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

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

April 18, 2000

Memorandum

SUBJECT: Toxicology Chapter for Chlorpyrifos. DP Barcode D263892, Case 818975, Submission S576466, PC Code 059101.

FROM: Deborah Smegal, M.P.H. Toxicologist
Re-Registration Branch 3
Health Effects Division (7509C)

THRU: Jess Rowland, M.S., Branch Chief
Re-registration Branch 3
Health Effects Division (7509C)

TO: Mark Hartman, Chemical Review Manager
Special Review and Reregistration Division (7508C)

This memorandum summarizes the guideline studies submitted by the registrant, and other relevant toxicity studies considered by HED in developing the acute and chronic reference doses (RfDs) and toxicity endpoints for use in risk assessment.

I. HAZARD PROFILE SUMMARY

The toxicological database is complete and adequate to support reregistration. in accordance with the Subdivision F Guidelines for a food use chemical.

Chlorpyrifos is moderately toxic following acute oral, dermal and inhalation exposures and is classified in toxicity category II for all exposure routes. Chlorpyrifos affects the nervous system by reversibly inhibiting the activity of cholinesterase (ChE), an enzyme necessary for the proper functioning of the nervous system. Inhibition of ChE is the most sensitive effect in all animal species evaluated and in humans, regardless of exposure duration. In animals, significant inhibition of plasma and red blood cell (RBC) ChE occur at doses below those that cause brain ChE inhibition. In animals, significant plasma and RBC ChE have been observed at oral doses as low as 0.025 to 0.3 mg/kg/day following exposure for two weeks to two years, while significant brain ChE inhibition has been observed at oral doses as low as 1 mg/kg/day following exposure for two weeks in pregnant rats (Hoberman 1998a,b). Female rats and especially pregnant rats appear to be more sensitive than adult male rats to cholinesterase inhibition (Moser et al. 1998, Hoberman 1998a,b, Mattsson et al. 1998). Data from two human studies suggest that humans (adult males) are similarly sensitive and possibly more sensitive than rats and dogs following acute and short-term oral exposure and acute dermal exposure based on plasma ChE inhibition and/or possible clinical signs. It is likely that the human sensitivity for ChE inhibition relative to rats (but not dogs) is due to species differences in the constituents of plasma ChE between rats and humans. For example, in rats, plasma ChE consists of approximately a 60:40 ratio of acetyl cholinesterase (AChE) and butyryl cholinesterase (BuChE), while in most humans and dogs, plasma ChE is predominately as BuChE, which is more sensitive to inhibition than AChE.

TCP. HED has concluded that the primary metabolite of chlorpyrifos, 3,5,6-trichloro-2-pyridinol (3,5,6-TCP), does not induce cholinesterase inhibition, and therefore is less toxic than chlorpyrifos (58 FR 19354, April 14, 1993). However, because of the potential exposure to TCP in food and residential settings, and evidence of increased susceptibility of rabbit fetuses relative to dams, HED conducted a screening-level risk assessment for TCP.

Neurotoxicity. Adult male rats acutely exposed to chlorpyrifos exhibited peak plasma ChE inhibition of 28-40% 3-6 hours after exposure at 1 mg/kg (Mendrala and Brzak 1998), while significant 30% RBC ChE inhibition was noted 4 hours following a single oral dose of 1.5 mg/kg (Zheng et al. 2000). Plasma, RBC and heart ChE inhibition of 45%, 17% and 19%, respectively were observed in female rats 24 hours following a single dose of 5 mg/kg (Dittenber 1997). The acute oral NOAEL for plasma ChE inhibition in male rats is 0.5 mg/kg/day. Clinical signs of neurotoxicity, in the absence of neuropathology, were observed in rats exposed to a single oral dose of 50 mg/kg as evidence by decreased motor activity, and increased incidence of clinical signs consistent with organophosphate intoxication. Chlorpyrifos was negative in the delayed neurotoxicity study in hens at single doses of 50, 100 or 110 mg/kg. Acute oral exposure to hens at 60 to 150 mg/kg caused 59-87% inhibition of neurotoxic esterase (NTE) 4-6 days after

exposure (Capodicasa et al. 1991). In addition, delayed neuropathy was noted at 60-90 mg/kg which corresponded to 4-6 times the LD_{50} and required aggressive antidotal treatment. In rats, chlorpyrifos failed to inhibit NTE at single doses up to 100 mg/kg. There is evidence that NTE inhibition is related to organophosphate-induced delayed neuropathy (OPIDN).

Following longer-term exposures, there was no evidence of neurotoxicity or neuropathology in rats exposed at doses up to 15 mg/kg/day for 13 weeks. However, in the developmental neurotoxicity study, pregnant dams exposed to chlorpyrifos for approximately 2 weeks exhibited 43% and 41% inhibition of plasma and RBC ChE activity at 0.3 mg/kg/day, significant 18% brain ChE inhibition at 1 mg/kg/day, and clinical signs of neurotoxicity, including fasciculations (muscle twitching), hyperpnea (increased respiration), and hyperactivity in addition to decreased body weight gain at 5 mg/kg/day (Hoberman 1998a,b). Cholinesterase inhibition (68% plasma, 56% RBC and 8% brain) was also noted in rats exposed to 1 mg/kg/day chlorpyrifos for 4 weeks in the cognitive study, while clinical signs of toxicity were not observed until higher doses of 3 mg/kg/day for miosis (pupil contraction) and 10 mg/kg/day for salivation and tremors (Maurissen et al. 1996).

Adult hens exposed to oral doses of 10 mg/kg/day for 20 days experienced significant body weight loss and marked inhibition of brain and plasma ChE, 18% brain NTE inhibition, but no significant change in lymphocyte NTE activity, and no behavioral signs of OPIDN during the dosing interval or during the subsequent 4 week observation period (Richardson et a. 1993).

Subchronic Toxicity. Several subchronic studies are available for chlorpyrifos including two oral rat studies, one oral dog study, a 21 day dermal toxicity study in rats, and two inhalation studies in rats. The most sensitive effect following subchronic oral exposure is inhibition of plasma ChE in rats and dogs at 0.025 to 0.03 mg/kg/day, and RBC ChE inhibition in dogs and rats at 0.22 to 0.3 mg/kg/day. Rats exposed to higher doses exhibited hematological effects at doses of 10 mg/kg/day and increased brain and heart weight, adrenal gland effects and decreased body weight gain at 15 mg/kg/day. No adverse effects were noted in rats exposed via inhalation to the highest attainable vapor concentration of 20.6 ppb (287 $\mu g/m^3$) (0.1 mg/kg/day). No adverse effects were observed in the 21-day dermal study in rats at doses as high as 5 mg/kg/day. However, in a 4-day dermal probe study, rats dermally exposed to doses of 0, 1, 10, 100, or 500 mg/kg/day exhibited reductions in plasma and RBC ChE activities at doses of 10 to 500 mg/kg/day. The 21-day dermal NOAEL is 5 mg/kg/day based on a 45% and 16% inhibition of plasma and red blood cell cholinesterase, respectively in rats dermally exposed to 10 mg/kg/day for 4 days.

Carcinogenicity/Genotoxicity. Chlorpyrifos was evaluated for carcinogenic potential in both rats (2 studies), and mice (2 studies). There was no evidence of carcinogenicity. Chlorpyrifos is not mutagenic in bacteria, or mammalian cells, but did cause slight genetic alterations in yeast and DNA damage to bacteria. In addition, chlorpyrifos did not induce chromosome aberrations in vitro, was not clastogenic in the mouse micronucleus test in vivo, and failed to induce unscheduled DNA synthesis in isolated rat hepatocytes.

Chronic Toxicity. Chlorpyrifos was evaluated for chronic toxicity in rats, mice and dogs. In all animal species, the most sensitive effect is inhibition of plasma, RBC and brain ChE that occurred at levels in the range of 0.03 to 3 mg/kg/day. Following chronic exposure dogs appear to be the most sensitive species for cholinesterase inhibition and systemic effects, as noted by increased liver weights in dogs exposed to 3 mg/kg/day that could be an adaptive response. Rats exposed to 7-10 mg/kg/day had decreased body weight and decreased body weight gain, ocular effects, adrenal gland effects and altered clinical chemistry and hematological parameters. Mice appear to be the least sensitive to chronic oral doses of chlorpyrifos, as exposure to 45-48 mg/kg/day resulted in decreased body weight and an increased incidence of non-neoplastic lesions (i.e., keratitis, hepatocyte fatty vacuolation).

Developmental Toxicity. Chlorpyrifos was evaluated for developmental toxicity in rats, mice and rabbits. In one rat study, developmental effects (increased post-implantation loss) were noted at 15 mg/kg/day (highest dose tested, HDT), that were also associated with maternal toxicity, while another rat study failed to observe developmental effects at 15 mg/kg/day. Developmental effects were also noted at higher doses in mice at 25 mg/kg/day (minor skeletal variations, delayed ossification and reduced fetal weight and length) and rabbits at 140 mg/kg/day (decreased fetal weights and crown rump lengths, and unossified xiphisternum and/or 5th sternebra). However, in both mice and rabbits, the developmental effects occurred at maternally toxic doses as indicated by reduced weight gain, and food consumption in both species, and increased mortality in mouse dams.

