling or cell-to-cell communication during critical periods of brain development (Song *et al.,*1997; Dam *et al.,* 1999b). At doses known to affect DNA and protein synthesis in postnatal rats, chlorpyrifos affected the adenylyl cyclase pathway, which is important for regulation of cell replication and differentiation (Song *et al.,* 1997). Effects on adenylyl cyclase activity (deficits of 25-35% compared to controls) were most evident in the forebrain after a delayed period (day 10 of age) when chlorpyrifos was administered during the early postnatal period (1 mg/kg during PND 1-4) compared to dosing during late postnatal development (5 mg/kg during PND 11-14). Thus, younger animals were more susceptible to chlorpyrifos, and the delayed effects (*i.e.*, the deficits in adenylyl cyclase activity continued to increase after the last dose) are found after nearly complete recovery of brain ChE inhibition. Chlorpyrifos also altered adenylyl cyclase activity in cerebellum and heart, but to a lesser extent compared to the forebrain.

Hyperstimulation of cholinergic activity because of inhibition of ChE often results in the down regulation of muscarinic receptors. Chlorpyrifos treatment was found to decrease M1- and M2-receptor binding in the forebrain, but the effect was opposite of that for deficits in adenylyl cyclase activity (Song *et al.*, 1997). Decreases in muscarinic receptor binding were most evident with late postnatal dosing (5 mg/kg, PND 11-14) and diminished with time, thus paralleling the recovery of ChE inhibition. Given the regional and temporal pattern of effects, the authors proposed that cholinergic hyperstimulation may not appear to account for the effects on the adenylyl cyclase signaling cascade. Measures of adenylyl cyclase activity in the presence and absence of GTP suggest that chlorpyrifos effects are on G-protein mediated signaling, including that operating through neurotransmitter receptors known to play roles in cell replication and differentiation (Song *et al.,* 1997).

2.2.6 Mechanism of Action

There are certain aspects of the actions of chlorpyrifos (and its metabolites) in the developing brain that suggest that receptor-mediated cholinergic overstimulation alone cannot account for the chlorpyrifos-induced changes found during postnatal rat brain development. From a neurobiological standpoint, this should not be surprising. In the developing nervous system, cholinesterases and the neurotransmitter acetylcholine all are thought to have additional functions as compared to the adult brain. Therefore, changes in the structure, activity or concentration of these trophic molecules by chlorpyrifos (or its metabolites) may elicit novel effects on the developing brain. Evidence for these qualitatively different effects of developmental exposure versus adult treatment to this pesticide is supported by the examples given above and fall into three general categories: (1) when tested, cholinergic receptor blockers do not inhibit the chlorpyrifos-induced effects; (2) chlorpyrifos alone elicits the expected effect when injected directly into the neonatal brain or when used *in vitro*; and (3) effects may still be noted after cholinesterase activity has returned to normal levels. This latter pattern may simply imply a persistent effect requiring primary cholinesterase inhibition or it could also be completely unrelated to cholinesterase inhibition. In order to assure that this is the case, *i.e.*, that chlorpyrifos is toxic to the developing nervous system in its own right, without cholinesterase inhibition, one would have to assure that there is not chlorpyrifos-oxon contamination of the chlorpyrifos and that there is no measurable cholinesterase inhibition. At this point, the available scientific literature does not contain these assurances, causing concern that chlorpyrifos itself and possibly TCP is toxic to the developing brain. There is also the possibility that chlorpyrifos-oxon is acting in the "traditional manner," *i.e.*, inhibiting acetylcholinesterase activity, resulting in higher levels of acetylcholine in the developing brain. These high levels of acetylcholine or low levels of acetylcholinesterase activity may not, however, result in the expected cholinergic crisis (as they do in the adult brain); rather, these changes could cause novel effects on neurological development, because of the atypical functions of these molecules in the developing brain. It may be that even if cholinesterase inhibition is not causally related to some of the changes induced by chlorpyrifos in the developing brain, it (*i.e.*, cholinesterase inhibition) may still be a sensitive biomarker for potential effects. Nevertheless, careful studies must be conducted with cholinesterase inhibition surveyed at all doses and time points, so that the correlation between cholinesterase inhibition and other effects may be established.