In the rat developmental neurotoxicity study, chlorpyrifos was associated with delayed alterations in brain development in offspring of exposed mothers. Specifically, pups of the 1 mg/kg/day group exhibited significant dose- and treatment-related decreases in measurements of the parietal cortex in female offspring at postnatal day 66. The only maternal effect at this dose was plasma and RBC ChE inhibition. At higher doses, pups of the 5 mg/kg/day group exhibited decreased body weight/body weight gain and food consumption in both sexes, reductions in pup viability, delays in development, decreased brain weight and morphometric alterations in the brain. However, these effects were observed in the presence of maternal toxicity as evidenced by fasciculations, hyperpnea and hyperactivity, in addition to reduced body weight gain.

Several studies in the peer reviewed literature and results of the guideline developmental neurotoxicity study 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., altered synaptic development, alterations in DNA, RNA, and protein synthesis, inhibition of mitosis and mitotic figures, and disruption of the structural architecture of the brain), and which may result in irreversible alterations of nervous system structure and/or function (Whitney et al. 1995, Campbell et al. 1997, Song et al. 1997, Johnson et al. 1998, Das and Barone 1999, Dam 1999, Roy et al. 1998, Hoberman 1998a,b).

Reproductive Toxicity. Chlorpyrifos induced reproductive toxicity in one generation of rats, but only at dose levels that induced parental toxicity. Reproductive effects included reduced pup

weights and increased pup mortality that corresponded to slightly by significantly reduced body weight gain in F0 dams during lactation days 1-21, in addition to parental toxicity as evidenced by inhibition of plasma, RBC and brain cholinesterase activities as well as histological lesions of the adrenal gland (vacuolation of cells of the zona fasciculata).

Human Studies. HED has reviewed two human studies conducted with chlorpyrifos submitted by the registrant (MRID 95175, Accession No. 249203). A third human study (Kisicki et al. 1999) that evaluated a single dose exposure was submitted on April 27, 1999 but is an incomplete submission because two Appendices with critical data were omitted. This study will be reviewed by the Agency once the submission is complete. In the first study (MRID No. 95175; Coulston et al., 1972), male volunteers from Clinton Correctional Facility (4/dose group) were given daily oral (tablet) doses of 0, 0.014, 0.03, or 0.1 mg/kg chlorpyrifos technical for 7 weeks, 9 days, 21 days and 28 days, respectively. Significant 36-82% plasma ChE inhibition relative to baseline was observed after 9 days of treatment with 0.1 mg/kg/day chlorpyrifos. In addition, one of the four men in the 0.1 mg/kg/day developed blurred vision, runny nose and a feeling of faintness on day 9. Exposure was discontinued on day 9 in this dose group however, due to plasma cholinesterase inhibition that exceeded the study investigator's guideline of 20%-30%. No significant plasma ChE inhibition was observed in the men exposed to 0.03 mg/kg/day for 21 days or at any other dose that could be attributed to treatment. No effects on RBC ChE were found at any dose that could be attributed to treatment. A gradual recovery was observed in plasma ChE values equaling baseline values by day 25 of the recovery period. The registrant and study director contend that the clinical signs were attributed to a cold, and not chlorpyrifos exposure. HED believes that blurred vision is a typical cholinergic sign of ChE inhibition, and can not be attributed to a common cold (February 2, 1998 HIARC Report, HED Doc No. 012471). In addition, there is no reason to believe that other clinical signs would not have appeared if the dosing had continued for 21 or 28 days as it did for the other groups. While the study director claims that exposure to the high dose group was discontinued on day 9 because plasma ChE inhibition was 20-30%, rather than because of concern for the clinical signs, this reason is inconsistent with the study findings of 46% mean plasma ChE inhibition following day 6 of treatment in the 0.1 mg/kg/day group, and 41% plasma ChE inhibition in one individual on day 3. HED notes that the relatively long recovery period of 25 days is unusual for plasma ChE, and is more characteristic of recovery for RBC acetyl ChE inhibition based on the 2 year dog data (McCollister et al. 1971, Kociba et al. 1985).

An acute oral and dermal pharmacokinetic study (Nolan et al. 1982, Accession No. 249203) dosed six men once with 0.5 mg/kg orally and four weeks later dosed five of these same men with 5 mg/kg dermally, and one man with 0.5 mg/kg dermally. No clinical signs or symptoms were observed in any of the subjects, but unlike the previous study, the primary focus of this study was pharmacokinetics. Men orally exposed to 0.5 mg/kg chlorpyrifos exhibited peak plasma ChE inhibition of 64-85%, 12 to 24 hours post-exposure. Peak RBC ChE inhibition of 11-52% occurred on post-exposure day 4. Men dermally exposed to 5 mg/kg chlorpyrifos exhibited peak plasma ChE inhibition of 27-45% on day 3, and mean RBC ChE inhibition of 8.6% on day 4. The return of plasma ChE activity to pre-dose levels required about 30 days. The registrant stated

that the inhibition noted on days 3 and 4 is an analytical artifact based on chlorpyrifos pharmacokinetics. If this is the case, it raises concerns about the quality and reliability of the study data. Again, HED notes that the relatively long recovery period of 30 days is unusual for plasma ChE, and is more characteristic of recovery for RBC acetyl ChE inhibition based on the 2 year dog data (McCollister et al. 1971, Kociba et al. 1985). On the basis of urinary excretion of the 3,5,6-trichloro-2-pyridinol (3,5,6-TCP) metabolite, the minimum oral absorption of chlorpyrifos was estimated at 70% and the minimal dermal absorption at 1-3%. Because the proportion of the administered dose metabolized to this pyridinol is unknown, these estimates are considered minimum values (i.e., absorption could be higher). The mean pharmacokinetic half-life for 3,5,6-TCP in the urine was approximately 27 hours following both oral and dermal exposure.

As noted previously, data from the two human studies suggest that humans are as sensitive and possibly even more sensitive than animals based on plasma ChE inhibition and possible clinical signs. For example, in animals (rats), the acute oral (single dose) NOAEL is 0.5 mg/kg/day, while humans exposed to a single oral 0.5 mg/kg/day dose exhibited 64-85% plasma ChE inhibition. Based on an overall assessment of the plasma and RBC ChE inhibition data, the HIARC identified an animal NOAEL and LOAEL of 0.03 mg/kg/day and 0.22-0.3 mg/kg/day, respectively for longer term exposures (several months), while humans exposed to 0.1 mg/kg/day for only 9 days exhibited 36-82% plasma ChE inhibition and possible clinical signs (blurred vision). The short-term dermal NOAEL in rats is 5 mg/kg/day based on plasma and RBC ChE inhibition observed at 10 mg/kg/day, while humans exposed dermally for one day to 5 mg/kg/day exhibited 27-45% plasma ChE inhibition. For all endpoints based on rat data, it is likely that this sensitivity can be attributed to species differences in plasma ChE between the rat and humans. For example, in rats, plasma ChE consists of approximately a 60:40 ratio of acetyl cholinesterase (AChE) and butyryl cholinesterase (BuChE), while in most humans and dogs, plasma ChE is predominately as BuChE, which is more sensitive to inhibition than AChE.

Metabolism/Pharmacokinetic Studies. In the rat, chlorpyrifos is excreted primarily in the urine (84%) with lesser amounts excreted in the feces (5%) within 72 hours. The metabolism of chlorpyrifos was extensive, and no unchanged parent compound was found in the urine. The major urinary metabolites were 3,5,6-TCP, as well as glucuronide and sulfate conjugates of TCP. As noted previously, in humans (adult males) approximately 70% of chlorpyrifos is excreted in the urine as TCP within 5 days following acute oral exposure, and the minimum dermal absorption is 1 to 3% (Nolan et al. 1982, Accession No. 249203). The mean pharmacokinetic half-life for 3,5,6-TCP in the urine was approximately 27 hours following both oral and dermal exposure.

Sensitivity/Susceptibility of the Young. A number of studies published in the scientific literature have also been considered by the Agency and are discussed in the Hazard Identification and Assessment Review Committee (HIARC) April 6, 2000 report (HED No. 014088), February 2, 1998 report (HED No. 012471) and December 7, 1998 report (HED No. 013004). Summaries of several of these studies are presented in the attached Toxicology Chapter memorandum from D. Smegal to M. Hartman, April 18 2000, D263892, and in the report "Chlorpyrifos Children's Hazard: Sensitivity and Susceptibility" March 28, 2000, HED No.

014074 (which is an appendix to the April 6, 2000 HIARC report). The HIARC concluded that there is sufficient evidence in the scientific literature to conclude that exposure to chlorpyrifos results in increased sensitivity and susceptibility to neonates as compared to adult rats. The Weight of Evidence Characterization and Conclusions of the "Chlorpyrifos Children's Hazard: Sensitivity and Susceptibility" document (March 28, 2000, HED No. 014074) are presented below.

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 cholinesterase inhibition (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 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 cholinesterasse 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, 1998a,b), 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 of the high dose included, 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 (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 increase 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.

Paraoxonase. Chlorpyrifos, and some other organophosphate (OP) compounds, are detoxified via a two-step pathway involving bioactivation of the parent compound to an oxon by the cytochrome P450 systems, and then hydrolysis of the resulting oxon compounds by esterases such as liver or serum paraoxonase (PON1) (located in the plasma) (Davies et al. 1996, Furlong et al. 1998, Shih et al. 1998). In the human population, serum PON1 activity is genetically determined (polymorphic) and individuals express widely different levels of this enzyme (Davies et al. 1996).

Therefore, it is possible that some individuals may be more sensitive to chlorpyrifos toxicity based on genetic factors that regulate serum PON1 activity resulting in a reduced capacity to detoxify chlorpyrifos-oxon. Paraoxonase data were collected for individuals in a recent single dose human study (Kisicki et al. 1999). HED will evaluate these data once they are submitted to the Agency.

In animals, there is evidence that serum paraoxonase is protective against poisoning by OPs. Animals with low PON1 levels were more sensitive to specific OP compounds than animals with high enzyme levels. For example, birds, which have very low to undetectable PON1 activity are more sensitive than various mammals to the acute toxicity of oxons for other OPs (paraoxon, diazinon oxon and pirimiphos oxon). Further rabbits, which have a sevenfold higher serum PON1 activity than rats, are more resistant to the acute toxicity of chlorpyrifos (approximately 9 and 25 fold for acute oral and dermal toxicity, respectively). Rabbit paraoxonase hydrolyzes chlorpyrifos-oxon with a much higher turnover number than does rat paraoxonase (Costa et al. 1999, Li et al. 1993).

II HAZARD ASSESSMENT

The hazard assessment addresses issues and data related to chlorpyrifos which is currently registered with EPA and has been supported for reregistration. The toxicology data in support of the reregistration case are complete and adequate in accordance with the Subdivision F Guidelines for a food use chemical.

a. Acute Toxicity

Chlorpyrifos is moderately toxic following acute oral, dermal and inhalation exposures. The oral LD_{50} values for technical chlorpyrifos are higher in rats (223 mg/kg) than mice (62.5 mg/kg, toxicity category II) or chicks (32 mg/kg, toxicity category 1). Female rats are more sensitive (i.e., lower LD_{50}) than male rats for both technical chlorpyrifos and formulated products. Guinea pigs and rabbits are less sensitive to acute toxicity than rats as noted by the oral LD_{50} values of 504 mg/kg and 1000-2000 mg/kg, respectively (both category III, Accession No. 112115). Chlorpyrifos was not acutely neurotoxic when given to hens at a single oral dose of 50 mg/kg (the LD_{50}), 100 or 110 mg/kg.

In rats, the LC_{50} was greater than 0.2 mg/L (or 200 mg/m^3), which is normally assigned toxicity category II (Accession No. 257590). This study is classified as Supplementary because only nominal concentrations were measured. This study does not need to be repeated, however, because the physical state of technical chlorpyrifos is a waxy solid, and the highest attainable vapor concentration of 0.287 mg/m^3 achieved in the 90-day rat inhalation study did not cause adverse effects, and therefore, is unlikely to cause a significant degree of acute toxicity in humans. Acute toxicity values and categories for the technical grade of chlorpyrifos are summarized in the following table.

Table 1. Acute Toxicity Results for Technical Chlorpyrifos			
STUDY	**MRID Number**	**RESULTS**	**CATEGORY**
Acute Oral LD_{50} - rat	44209101	223 mg/kg M&F	II
Acute Dermal LD_{50} - rat Acute Dermal LD_{50} - rabbit	Accession No. 112115 44209102	202 mg/kg >5000 mg/kg	II IV
Acute Inhalation LC_{50}; rat Supplementary	00146507 and Accession No. 257590	LC_{50} > 0.2 mg/L (200 mg/m^3) (nominal concentration)	II
Eye Irritation - rabbit	44209103	slight irritation resolved within 24 hours	IV
Dermal Irritation - rabbit	44209104	mild irritant; (irritation resolved within 7 days)	IV
Dermal Sensitization - guinea pig	44209105	non-sensitizing	NA
Acute Delayed Neurotoxicity in hens	00097144 00405106	not neurotoxic at 50, 100 or 110 mg/kg	NA

NA = not applicable

b. Subchronic Toxicity

Several subchronic studies are available for chlorpyrifos including two oral rat studies, one oral dog study, a 21 day dermal toxicity study in rats, and two inhalation studies in rats. The most sensitive effect following subchronic oral exposure is inhibition of plasma ChE in rats and dogs at 0.025 to 0.03 mg/kg/day (Crown et al. 1985, McCollister et al. 1971, Kociba 1985), and RBC ChE inhibition in dogs and rats at 0.22 to 0.3 mg/kg/day (Barker 1989, Hoberman 1998a,b). Rats exposed to higher doses exhibited hematological alterations at doses of 10 mg/kg/day (Crown et al. 1985) and increased brain and heart weight, adrenal gland effects and decreased body weight gain at 15 mg/kg/day (Szabo et al. 1988). No adverse effects were noted in rats exposed via inhalation to the highest attainable vapor concentration of 20.6 ppb (287 μg/m^3) (Corley et al. 1986a,b, Newton 1988). No adverse effects were observed in the 21-day dermal study in rats at doses as high as 5 mg/kg/day. However, in a 4-day dermal probe study, rats dermally exposed to doses of 0, 1, 10, 100, or 500 mg/kg/day exhibited reductions in plasma and red cell cholinesterase activities at doses of 10 to 500 mg/kg/day. The 21-day dermal NOAEL is 5 mg/kg/day based on a 45% and 16% inhibition of plasma and red blood cell cholinesterase, respectively in rats dermally exposed to 10 mg/kg/day for 4 days (MRID No. 40972801; Calhoun and Johnson, 1988). The following table summarizes the subchronic toxicity studies for

chlorpyrifos:

Table 2. Subchronic Toxicity of Technical Chlorpyrifos

GDLN	STUDY	DOSE (mg/kg/day) (1)	RESULTS (mg/kg/day) (1)
82-1(a)	Subchronic Feeding in Rats (90 days) MRID #: 40436406 Makhteshim-Agan; Crown et al. 1985 Core Grade: acceptable guideline	0, 0.025, 0.5, or 10 (0, 0.5, 10 or 200 ppm)	**95.5% a.i. chlorpyrifos** NOAEL ChEI: none for plasma ChEI due to reductions in male plasma enzymes at 0.025 LOAEL ChEI: 0.025 (significant 22%↓ in plasma ChE activity that was dose-related) NOAEL (systemic): 0.5 LOAEL (systemic): 10 Effects: decreased weight gain and slight decreases in packed cell volume, red cells and hemoglobin Note: Female ChEI data is unreliable due to a possible reporting error. RBC and brain ChE activity were not measured.
82-1(a)	Subchronic Feeding in Rats (90 days) MRID #: 40952801 Szabo et al. 1988 Core Grade: acceptable guideline	0, 0.1, 1, 5 or 15	**95.7 - 98.5% a.i. chlorpyrifos** NOAEL: 0.1 (plasma and RBC ChEI) LOAEL: 1 (significant plasma and RBC ChEI in both sexes) Effects: increased organ weights (brain and heart), and reduced weight gain at 15 mg/kg/day and increased adrenal gland vacuolation and significant brain ChEI in both sexes 5 and 15 mg/kg/day.

Table 2. Subchronic Toxicity of Technical Chlorpyrifos			
GDLN	**STUDY**	**DOSE (mg/kg/day) (1)**	**RESULTS (mg/kg/day) (1)**
82-1(b)	Subchronic Oral (capsule) in Beagle Dogs MRID #: 42172801 Barker 1989 Core Grade: acceptable guideline	0, 0.01, 0.22, or 5	**95.8% a.i. chlorpyrifos** NOAEL: 0.01 LOAEL: 0.22 (significant 33-67% ↓ plasma and 24-46% ↓ RBC ChEI) Effects: Brain ChEI (46% ↓) occurred at 5 mg/kg/day. Comments: At 0.01 mg/kg/day, plasma ChEI noted in females (significant 20-24% at week 6, and non-significant 24% at week 12) and males (15% at week 13) that was not considered of sufficient magnitude and consistency to be biologically and toxicologically meaningful.
82-2	21-Day Dermal Toxicity Study in Rats and 4-day Dermal Probe Study MRID #: 40972801 Calhoun and Johnson 1988 Core Grade: acceptable guideline	0, 0.1, 0.5, 1 or 5 (21 day study) 0, 1, 10, 100 or 500 (4-day dermal probe study)	**100% pure chlorpyrifos** NOAEL: 5 (plasma and RBC ChEI) LOAEL: 10 (45% plasma and 16% RBC ChEI following 4 days of exposure) NOAEL (systemic): not identified LOAEL (systemic): not identified (>5) Effects: Slight erythema in 2/4 females at 1 and 10 mg/kg/day, respectively.
82-4	Subchronic Inhalation in Rats (90 days) (nose only) MRID #: 40013901 & 40166501 Corley et al. 1986a,b Core Grade: acceptable guideline	0, 5.2, 10.3 or 20.6 ppb (0, 72, 143 or 287 $\mu g/m^3$) (maximum dose equivalent to 0.044-0.082 mg/kg/day)	**100% pure chlorpyrifos** NOAEL: not identified (ChEI and systemic) LOAEL: not identified at highest attainable vapor concentration (>20.6 ppb or 287 $\mu g/m^3$) (ChEI and systemic)
82-4	Subchronic Inhalation in Rats (90 days) (nose only) MRID #: 40908401 Makhteshim-Agan; Newton 1988 Core Grade: acceptable guideline	0, 5, 10 or 20 ppb (0, 70, 143 or 287 $\mu g/m^3$) (equivalent to 0, 0.024, 0.048 or 0.097 mg/kg/day, respectively)	**95% a.i. chlorpyrifos** NOAEL: not identified (ChEI and systemic) LOAEL: not identified at highest attainable vapor concentration (>20 ppb) (ChEI and systemic)