3. Weight of Evidence Characterization and Conclusions

The weight of evidence provides appreciable support for the increased sensitivity of the young compared to adult rats to the neurotoxic effects of chlorpyrifos and for the susceptibility of the developing brain to chlorpyrifos treatment. A number of different rat studies clearly demonstrate that at a given oral dose the young rat will respond more to the anticholinesterase effects of chlorpyrifos (as defined biochemically and behaviorally) than adult animals. The differential found between pups and adult animals is a function of the treatment dose, duration of treatment, timing of treatment (*i.e.*, developmental stage) and of measurements (*i.e.*, time to peak effect), and the toxicological endpoint examined. At high acute doses, chlorpyrifos is fatal to the rat pup, but produces no lethality and little to no behavioral changes in the adult rat (*e.g.*, LD_{10} and MTD doses = neonate-15 mg/kg; adult-136 and 100 mg/kg, respectively). At the LD_{10} or MTD doses neonates are up to ~5-fold more sensitive than adult rats to ChEI (brain and blood) and clinical/behavioral effects. Furthermore, at a single treatment of 15 mg/kg, the down-regulation of the cholinergic (muscarinic) receptors was more extensive in the pups than in adults treated with 80 mg/kg. The magnitude of change, the effective time points, and the brain regions involved were different in pups versus adult rats. This suggests that the cholinergic receptors are more readily altered in the pup following chlorpyrifos treatment. Although the consequence of this is unknown, cholinergic receptors play an important role in normal brain development.

The increase in sensitivity between young and adult animals appears to occur at acute doses below 15 mg/kg. The study by Zheng *et al.* (2000) using lower dose levels (ranging from 0.15 mg/kg to 15 mg/day) provides ChEI data in 7-day old animals and adult male rats showing a greater sensitivity (up to ~3-fold for RBC and plasma, and perhaps at least 5-fold for brain) of pups compared with adult males. In the Zheng *et al.* study, the adult did not respond at the high dose of 15 mg/kg for brain ChEI. Thus, a difference in response greater than 5-fold can not be ruled out. Because of the lack of data, the extent of differences in brain ChEI between pups and the pregnant female rat remains uncertain. Although the young animal appears to recover at least two times faster than the adult animal from the ChEI induced by acute chlorpyrifos treatment, other toxicities (*e.g.*, delays in brain development, behavioral effects) may persist or appear at later times.

Repeated dosing with chlorpyrifos does not appear to result in an increase in brain or blood ChEI in neonates relative to adults with one exception. Based on $ED_{50's}$, there is a 1.5-fold difference in the response of PND 7 pups to brain ChEI compared to adult males (Zheng *et al.*, 2000). In contrast to the rapid recovery from ChEI observed with acute chlorpyrifos treatments of neonates (Pope and Liu, 1997), repeated dosing with chlorpyrifos (every other day, 11 treatments during PND 1 to PND 21) indicates ChEI persists for ~9 to >19 days depending on the dose administered (Tang *et al.*, 1999). Body weight changes and behavioral effects occur at ~3-fold lower doses in neonates versus adult rats with repeated treatments of chlorpyrifos doses equal to or above 3 mg/kg/day.

It is apparent that cholinesterase activity is inhibited in the fetus is if the dam is treated with a chlorpyrifos dose which can be absorbed by the fetus. The magnitude of brain, plasma, and RBC ChEI in the fetus is less or equal to that observed in dams with acute or repeated treatments of dams with chlorpyrifos. The lack of an apparent differential response of the fetus (or neonate with repeated dosing) versus the maternal system to treatment of dams with chlorpyrifos may be due to the increased new synthesis or more rapid turnover of inhibited molecules of cholinesterases in the fetal brain than in the adult (Lassiter *et al.*, 1998; Mortensen *et al.*, 1998).

Differences in detoxification between the young and adults may explain the increased

sensitivity of exposed pups to chlorpyrifos toxicity. Chlorpyrifos and its oxon *(i.e.*, the anticholinesterase metabolite) are detoxified by binding to carboxlyesterases and hydrolysis by A-esterases. The young animal has minimal activity of these detoxification enzymes compared to adult animals. The precise influence of these enzymes on sensitivity to chlorpyrifos treatment has not been established. Because detoxification enzyme activities increase with age, the enzymatic profile of newborn rats raises concern that the newborn may be even more sensitive than older neonates to an acute chlorpyrifos treatment. There is some evidence (albeit at high doses) that suggests that the magnitude of the differential sensitivity between young and adult animals depends on the age of the animal. Based on the LD_{10} data in Zheng *et al.* and from the ChEI data in Zheng *et al.* and Moser and Padilla (1998), the order of sensitivity is PND 7 > PND 17 > PND 27 > adult female > adult male. Therefore, given that 7-day old rats are the youngest animals evaluated to date, it is uncertain whether the magnitude of differential sensitivity would be greater with pups exposed earlier than 7 days.