(1) Unless specified.
ChEI = Cholinesterase Inhibition
RBC = red blood cell
NOAEL = No Observable Adverse Effect Level
LOAEL = Lowest Observable Adverse Effect Level

c. Chronic Toxicity and Carcinogenicity

Chlorpyrifos was evaluated for carcinogenic potential in both rats (2 studies), and mice (2 studies). There was no evidence of treatment-related tumors or carcinogenicity. In addition, chlorpyrifos was evaluated for chronic toxicity in dogs. In all animal species, the most sensitive effect is inhibition of plasma, red blood cell and brain cholinesterase that occurred at levels in the range of 0.03 to 3 mg/kg/day. Following chronic exposure, dogs appear to be the most sensitive species for cholinesterase inhibition and systemic effects, as noted by increased liver weights that could be an adaptive response in dogs exposed to 3 mg/kg/day. Rats exposed to 7-10 mg/kg/day had decreased body weight and decreased body weight gain, ocular effects, adrenal gland effects and altered clinical chemistry and hematological parameters. Mice appear to be the least sensitive to chronic chlorpyrifos exposure, as exposure to 45-48 mg/kg/day resulted in decreased body weight and an increased incidence of non-neoplastic lesions (i.e., keratitis, hepatocyte fatty vacuolation) (Gur 1992). The following table summarizes the chronic toxicity/carcinogenicity studies for chlorpyrifos:

Table 3. Chronic Toxicity/Carcinogenicity of Technical Chlorpyrifos

GDLN	STUDY	DOSE (mg/kg/day)	RESULTS (mg/kg/day)
83-1& 2	Chronic feeding/ carcinogenicity study in F344 rats (2 yrs) MRID # 42172802 Crown et al. 1990 Core Grade: acceptable guideline	Males: 0, 0.0132, 0.33 or 6.99 and Females: 0, 0.0146, 0.365 or 7.78 (0, 0.2, 5 or 100 ppm)	**96.1% a.i. chlorpyrifos** NOAEL:0.0132 LOAEL: 0.33 (significant 15-51% plasma ChEI in both sexes, 19-31% RBC ChEI at 104 weeks vs. controls and 11-17% RBC ChEI vs. vehicle controls) NOAEL (systemic):0.33 LOAEL (systemic): 6.99 Effects: decreased body weights in males and females, and cataracts, and diffuse retinal atrophy in females. No evidence of carcinogenicity.

Table 3. Chronic Toxicity/Carcinogenicity of Technical Chlorpyrifos			
GDLN	**STUDY**	**DOSE (mg/kg/day)**	**RESULTS (mg/kg/day)**
83-1 & 2	Chronic feeding/ carcinogenicity study in F344 rats (2 yrs) MRID # 40952802 Young and Grandjean 1988 Core Grade: acceptable guideline	0, 0.05, 0.1, 1 or 10	**Lorsban 98.5% pure** NOAEL: 0.1(plasma and brain ChEI) LOAEL: 1 (significant 39-86% plasma, 14-34% RBC and 5-9% brain ChEI) NOAEL (systemic): 1 LOAEL (systemic): 10 Effects: decreased body weight gain, red blood cells, hemoglobin, cholesterol, protein, and globulin, and increased platelets and specific gravity, increased adrenal gland weight, and fatty vacuolation of the zona fasciculata. No evidence of carcinogenicity.
83-1b	Chronic feeding study in beagle dogs (2 yrs) MRID # 00064933 & 00146519 McCollister et al. 1971, Kociba 1985 Core Grade: acceptable guideline	0, 0.01, 0.03, 0.1, 1 or 3	**97.2-98.8% a.i. chlorpyrifos** NOAEL: 0.01, 0.03, & 1 for plasma, RBC and brain ChEI, respectively LOAEL (plasma ChEI): 0.03 (mostly significant mean of 23-29% ↓ at 1 year and 10-24% ↓ at 2 years) LOAEL (RBC ChEI): can not be established due to data quality issues LOAEL (brain ChEI): 3 (19.4-20.8% ↓ at 2 yr) NOAEL (systemic): 1 LOAEL (systemic): 3 Effects: increased absolute and relative liver weights that could be an adaptive response
83-2	Chronic feeding study in CD-1 mice (2 yrs) MRID # 00054352 & 00142902 (Accession No. 242059) Warner et al. 1980 Core Grade: acceptable guideline	0, 0.5, 5 or 15 ppm (highest dose tested is 2.25 mg/kg/day)	**99.6% a.i. chlorpyrifos** LOAEL: 2.25 (90%↓plasma, and 50%↓ RBC ChE activity relative to controls after 1 week) NOAEL(systemic) = 2.25 LOAEL (systemic): none observed (>2.25) Effects: no systemic effects observed at highest dose tested (HDT). No treatment-related tumors. ChE only measured at 15 ppm (2.25 mg/kg/day) after 1 and 4 weeks.

Table 3. Chronic Toxicity/Carcinogenicity of Technical Chlorpyrifos			
GDLN	**STUDY**	**DOSE (mg/kg/day)**	**RESULTS (mg/kg/day)**
83-2	Chronic feeding/ carcinogenicity study in CD-1 mice (78 weeks) MRID # 42534201 Gur 1992 Core Grade: acceptable guideline	Males: 0, 0.89, 8.84, 45.2 Females: 0, 0.938, 9.79, or 48.1 (0, 5, 50 or 250 ppm)	**95.5% a.i. chlorpyrifos** NOAEL: none for ChEI LOAEL: 0.89 males; 0.938 females (significant 45-51% plasma ChEI in both sexes) NOAEL (systemic): 8.84 males, 9.79 females (50 ppm) LOAEL (systemic): 48.1 females, 45.2 males (HDT; 250 ppm) Effects: decreased body weight gain and food consumption in males, decreased water consumption in females, increased incidences of keratitis and hepatocyte fatty vacuolation, and increased incidence of gross clinical findings (ocular opacity and hair loss) in both sexes. Brain cholinesterase was inhibited at the high dose in both sexes. No evidence of carcinogenicity. Brain ChEI at high dose. Note: The validity of the RBC ChE assay is questionable.

ChEI = Cholinesterase inhibition

d. Developmental Toxicity

Chlorpyrifos was evaluated for developmental toxicity in rats, mice and rabbits. In one rat study, developmental effects (increased post-implantation loss) were noted at 15 mg/kg/day (HDT), that were also associated with maternal toxicity (Rubin et al. 1987a), while another rat study failed to observed developmental effects at 15 mg/kg/day (Ouellette et al. 1983). Developmental effects were also noted at higher doses in mice at 25 mg/kg/day (minor skeletal variations, delayed ossification and reduced fetal weight length) and rabbits at 140 mg/kg/day (decreased fetal weights and crown rump lengths, and unossified xiphisternum and/or 5th sternebra). However, in both mice and rabbits, the developmental effects occurred at maternally toxic doses as indicated by reduced weight gain, and food consumption in both species, and increased mortality in mouse dams.

In the rat developmental neurotoxicity study, chlorpyrifos was associated with delayed alterations in brain development in offspring of exposed mothers. Specifically, pups of the 1 mg/kg/day group exhibited significant dose- and treatment-related decreases in measurements of the parietal cortex in female offspring at postnatal day 66. The only maternal effect at this dose was plasma

and RBC ChE inhibition. At higher doses, pups of the 5 mg/kg/day group exhibited decreased body weight/body weight gain and food consumption in both sexes, reductions in pup viability, delays in development, decreased brain weight and morphometric alterations in the brain. However, these effects in pups were observed only at a dose level that caused a significant decrease in maternal body weight gain on gestational days 17-20 and post natal days 0-3, and also caused clinical signs of cholinergic toxicity in the dams as evidenced by fasciculations, hyperpnea and hyperactivity. At the lowest dose tested of 0.3 mg/kg/day, dams exhibited 43% and 41% plasma and red blood cell cholinesterase inhibition, respectively. This study is discussed in greater detail in the developmental neurotoxicity section below.

Several studies in the peer reviewed literature and results of the guideline developmental neurotoxicity study 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., altered synaptic development, alterations in DNA, RNA, and protein synthesis, inhibition of mitosis and mitotic figures, and disruption of the structural architecture of the brain), and which may result in irreversible alterations of nervous system structure and/or function (Whitney et al. 1995, Campbell et al. 1997, Song et al. 1997, Johnson et al. 1998, Das and Barone 1999, Dam 1999, Roy et al. 1998, Hoberman 1998a,b).