The developmental neurotoxicity study, which involved treatment of dams with 5, 1, or 0.3 mg/kg/day chlorpyrifos from GD 6 through lactation day 11 (Hoberman, 1998), offspring were observed to have alterations in brain structure that are suggestive of a developmental defect that may predispose the neonate to unique adverse consequences. In this study, morphometric measurements in PND 11 pups showed at the high dose, decreases in anterior to posterior measurements of the cerebellum, reduced height of the cerebellum, decreased thickness of the parietal cortex, and decreased thickness of the hippocampal gyrus. These effects at the high dose occurred in the presence of maternal toxicity (*e.g.*, maximum brain, RBC and plasma ChEI) but in the absence of effects on body weights, food consumption, pregnancy parameters, or deaths among the dams. In mid- and high-dose PND 66 offspring, effects on brain structure included marginal but statistically significant decreases in the thickness of the parietal cortex and non-significant decreases in the thickness of the hippocampal gyrus. This difference in the qualitative severity of the findings seen in adult and neonatal animals is indicative of susceptibility of the offspring. It is also important to note that morphometric evaluation of the low-dose brains was not conducted. So it is not known whether alterations are occurring at lower doses. Additionally, a number of the treatment-related findings in the offspring appear to be delayed in expression of perturbations in earlier neurological development, because functional and morphological changes are observed at study termination (~PND 61 - 66), approximately 50 - 55 days after cessation of maternal dosing. At the high dose, these findings included increased motor activity in females at PND 61, alterations in auditory startle measurements (increased latency to peak response and decreased peak response amplitudes) at PND 62, and morphometric alterations in the parietal cortex and hippocampal gyrus on PND 66.

A variety of *in vitro* and *in vivo* studies published in the peer reviewed literature show that chlorpyrifos can alter macromolecular synthesis, neuronal activity, neurotransmitter levels, neurite outgrowth and branching, and cell signaling in the developing rat brain (as cited in Table 4 and reviewed by Slotkin, 1999). Although these studies did not include accompanying measures of direct adverse effects (*e.g.*, functional effects) but rather used biomarkers, they nevertheless raise concern that chlorpyrifos potentially can affect processes occurring in both early and late developmental periods of brain growth that influence cell replication and differentiation needed for normal function. Although the data primarily come from one laboratory, multiple studies from this group have shown a consistency in the different responses measured. Furthermore, several of the key responses observed are highly significant and robust (*e.g.*, effects on norepinephrine turnover, DNA synthesis, adenylyl cyclase transduction). Also, the responses reported tend to have little variability in the data. Finally, effects on the developing brain reported in the literature are consistent with the morphometric changes observed in the guideline developmental neurotoxicity

study by Hoberman (1998) even though a direct linkage of effects can not be made. The available data suggest a selective action of chlorpyrifos on the developing brain, given the regional and temporal pattern of responses. Thus, it seems unlikely that the observed effects are due to nonspecific toxicity.

Although there are strengths of these studies, there are also some limitations and questions raised which are not addressed by the results. As discussed above, the mechanism of action for chlorpyrifos in the developing brain is unclear. Also, the *in vivo* studies using macromolecular biomarkers have primarily been conducted using the subcutaneous injection (SC) route of exposure and DMSO as the vehicle. It should be noted that DMSO controls were conducted in all the studies. DMSO would result in a rapid uptake and full absorption of the compound. Compounds administered via SC injection enter directly into the general circulation and bypass hepatic metabolism once, thus bypassing hepatic activation of chlorpyrifos to its active metabolite chlorpyrifos-oxon. The SC route of exposure can not be reliably compared to the oral route given the lack of pharmacokinetic data on this dosing regime. Also, this is not a pathway of human exposure. Thus the DMSO-SC dosing regime makes quantitative interpretation and extrapolation of the results problematic. Nevertheless, these studies still provide important qualitative information on the potential for chlorpyrifos to affect neurodevelopmental processes. Cholinesterase inhibition was not measured in most of these studies except for Song *et al.* (1997). In that study, no extreme cholinesterase inhibition is found in the brainstem at the low dose used in the study: approximately 20-25% cholinesterase inhibition is found when 1 mg/kg of chlorpyrifos is administered during PND 1-4 and cholinesterase activity (measured 24 hours after the last dose) is almost completely recovered by 10 days of age (Song *et al.*, 1997). Given that key effects in the postnatal brain are found at the low dose, the concern of a rapid delivery of a toxic dose with this standard dosing regime is reduced. Also, no significant changes in body or brain weight and no mortality occurs with this dosing regime (1 mg/kg at PND 1-4 or 5 mg/kg at PND 11-14). Additionally, it should be noted that chlorpyrifos is rapidly absorbed and transported to the brain with oral dosing (Mendrala and Brzak, 1998). Thus, the findings derived from the SC/DMSO dosing regime can not be discounted as an artifact of the vehicle and route of exposure and raise concerns for the unique susceptibility of the young.

The mechanism(s) of action for the chlorpyrifos-induced changes (*e.g.*, macromolecular synthesis, cell signaling) is/are unclear. However, given that these effects can be found after intracisternal injection of chlorpyrifos, with *in vitro* TCP treatment, and *in vitro* PC12 cell cultures with limited capability to activate chlorpyrifos to its ChE-inhibiting oxon, raises the issue of whether these effects can occur independent of cholinesterase inhibition. Although it is not possible to link each effect reported with another effect or with a functional outcome, the data show a consistent pattern of the potential for chlorpyrifos to produce qualitatively different effects in the central nervous system (CNS) of young versus adult animals. Potential implications of the effects include alteration of synaptic responses that are programmed by neural input, disruption of cell replication and differentiation, and temporary or persistent delays in the development of CNS structures.

In conclusion, the weight of the evidence raises concern for an increase in both the sensitivity and susceptibility of the fetus or young animal to adverse biochemical, morphological, or behavioral alterations from chlorpyrifos treatment during brain development. With respect to cholinesterase inhibition, an increased in sensitivity of the young compared to adults was seen all along the dose response curve, even at relatively low doses. There is a clear differential response (2- to ~5-fold) in the young compared to the adult animal after an acute treatment to a relatively low dose of chlorpyrifos. There is also increased sensitivity found after

repeated dosing (up to 9-fold), but at the LD_{10} and MTD. It is important to point out that an uncertainty remains concerning the magnitude of the differential response, given that newborn animals (less than PND 7) have not been characterized for sensitivity. Results of multiple studies have consistently shown that the developing brain is susceptible to chlorpyrifos treatment. Effects on the developing CNS that are indicative of the unique susceptibility to the young animal include changes in macromolecular synthesis, altered cell signaling and muscarinic receptor down-regulation, as well as morphological alterations in brain development. An uncertainty remains regarding the NOAELS for the susceptibility effects. The effects observed raise a high degree of concern that the fetus or young animal is particularly susceptible to adverse outcome if exposed to chlorpyrifos.

Table 1. Responses of male, female, juvenile, neonatal and fetal rats to acute gavage treatments with chlorpyrifos

Endpoint	Response	Comments
Acute lethality - LD_{10} (Zheng *et al.*, 2000)	7-day neonate-15 mg/kg; 21-day juveniles - 47 mg/kg; adults-136 mg/kg	Neonate more sensitive: 9-fold adult and 3-fold juvenile
MTD - less than 10% mortality (Moser and Padilla,1998)	PND 10 neonate-15 ,mg/kg; adult-100 mg/kg	Neonate 6.7-fold more sensitive than adult
ChEI - male and female rats (Mendrala and Brzak, 1998; Lassiter *et al.*, 1998; Moser *et al.*, 1998; Zheng *et. al.*, 2000)	Male rats: slight (about 15%) brain ChEI at 10 mg/kg (2 studies); Male rats: 40% brain ChEI at 20 mg/kg; Female rats: 70% brain ChEI at 20 mg/kg; Female pregnant rats: 50% brain ChEI at 10 mg/kg	Pregnant female rats about 2-fold more sensitive than male rats to brain ChEI
ChEI - ED_{50}* (Zheng *et al.*, 2000) Brain RBC Plasma	 PND 7 neonate-2.9 mg/kg; adults N.S. at 15 mg/kg PND 7 neonate -2.2 mg/kg; adults-4.4 mg/kg PND 7 neonate-1.5 mg/kg; adults -3.9 mg/kg	<u>Sensitivity:</u> Neonate 5-fold > adult Neonate 2-fold >adult Neonate 2.6-fold >adult
Acute dose resulting in 80-90% ChEI in blood and brain (Moser and Padilla, 1998a)	PND17 juvenile-15 mg/kg; adult females-80 mg/kg	Juvenile 5.3-fold more sensitive than adult
Acute neurotoxicity (Moser *et al.*, 1998)	PND17 juvenile- neurotoxicity at 20mg/kg; adult females-neurotoxicity at 50 mg/kg	Juvenile 2.5-fold more sensitive than adult
Muscarinic down regulation-acute dose (17 day juveniles) (Moser *et al.*, 1998)	PND17 juvenile-down regulation at 15 mg/kg; adult females-down regulation at 80 mg/kg	Juvenile 5.3-fold adult (at the respective doses, down regulation was more extensive in young rats)
Recovery from single dose; (Moser and Padilla, 1998a)	PND17-1 week (15 mg/kg); adult females >2 weeks (80 mg/kg)	Juveniles recover 2-fold faster than adults to an MTD dose
ChEI *in utero;* (Lassiter *et al.*, 1998)	GD-18 fetal brain ChEI ~20% at 7 mg/kg and 40% at 10 mg/kg; dam brain ChEI ~ 30% at 7 mg/kg and ~50% at 10 mg/kg	Fetus sensitive to ChEI when dam treated with chlorpyrifos