The following table summarizes the developmental studies for chlorpyrifos:

Table 4. Developmental Toxicity of Technical Chlorpyrifos

GDLN	STUDY	DOSE (mg/kg/day)	RESULTS (mg/kg/day)
83-3a	Developmental Study in CD rats (gavage) MRID# 40436407 Makhteshim-Agan; Rubin et al. 1987a Core Grade: acceptable guideline	0, 0.5, 2.5 or 15 (gestation day 6-15)	**96.1% a.i. chlorpyrifos** Maternal NOAEL: none observed for plasma ChEI; 2.5 for systemic Maternal LOAEL: 0.5 (decreased plasma ChEI); 15 (systemic) based on decreased food consumption (only the first few days of dosing) and body weight during dosing. Developmental NOAEL: 2.5 Developmental LOAEL: 15 (HDT) based on an increase in post-implantation loss. Comments: RBC and brain ChE were not measured.

Table 4. Developmental Toxicity of Technical Chlorpyrifos

GDLN	STUDY	DOSE (mg/kg/day)	RESULTS (mg/kg/day)
83-3a	Developmental Study in F344 rats (gavage) MRID# 00130400 Ouellette et al. 1983 Core Grade: acceptable guideline	0, 0.1, 3, or 15 (gestation day 6-15)	**96.6% a.i. chlorpyrifos** Maternal NOAEL: 0.1 (plasma and RBC ChEI) Maternal LOAEL: 3 (90.3% plasma and 74.3% RBC ChEI) Developmental NOAEL: none identified Developmental LOAEL: none identified (>15; HDT)
83-3a	Developmental Study in CF-1 mice (gavage) MRID# 00095268 Deacon et al. 1979 Core Grade: Not acceptable guideline	0, 0.1, 1, 10, or 25 (gestation day 6-15)	**96.8% a.i. chlorpyrifos** Maternal NOAEL: 0.1 (plasma and RBC ChEI); 10 (systemic toxicity) Maternal LOAEL: 1 (plasma and RBC ChEI); 25 (systemic toxicity) based on decreased body weight, food and water consumption, and increased mortality. Developmental NOAEL: 1 (plasma and RBC ChEI); 10 for systemic toxicity Developmental LOAEL: 10 (plasma and RBC ChEI); 25 (systemic toxicity) based on minor skull variations, delayed ossification of skull bones and sternebrae and reduced fetal body length. Comments: Brain ChE not measured.
83-3 (b)	Developmental Study in New Zealand rabbits (gavage) MRID# 40436408 Makhteshim-Agan; Rubin et al. 1987b Core Grade: acceptable guideline	0, 1, 9, 81, or 140 (gestation day 7-19)	**96.1% a.i. chlorpyrifos** Maternal NOAEL: none observed for plasma ChEI; 81 for systemic toxicity Maternal LOAEL: 1 (plasma ChEI); 140 for systemic toxicity based on reduced food consumption, body weight loss, and apparent post-implantation loss. Developmental NOAEL (systemic): 81 Developmental LOAEL(systemic): 140 based on slightly decreased fetal weights and crown-rump lengths, and an increased incidence of unossified xiphisternum and/or 5th sternebra.

Table 4. Developmental Toxicity of Technical Chlorpyrifos			
GDLN	**STUDY**	**DOSE (mg/kg/day)**	**RESULTS (mg/kg/day)**
83-6	Developmental Neurotoxicity Study in Rats MRID: 44556901 Hoberman. 1998a,b Core Grade: unacceptable guideline, but upgradeable	0, 0.3, 1, or 5 (gestation day 6 through lactaction day 11)	**99.8% a.i. chlorpyrifos** Maternal NOAEL: none observed for plasma or RBC ChEI Maternal LOAEL: ≤0.3 (43%↓ plasma and 41%↓% RBC ChE activity relative to controls) Developmental NOAEL (systemic): can not be determined Developmental LOAEL(systemic):can not be determined Comments: at 1mg/kg/day 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. Morphometric data for low-dose (0.3 mg/kg/day) female rats at postnatal day 66 have been requested.
NA	Cholinesterase and Metabolite Determination Study in Rats (Companion Study of the Developmental Neurotoxicity Study) MRID: 44648101 Mattsson et al. 1998 Core Grade: Acceptable Non-guideline	0, 0.3, 1, or 5 (gestation day 6 through lactaction day 11)	**99.8% a.i. chlorpyrifos** Maternal Effects: Dams in the 0.3 mg/kg/day group exhibited a 33%↓ plasma and 26↓% RBC ChE activity relative to controls Developmental Effects: Pups in the 5 mg/kg/day group exhibited an 85%↓ plasma, 92↓% RBC, 82%↓ heart and 60%↓ brain ChE activity relative to controls Note: This is a pharmacokinetic study, and therefore, NOAELs and LOAELs were not identified

RBC = Red blood cell
NA= Not applicable
ChEI = Cholinesterase Inhibition

e. Reproductive Toxicity

Chlorpyrifos induced reproductive toxicity in one generation of rats, but only at dose levels that induced parental toxicity. Reproductive effects in 5 mg/kg F1 pups included reduced pup weights and increased pup mortality that corresponded to slightly but significant reduced body weight gain in F0 dams during lactaction days 1-21 in addition to parental toxicity as evidenced by

inhibition of plasma, red blood cell and brain cholinesterase activities as well as histological lesions of the adrenal gland (vacuolation of cells of the zona fasciculata). The following table summarizes the reproduction study for chlorpyrifos:

Table 5. Reproductive Toxicity of Technical chlorpyrifos

GDLN	STUDY	DOSE (mg/kg/day)	RESULTS (mg/kg/day)
83-4	2-Generation Reproduction Toxicity in SD Rats MRID No: 41930301 Breslin et al. 1991 Core Grade: acceptable guideline	0, 0.1, 1, or 5 for 10 (F0) or 12 (F1) weeks prior to mating, through lactation and weaning	**97.8-98.5% a.i. chlorpyrifos** Parental NOAEL: 0.1 Parental LOAEL: 1 (significant 43-59% plasma, and 65-69% RBC ChEI at 1 mg/kg/day; and 48-49% brain ChEI and histological lesions of the adrenal gland at 5 mg/kg/day). Reproductive NOAEL: 1 Reproductive LOAEL: 5 (HDT) based on reduced pup weight and increased pup mortality in F1 generation only.
83-4	3-Generation Reproduction Toxicity in SD Rats MRID No: 00029064, 00064934 Thompson 1971 Core Grade: acceptable guideline	0, 0.03, 0.1,or 0.3 for first generation, and 0.1, 0.3 or 1 for second and third generation	Parental NOAEL: 0.1 Parental LOAEL: 0.3 (plasma and RBC ChEI) Reproductive NOAEL: >1 (HDT) Reproductive LOAEL: not identified

f. Mutagenicity Studies

Chlorpyrifos is not mutagenic in bacteria, or mammalian cells, but did cause slight genetic alterations in yeast and DNA damage to bacteria. Chlorpyrifos did not produce gene mutation in Ames reversion assays (MRID Nos.00157058 and 40436411) or in Chinese Hamster Ovary (CHO)/HGPRT assays in vitro (MRID Nos. 00152683 and 40436410). Also, it did not induce chromosome aberrations in vitro (MRID No. 40436409, 44533401) and was not clastogenic in the mouse micronucleus test in vivo (MRID No. 00152684). Chlorpyrifos did not induce unscheduled DNA synthesis in isolated rat hepatocytes (MRID No. 00157057). A slight increase in recombination frequency in the Saccharomyces mitotic recombination assay (Accession No. 256040) and direct damage to DNA in a DNA repair assay using B. subtilis H17/m45 and E. coli pol A+/pol A- (Accession No. 256040) were noted. (These studies fulfill guidelines 84.)

g. Neurotoxicity

As noted previously, chlorpyrifos affects the nervous system by reversibly inhibiting the activity of cholinesterase (ChE), an enzyme necessary for the proper functioning of the nervous system. The principle and most sensitive toxic effect of chlorpyrifos is the inhibition of cholinesterase activity. Adult male rats acutely exposed to chlorpyrifos exhibited peak plasma ChE inhibition of 28-40% 3-6 hours after exposure at 1 mg/kg (Mendrala and Brzak 1998), while significant 30% RBC ChE inhibition was noted 4 hours following a single oral dose of 1.5 mg/kg (Zheng et al. 2000). Plasma, RBC and heart ChE inhibition of 45%, 17% and 19%, respectively were observed in female rats 24 hours following a single dose of 5 mg/kg (Dittenber 1997). The acute oral NOAEL for plasma ChE inhibition in male rats is 0.5 mg/kg/day. Clinical signs of neurotoxicity, in the absence of neuropathology, were observed in rats exposed to a single oral dose of 50 mg/kg as evidence by decreased motor activity, and increased incidence of clinical signs consistent with organophosphate intoxication.

Chlorpyrifos was negative in the delayed neurotoxicity study in hens at single doses of 50, 100 (MRID 00097144) or 110 mg/kg (MRID 00405106). Acute oral exposure to hens at 60 to 150 mg/kg caused 59-87% inhibition of neurotoxic esterase (NTE) 4-6 days after exposure. In addition, delayed neuropathy was noted at 60-90 mg/kg, which corresponded to 4-6 times the LD_{50} and required aggressive antidotal treatment (Capodicasa et al. 1991). In rats, chlorpyrifos failed to inhibit NTE at single doses up to 100 mg/kg. There is evidence that NTE inhibition is related to organophosphate-induced delayed neuropathy (OPIDN).

Following longer-term exposures, there was no evidence of neurotoxicity or neuropathology in rats exposed at doses up to 15 mg/kg for 13 weeks (MRID No. 4292801). However, in the developmental neurotoxicity study, pregnant dams exposed to chlorpyrifos for approximately 2 weeks exhibited 43% and 41% inhibition of plasma and RBC ChE activity at 0.3 mg/kg/day, significant 18% brain ChE inhibition at 1 mg/kg/day, and clinical signs of neurotoxicity, including fasciculations (muscle twitching), hyperpnea (increased respiration), and hyperactivity in addition to decreased body weight gain at 5 mg/kg/day (Hoberman 1998a,b). Cholinesterase inhibition (68% plasma, 56% RBC and 8% brain) was also noted in rats exposed to 1 mg/kg/day chlorpyrifos for 4 weeks in the cognitive study (MRID 4402901), while clinical signs of toxicity were not observed until higher doses of 3 mg/kg/day for miosis and 10 mg/kg/day for salivation and tremors. A number of neurotoxicity studies published in the scientific literature have also been considered by the Agency, and these studies are discussed in the February 2, 1998 Hazard Identification Assessment Review Committee (HIARC) Report and are presented later in Section m.

Adult hens exposed to oral doses of 10 mg/kg/day for 20 days experienced significant body weight loss and marked inhibition of brain and plasma ChE, 18% brain NTE inhibition, but no significant change in lymphocyte NTE activity, and no behavioral signs of OPIDN during the dosing interval or during the subsequent 4 week observation period (Richardson et a. 1993).

The following Table summarizes the registrant-submitted neurotoxicity studies for chlorpyrifos:

Table 6. Neurotoxicity of Technical Chlorpyrifos			
GDLN	**STUDY**	**DOSE (mg/kg/day)**	**RESULTS (mg/kg/day)**
81-7	Delayed Neurotoxicity Study in Hens MRID No: 00097144 and 00405106 Huntingdon Research Center (UK) 1987 Core Grade: acceptable guideline	0, 50, 100 or 110	**96.8% a.i. chlorpyrifos** NOAEL: 110 (HDT) ; Not neurotoxic
81-8	Acute Neurotoxicity Study in Rats MRID 42669101and 42943101 Wilmer et al. 1992 Core Grade: acceptable guideline	0, 10, 50 or 100	**98.2% a.i. chlorpyrifos** NOAEL (systemic): 10 LOAEL (systemic): 50 Effects: Decreased body weight, and motor activity and increased incidence of adverse clinical signs
NA	Acute Pharmacokinetic Study in Rats MRID 44648102 Mendrala and Brzak 1998 Core Grade: acceptable nonguideline	0.5, 1, 5, 10, 50, 100	**89.4-99.8% a.i. chlorpyrifos** NOAEL: 0.5 LOAEL: 1 (28-40% plasma ChEI at the peak time of inhibition, 3-6 hours post exposure) Other: significant brain ChEI at doses ≥10 Note: red blood cell ChE measurements were not collected.
82-8	13 Week Rat Neurotoxicity Study MRID 42929801 Shankar et al. 1993 Core Grade: acceptable guideline	0, 0.1, 1, 5, or 15	**98.2% a.i. chlorpyrifos** NOAEL (systemic): ≥15 LOAEL (systemic): none established Effects: Decreased motor activity and an increased incidence of urine incontinence in females. Note: This study did not measure cholinesterase activity.
NA	Special Acute Neurotoxic Esterase (NTE) Rat Study MRID 44273901 Dittenber 1997 Core Grade: acceptable non-guideline	0, 1, 5, 10, 50 or 100	**98.1% a.i. chlorpyrifos** NOAEL: 1 [plasma ChE, and RBC and heart acetyl cholinesterase (AChE)] LOAEL: 5 (45% plasma ChEI; 17% RBC AChEI; and 19% heart AChEI). Effects: NTE was not inhibited at any dose. Note: cholinesterase measurements were made 24 hours post exposure.

Table 6. Neurotoxicity of Technical Chlorpyrifos			
GDLN	**STUDY**	**DOSE (mg/kg/day)**	**RESULTS (mg/kg/day)**
NA	Cognitive Rat Study MRID 44020901 Maurissen et al. 1996 Core Grade: Acceptable non guideline	0, 1, 3, or 10 for 5 days/week for 4 weeks	**98.1% a.i. chlorpyrifos** NOAEL: none observed (plasma and RBC ChE), LOAEL: 1 (68% plasma ChEI; 56% RBC ChEI and 8% brain ChEI). NOAEL (systemic): 1 (miosis) LOAEL (systemic): 3 (miosis)
83-6	Developmental Neurotoxicity Study in Rats MRID: 44556901 Hoberman. 1998a,b Core Grade: unacceptable guideline, but upgradeable	0, 0.3, 1, or 5 (gestation day 6 through lactaction day 11)	**99.8% a.i. chlorpyrifos** Maternal NOAEL: none observed for plasma or RBC ChEI Maternal LOAEL: ≤0.3 (43%↓ plasma and 41%↓% RBC ChE activity relative to controls)

NA = Not applicable

Acute Neurotoxicity Studies

The acute neurotoxicity of chlorpyrifos was evaluated in rats. Rats were exposed once by gavage to 0, 10, 50 or 100 mg/kg/day chlorpyrifos and evaluated for neurotoxicity on days 1 (at peak time of toxicity, approximately 6 hours post dosing), 8 and 15. Systemic toxicity consisted of decreased body weights of animals in the 50 and 100 mg/kg dose groups. Neurotoxic effects consisted of decreased motor activity following the 50 and 100 mg/kg dose level on day 1 (both sexes) and day 8 (females only). Significant FOB changes were limited to the high dose females, of which 6 out of 10 could not perform the landing hind leg splay on day 1 of the study. Grip performance on day 1 revealed a possible treatment-related decrease with increasing dose in the 50 and 100 mg/kg groups. Neuropathological examinations did not reveal any treatment-related effects. The systemic NOAEL and LOAEL are 10 and 50 mg/kg, respectively based on decreased body weight and motor activity and increased incidence of adverse clinical signs consistent with organophosphorus intoxication. This study, in conjunction with the supplemental data on positive controls satisfies the 81-8 guideline (MRID 42669101).

In a special acute oral study that evaluated the potential of chlorpyrifos to inhibit cholinesterase and neurotoxic esterase (NTE), chlorpyrifos was administered by gavage to six groups of Fischer 344 strain female rats at dose levels of 0, 1, 5, 10, 50 or 100 mg/kg and sacrificed 24 hours later. Dosing was by gavage at a dosing volume of 10 ml/kg. The rats were also assessed for

cholinesterase inhibition in the plasma, red blood cells (RBCs), heart and brain and there was an additional group dosed at 0.5 mg/kg included for assessment of cholinesterase only. The cholinesterase inhibition data indicated a NOAEL and LOAEL for plasma cholinesterase (ChE) and RBC and heart acetyl cholinesterase (AChE) of 1 and 5 mg/kg, respectively. At 5 mg/kg, plasma ChE, RBC AChE and heart AChE were significantly inhibited approximately 45%, 17% and 19%, respectively. Brain AChE demonstrated a NOAEL and LOAEL of 10 and 50 mg/kg, respectively and at 50 mg/kg inhibition was approximately 53%. NTE was not inhibited at the highest dose level of 100 mg/kg and there was an apparent 9% increase in activity at this dose level.

Subchronic Neurotoxicity Studies

In a 13 week study, rats (10/sex/dose) were fed doses of 0, 0.1, 1, 5 or 15 mg/kg/day. Body weights, clinical signs, FOB, motor activity and neuropathology were examined. FOB, performed at pre-study and weeks 4, 8 and 13 consisted of hand-held and open field observations and measurement of grip performance and landing foot splay. The study indicated that treatment-related effects included decreased motor activity and an increased incidence of urine incontinence in females. Although a statistically significant depression in motor activity was present in high-dose animals at week 4, the transitory nature of the effect suggest that the differences were not treatment-related. In addition, a low, and statistically non-significant increase in the incidence of urine incontinence was observed in several 5 and 15 mg/kg/day females during the clinical examinations and FOB evaluations. One high-dose female showed urine incontinence at weeks 4, 8 and 13 and another, only at weeks 4 and 8. None of the other animals showed urine incontinence in more than one FOB session. There was no clear dose- or time-relationships which would suggest that the incontinence was treatment-related. Body weights of the treated animals were comparable to controls. Neuropathological examination did not reveal any differences which might be attributed to treatment. No neurotoxicity was noted at 15 mg/kg/day, a dose previously shown to markedly inhibit plasma (>80%), RBC (>45%) and brain (>62%) cholinesterase activities. This study identifies a NOAEL $\geq$15 mg/kg, while a LOAEL was not established. (MRID 42929801). However, it should be noted that this study did not measure cholinesterase levels. This study satisfies guideline 82-7.