* ED_{50s} for neonate estimated from Figure 3 of Zheng *et al.* (2000)

Table 2. Responses of male, female, neonatal and fetal rats to repeated chlorpyrifos treatments*

Endpoint/Treatment duration	Response	Comments
ChEI ED_{50}-14 days treatment; SD rats; (Zheng *et al.*, 2000) Brain RBC Plasma	 Neonate-2.2 mg/kg/day; adults-3.3 mg/kg/day Neonate-1.2 mg/kg/day; adults-0.5 mg/kg/day Neonate-1.5 mg/kg/day; adults-1.5 mg/kg/day	<u>Sensitivity:</u> Neonate 1.5-fold >adult Adult 3-fold > neonate No increase in sensitivity
ChEI GD6-GD20; SD rat; 5 mg/kg/day (Mattsson *et al.*, 1998)	Fetus -60% forebrain; dam - 87% forebrain; Fetus -54% hindbrain; dam 76% I hindbrain. RBC and plasma ChEI in maternal animals at 1 mg/kg/day but none in fetus	Substantial inhibition in fetal brain when dam treated with chlorpyrifos
ChEI GD6-20; SD rats (Mattsson *et al.*, 1998)	Dams: 33% plasma at 0.3 mg/kg/day; 26% RBC at 0.3 mg/kg/day	No NOAEL - suggests pregnant SD rat more sensitive than male
ChEI 14 days SD male rats (Zheng *et al.*, 2000)	23% plasma ChEI at 0.45 mg/kg/day; 39% RBC at 0.45 mg/kg/day	Suggests pregnant SD rat more sensitive to ChEI than male SD rat
ChEI GD6-20; SD pregnant rat; (Hoberman, 1998)	43% plasma ChEI at 0.3 mg/kg/day; 41% RBC ChEI at 0.3 mg/kg/day	Suggests pregnant rat more sensitive to ChEI than male rat
ChEI GD14-GD18; LE rat (Lassiter *et al.*, 1998)	Fetal brain-20% (7mg/kg/day) Maternal brain-80% (7mg/kg/day Fetal brain control ChE activity increased 4.3 fold from GD 14 to GD 18	Fetal brain ChEI with repeated exposure of dam
ChEI-GD14-18; LE rat (Hunter *et al.*, 1999)	Fetal brain ChEI- 3 mg/kg/day, ~3% Maternal brain ChEI at 3 mg/kg/day, ~41%; Fetal brain ChEI at 7 mg/kg/day, ~32% ChEI Maternal brain ChEI at 7 mg/kg/day, ~ 87% TCP metabolite ~4.5 fold higher in fetus than dam	Negligible ChEI in fetal brain at 3 mg/kg/day.
ChEI-14 days <u>subcutaneous</u> treatment (Liu *et al.*, 1999)	7 Day neonate and 90 day adult males - similar ChEI & similar muscarinic receptor binding at day 15; neonatal brain ChEI at day 8 somewhat greater at low dose; doses 5 & 10 mg/kg/day; neonates recovery from effects faster	No increase in sensitivity of neonate with 14 days treatment; some increase in neonatal sensitivity to brain ChEI with 8 days treatment at 5 mg/kg/day.
ChEI-treatment of newborn SD rats (Tang *et al*., 1999)	Dose: 3 mg/kg/day every other day from PND 1-PND 21; 37% Brain ChEI on PND 6 and PND 14; brain ChEI persisted for nine days following last treatment.	Direct dosing of newborn rats results in brain ChEI at relatively low doses; brain ChEI persists after cessation of dosing

* Dosing by gavage unless otherwise noted.