In a cognitive study (MRID 44020901) the effects of repeated oral administration of chlorpyrifos technical (purity, 98.1%) on the cognitive function of rats were evaluated with a delayed matching to position (DMTP) test. Groups of 10 *female* Long-Evans rats, pretrained in a DMTP apparatus were administered oral doses of chlorpyrifos in corn oil of 0, 1, 3, or 10 mg/kg/day for 5 days/week for 4 weeks. DMTP testing was conducted 6 days/week during treatment and continued post-dosing for 4 weeks. Testing for short-term memory (as evidenced by the retention rate) and attention/encoding deficits was based on the percent correct accuracy on several time delays. Slope over delay and intercept at time zero were calculated from these data for each rat and represented the "forgetting curve."

A satellite group of 6 rats/dose was sacrificed after the 4-week dosing period and plasma,

erythrocyte and brain cholinesterase (ChE) were determined. Neurotoxic esterase (NTE) activity was determined in satellite rats from the control and high-dose groups one day after the last dose administration. Plasma (68%), RBC (56%) and brain (8%) ChE were inhibited at 1 mg/kg/day. At 3 mg/kg/day, plasma (83%), RBC (65%) and brain (63%) ChE inhibition was increased. At 10 mg/kg/day plasma (93%), RBC (65%) and brain (86%) ChE inhibition was further increased. NTE was minimally decreased (6%) in the high-dose group but this was not considered toxicologically significant. The LOAEL for ChE inhibition is < 1 mg/kg/day. No NOAEL was established.

The clinical sign of miosis was observed in rats that received 3 and 10 mg/kg/day particularly at weeks 3 and 4. Salivation and tremors were observed primarily at 10 mg/kg/day with the tremors usually disappearing by the following morning. The LOAEL for overt cholinergic signs is 3 mg/kg/day based on miosis. The NOAEL is 1 mg/kg/day.

A statistical analysis of the actual percent correct data was provided (supplemental report dated February 10, 1999) and no statistical differences (i.e., $p < 0.05$) indicative of treatment related decreases in percent correct choices were established for any dose or delay time. Thus, cognitive function is not obviously impaired. No consistent pattern in the intercept of the retention gradient was noted since it was increased at week 2 and decreased at week 3 but equivalent to the control at weeks 1 and 4 at 10 mg/kg/day. The DMTP parameters of actual total delay (increased by as much as 2.5 sec in the 0 delay trial at week 2), void trials per session (increased from about 5 in the control to about 15) and nosepokes (decreased ~42% at week 1 for the 15 sec delay) were affected in the 10 mg/kg/day chlorpyrifos dose at most or all intervals during dosing. Although these effects can be possibly related to a decrease in motor activity known to be associated with organophosphates, the increase in void trials may also indicate a motivational or attention deficit. The LOAEL for DMTP performance (i.e., increase in void trials) is 10 mg/kg/day. The NOAEL is 3 mg/kg/day.

Neurotoxicity Studies in Progress

In addition, HED has recommended and the registrant has developed a protocol for a Repeated Exposure Neurotoxicity Study of Sensory Electrophysiology. This study will also include measurement of neurotoxic esterase (NTE). It is expected that this would be a 28 day 2 dose, oral exposure study. In addition to the neurophysiological and neurochemical measures, neuropathological assessment focused on central/peripheral axonopathic changes associated with OPIDN (organophosphate-induced delayed neuropathy should also be performed). This is special study for which no single EPA guideline provides complete guidance. EPA has a guideline for 28 day hen studies of organophosphates that may cause OPIDN that includes guidance for neuropathology and NTE measurements (US EPA 1998; 870.6100). EPA has a guideline for examining peripheral nerve function (US EPA 85-SS1998; 870.6850) and a guideline for sensory evoked potentials (US EPA 1998; 870.6855). The current protocol for this special study has been developed by the registrant working voluntarily in conjunction with EPA. While EPA has not required this study, EPA maintains the right to require further study, based on concerns for

potential health effects, consistent with its obligations under FIFRA.

h. Developmental Neurotoxicity Study and Companion Pharmacokinetic Study

In the developmental neurotoxicity study (MRID 44556901) (summarized on Tables 4 and 6), 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).

A second study (MRID 44648101) that complements the Developmental Neurotoxicity study (summarized previously on Table 4) was designed to evaluate cholinesterase inhibition and determine chlorpyrifos and its principal metabolites in dams and pups. Pregnant Sprague-Dawley CD® rats were administered chlorpyrifos (99.8% a.i.; Lot No. MM930503-17; TSN 100227) by gavage at doses of 0, 0.3, 1.0, or 5.0 mg/kg/day beginning on gestation day (GD) 6 and continuing through lactation day 10. Five dams, as well as 5 male and 5 female pups/dose, were sacrificed on GD 20 and lactation days 1, 5, and 11 for chlorpyrifos and metabolite determinations. Milk samples were taken from the dams for chlorpyrifos and chlorpyrifos-oxon

analyses. Blood samples were taken from dams and pups for chlorpyrifos, chlorpyrifos-oxon, and 3,5,6-trichloro-2-pyridinol (TCP) analyses. Cholinesterase (ChE) was determined in an additional 5 dams/dose and 5 pups/sex/dose on GD 20 and lactation days 1, 5, 11, 22, and 65 (pups only). ChE activity was determined in plasma, RBC, brain, and heart. For all analyses, samples were taken from dams and fetuses 4 hours postdosing, and from pups 2 hours postdosing of the dams.

No treatment-related clinical signs of toxicity in dams or pups and no differences in maternal body weights were observed at any time during the study. No differences in litter sizes at parturition or in the number of pups born dead were observed between the treated and control groups. Pup survival during lactation was similar for the treated groups as compared to the control.

Chlorpyrifos was detected in the blood of high-dose dams at a mean concentration of 108.78 ng/g on GD 20. Levels of chlorpyrifos then declined by 87% on lactation day 1, remained unchanged on lactation day 5, and were below the limit of detection by lactation day 11. Chlorpyrifos was detected at a low level (2.55 ng/g) in blood of mid-dose dams only on GD 20 and was not detected at any time in blood of low-dose dams. In milk, chlorpyrifos concentrations in the 0.3, 1.0, and 5.0 mg/kg/day groups were 20.57, 139.49, and 3022.00 ng/g, respectively on lactation day 1 and were 13.54, 81.76, and 1533.98 ng/g, respectively on lactation day 5. By lactation day 11, chlorpyrifos was detected only in the high-dose group at a level of 19.79 ng/g. Chlorpyrifos-oxon was not detected in the blood or milk of any dams at any time point.

Blood concentrations of chlorpyrifos in male and female fetuses from high-dose dams were 52.81 and 39.40 ng/g, respectively on GD 20. Concentrations in the pups declined to less than half of the GD 20 levels by lactation day 1 and were below the limit of detection by lactation day 5. Levels of chlorpyrifos in the blood of male and female fetuses from the mid-dose dams were 0.99 and 1.19 ng/g, respectively on GD 20, but were undetectable thereafter. Chlorpyrifos-oxon was detected in the blood of male and female fetuses from high-dose dams only on GD 20 at concentrations of 0.97 and 0.94 ng/g, respectively.

TCP was detected in the blood of dams from all treated groups on GD 20, lactation day 1, and lactation day 5. In the 0.3, 1.0, and 5.0 mg/kg/day groups, TCP levels in blood were 114.40, 322.01, and 1974.00 ng/g, respectively on GD 20, and were 142.93, 536.53, and 1449.92 ng/g, respectively on lactation day 5. On lactation day 11, TCP was detected only in the mid- and high-dose groups at levels of 9.87 and 71.40 ng/g, respectively.

TCP was detected in the blood of male and female fetuses from all dose groups in a dose dependent pattern on GD 20. In the 0.3, 1.0, and 5.0 mg/kg/day groups, TCP levels in blood on GD 20 were 93.93, 361.00, and 1680.00 ng/g, respectively for males, and were 99.49, 339.13, and 1884.00 ng/g, respectively for females. TCP was not detectable in the blood of low- and mid-dose pups by lactation day 5, but was detected in the mid-dose females at 9.5 ng/g on lactation day 11. On lactation day 11, TCP was detected in the high-dose male and female pups at levels of 42.29 and 47.01 ng/g, respectively.

ChE activity in fore- and hindbrain from high-dose dams was 11.1-22.7% and 19.5-42.8%, respectively of the control level on GD 20 through lactation day 11 and 57.9% and 80.4%, respectively of controls on lactation day 22. ChE activity in the heart of high-dose dams was 16.9% of controls on GD 20, but recovered to 93.6% of controls on lactation day 22 (12 days after the last dose). Mid-dose dams had brain ChE levels of 87.8-93.1% of controls from GD 20 through lactation day 11. In low-dose dams, brain and heart ChE activities were unaffected by treatment. In high-dose dams plasma and RBC ChE activities relative to the controls were 12.0% and 4.9%, respectively, on GD 20, and 48.9% and 7.4%, respectively on lactation day 11 (24 hours after the last dose). By lactation day 22, plasma ChE had returned to control level but RBC activity was still only 53.6% of the control level. In mid-dose dams plasma and RBC ChE activities relative to the controls were 38.5% and 17.6%, respectively on GD 20, and 65.5% and 23.3%, respectively on lactation day 11. By lactation day 22, plasma ChE had recovered but RBC activity was still only 66.6% of the control level. In the low-dose group, plasma and RBC activities were 67.2% and 73.7%, respectively of controls on GD 20, but recovered to 83.8% and 75.5%, respectively on lactation day 11 (24 hours after the last dose) and were similar to controls on lactation day 22 (12 days after the last dose).