Table 3. Results of Developmental Neurotoxicity Study (MRID 44556901)

Parameter	0.3 mg/kg/day	1.0 mg/kg/day	5.0 mg/kg/day
DAMS			
Clinical obs.			Fasciculations, hyperpnea, hyperactivity
ChE activity	Plasma (↓43%) RBC (↓41%)	Plasma (↓69%) RBC (↓84%) Brain (↓18%)	Plasma (↓92%) RBC (↓99%) Brain (↓90%)
OFFSPRING			
Growth and development			Preweaning: Decreased BW/BWG, terminal BW (PND 12); delayed pinna unfolding
			Postweaning: Decreased BW/BWG (males); decreased terminal BW (PND 66) (males);decreased FC (PND 23-30);delayed sexual maturation
Survival			Decreased survival
Motor activity			Decreased in both sexes PND 14 Increased in females PND 18 & 22
			Increased in males and females PND 61
Auditory startle			PND 23: Increased latency to peak response; decreased peak response amplitude (NS)
			PND 62: Increased latency to peak response; decreased peak response amplitude (NS)
Postmortem findings			PND 12: Decreased absolute brain weight (♂,♀) Increased relative brain weight (♂,♀) Decreased morphometric measures: Anterior/posterior cerebellum (♂) Cerebellum height (♂) Caudate putamen width (♀) Parietal cortex (♂,♀) Hippocampal gyrus (♂,♀)
	(Not examined)	PND 66: Decreased morphometric measures: Parietal cortex (♀) Hippocampal gyrus (♀) (NS)	PND 66: Decreased morphometric measures: Parietal cortex (♀) Hippocampal gyrus (♀) (NS)

Table 4. Summary of Key Literature of the Effects of Chlorpyrifos (CPF) on the Developing Rat Brain

Dose-Exposure Period	Neurodevelopmental Effects	Reference
IN VIVO STUDIES		
1mg/kg - PND1-4 5mg/kg - PND1-14 (subcutaneous injection in DMSO)	Altered synaptic development (ChAT activity) and activity (HC-3 binding,). The cerebellum was affected most for norepinephrine and dopamaine turnover.	Dam *et al.*, 1999b
As above	Inhibition of DNA synthesis	Dam *et al.*, 1998
As above	Altered adenylyl cyclase signal cascade in all brain regions several days after exposure.	Song *et al.*, 1997
As above	Altered RNA levels in brain stem and forebrain.	Johnson *et al.*, 1998
1 or 5 mg/kg - PND1-4 5or 25 mg/kg - PND11-14 (subcutaneous injection in DMSO)	Increase in DNA and protein levels. Significant mortality occurred at higher doses	Campbell *et al.*, 1997
2 mg/kg - PND1 11 mg/kg -PND6-11 (subcutaneous in DMSO)	Inhibition of DNA synthesis within 4 hr of treatment and at 8 days of age in all brain regions	Whitney *et al.*, 1995
IN VITRO STUDIES		
50 ug/ml CPF of rat brain synaptosome preparations	Increased release of norepinephrine (NE), which was not mediated via cholinergic receptors given that the blockers, atropine and mecamylamine did not interfere with the effect. Neonatal synaptosomes were effected to a lesser extent compared to adult	Dam *et al.*, 1999a
1,10, 100, or 1000 ug/ml CPF or TCP, or 0.1, 1, 10, 100, or 1000 ng/ml CPF-oxon treatment of pheochromocytoma (PC12) cells for 24 hr	1 ug/ml of CPF or TCP, and 1 ng/ml of CPF-oxon inhibited NGF-induced neurite outgrowth as determined microscopically by measuring branches per cell, fragments per cell, fragment length per cell, and total neurite outgrowth per cell.	Das & Barone, 1999
0.5 -1.5 ug/ml CPF treatment of PC12 cells	Concentration dependent inhibition of DNA synthesis; RNA and protein synthesis was less effected under similar conditions. Effect on DNA synthesis was not mediated via cholinergic receptors given that the blockers atropine and mecamylamine did not ameliorate the inhibitory effect.	Song *et al.*, 1998
0.5, 5, 50 ug/ml CPF treatment of 9.5 day rat embryo culture	Inhibition of mitosis and altered mitotic figures	Roy *et al.*, 1998

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