No effects on ChE activity were seen in tissues from pups from the low- or mid-dose dams. In pups from the high-dose group, forebrain activity was 40.2% of controls on GD 20 and 63.3% on lactation day 1; hindbrain activity was 46.1%, 67.2%, and 88.4% of control levels on GD 20, lactation day 1, and lactation day 5, respectively. ChE activity in the heart of high-dose pups was 18.4%, 34.7%, and 83.9% of control levels on GD 20, lactation day 1, and lactation day 5, respectively. ChE activity in plasma from the high-dose pups was 15.3% of controls on GD 20, 40.0% of controls on lactation day 1, and 81.5% of controls on lactation day 5. ChE activity in RBC from the high-dose pups was inhibited to 7.9% of controls on GD 20, 14.7% of controls on lactation day 1, and 86.4% of controls on lactation day 11. Complete recovery of ChE activity occurred in high-dose pups by lactation day 5 for forebrain, by lactation day 11 for hindbrain, heart, and plasma, and by lactation day 22 for RBC.

This study is classified as **ACCEPTABLE-NONGUIDELINE**. This is a special study intended to investigate specific parameters and does not fit into a guideline study classification. It is acceptable for the purposes for which it was intended.

i. Human Studies

HED has reviewed two human studies conducted with chlorpyrifos submitted by the registrant (MRID 95175, Accession No. 249203). A third human study (Kisicki et al. 1999) that evaluated a single dose exposure was submitted on April 27, 1999 but is an incomplete submission because two Appendices with critical data were omitted. This study will be reviewed by the Agency once the submission is complete.

In the first study (MRID No. 95175; Coulston et al., 1972), male volunteers from Clinton Correctional Facility (4/dose group) were given daily oral (tablet) doses of 0, 0.014, 0.03, or 0.1 mg/kg chlorpyrifos technical at the time of breakfast. Prior to exposure, all subjects received thorough physicals, which included electrocardiograms (ECG), urinalysis, chest x-ray, hematology and clinical chemistry. Treatment continued daily for a 7 week period for the control group, 9 days for the 0.1 mg/kg/day group, 21 days for the 0.03 mg/kg/day and 28 days for the 0.014 mg/kg/day dose group. Blood was drawn twice pre-exposure for baseline values; twice weekly for plasma and red blood cell cholinesterase measurements, and once weekly for clinical chemistry and hematology. Urinalysis was also conducted weekly. The volunteers were also evaluated from 7 to 35 days post exposure.

After 1 and 3 days of treatment, the mean plasma cholinesterase activity was not significantly decreased at any dose level (up to 0.1 mg/kg/day) as compared to the baseline measurement. In the high dose group, plasma cholinesterase activity for individual subjects, relative to baseline, ranged from an 8% increase to a 41% decrease (mean decrease of 10%). However, significant 36-82% plasma cholinesterase inhibition relative to baseline was observed after 9 days of treatment with 0.1 mg/kg/day chlorpyrifos. In addition, one of the four men in the 0.1 mg/kg/day developed blurred vision, runny nose and a feeling of faintness on day 9 that HED believes are possibly cholinergic in nature. Exposure was discontinued on day 9 in this dose group due to plasma cholinesterase inhibition that exceeded the guideline of 20%-30%. No significant plasma ChE inhibition was observed in the men exposed to 0.03 mg/kg/day for 21 days (mean inhibition relative to baseline was 29%, with a range of 16-50%). A gradual recovery was observed in plasma ChE values equaling baseline values by day 25 of the recovery period. No effects on RBC ChE were found at any dose that could be attributed to treatment. HED notes that the relatively long recovery period of 25 days is unusual for plasma ChE, and is more characteristic of recovery for RBC acetyl ChE inhibition based on the 2 year dog data (McCollister et al. 1971, Kociba et al. 1985).

This study failed to control for confounding factors, and provided limited details on the study methodology and study design (i.e., no details on the volunteers, such as age or race, and there was no explanation why the 0.03 mg/kg/day dose group exposure was truncated at 21 days). It should be noted that the registrant and the study director contend that the clinical signs were attributed to a cold, and not chlorpyrifos exposure. HED believes that blurred vision is a typical cholinergic sign of cholinesterase inhibition, and can not be attributed to a common cold (Personal communication by Dr. Brian Dementi, Toxicologist, HED, OPP, January 29, 1996 with Dr. Jean Hollingsworth and Dr. Joe Bresee of the Center for Disease Control and Prevention, see February 2, 1998 HIARC Report, HED Doc No. 012471). In addition, there is no reason to believe that other clinical signs would not have appeared if the dosing had continued for 20 or 28 days as it did for the other groups. While the study director claims that exposure to the high dose group was discontinued on day 9 because plasma ChE inhibition was 20-30%, rather than because of concern for the clinical signs, this reason is inconsistent with the study findings of 46% mean plasma ChE inhibition following day 6 of treatment in the 0.1 mg/kg/day group, and 41% plasma ChE inhibition in one individual on day 3.

An acute oral and dermal pharmacokinetic study (Nolan et al. 1982, Accession No. 249203) dosed six men once with 0.5 mg/kg orally and four weeks later dosed five of these same men with 5 mg/kg dermally, and one man with 0.5 mg/kg dermally. Blood was collected 2, 6, 12 and 24 hours, and up to 30 days (oral) and up to 9 days (dermal) post dosing for plasma and RBC ChE measurements. No signs or symptoms were observed in any of the subjects, but unlike the previous study, the primary focus of this study was pharmacokinetics. Men orally exposed to 0.5 mg/kg chlorpyrifos exhibited peak plasma ChE inhibition of 64-85%, 12 to 24 hours post-exposure and peak RBC ChE inhibition of 11-52% on post-exposure day 4. Men dermally exposed to 5 mg/kg chlorpyrifos exhibited peak plasma ChE inhibition of 27-45% on day 3, and mean RBC ChE inhibition of 8.6% on day 4. While RBC cholinesterase inhibition was judged not to be significantly affected in the orally dosed group, mean values for the group reached a low point on day 4 at 0.67 in comparison to pre-exposure control value of 0.92, a 27% decrease. Individual values varied on this day between 11-52% of their pre-dose controls and a paired t-test comparing days 3 and 4 shows a statistically significant difference at a level of p=0.0115. The return of plasma ChE activity to pre-dose levels required about 30 days. The registrant stated that the inhibition noted on days 3 and 4 is an analytical artifact based on chlorpyrifos pharmacokinetics. If this is the case, it raises concerns about the quality and reliability of the study data. Again, HED notes that the relatively long recovery period of 30 days is unusual for plasma ChE, and is more characteristic of recovery for RBC acetyl ChE inhibition based on the 2 year dog data (McCollister et al. 1971, Kociba et al. 1985).

On the basis of urinary excretion of the 3,5,6-trichloro-2-pyridinol (3,5,6-TCP) metabolite, the minimum oral absorption was estimated at 70% and the minimal dermal absorption at 1-3%. Because the proportion of the administered dose metabolized to this pyridinol is unknown, these estimates are considered minima.

j. Metabolism/Pharmacokinetic Studies

In the rat, chlorpyrifos is excreted primarily in the urine (84%) with lesser amounts excreted in the feces (5%) (Nolan et al., 1987). The major excretion product in the urine is 3,5,6-trichloro-2-pyridine (3,5,6-TCP) in both humans and rats. As noted previously, in humans (adult males) approximately 70% of chlorpyrifos is excreted in the urine as TCP within 5 days following acute oral exposure, and the minimum dermal absorption is 1 to 3% (Nolan et al. 1982, Accession No. 249203). The mean pharmacokinetic half-life for 3,5,6-TCP in the urine was approximately 27 hours following both oral and dermal exposure.

In a metabolism and tissue distribution study, Fischer 344 rats were administered a single dose of 0.5 or 25 mg/kg of ^{14}C labeled chlorpyrifos or 15 daily doses of 0.5 mg/kg unlabeled chlorpyrifos followed by one dose of 0.5 mg/kg of ^{14}C labeled chlorpyrifos. During 72 hours, more than 84% of the radioactivity was recovered in the urine, about 5% was found in the feces and less than 0.2% was found in the tissues and carcass. The metabolism of chlorpyrifos was extensive, and no unchanged parent compound was found in the urine. The major urinary metabolites were TCP, as well as glucuronide and sulfate conjugates of TCP (this study fulfills guideline 85-1; MRID No.

40458901; Nolan et al., 1987).

An acute pharmacokinetic 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 acetylcholinesterase activity, and its metabolites, chlorpyrifos-oxon (OXON), a strong cholinesterase inhibitor and 3,5,6-TCP. 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. 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 16% for the 1 mg/kg group. The decrease in plasma ChE activity of rats treated with 50 or 100 mg/kg began within 10 minutes of treatment. By 12 hours after treatment, plasma ChE activity in both groups were approximately 11% of the control group and had not shown signs of recovery.

Brain cholinesterase 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 cholinesterase 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 cholinesterase 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 5.0, 10.0, 50.0, 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, $\geq$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 (in vitro half life in rat blood of 10 seconds and 55 seconds in human blood (Brzak et al. 1998)) and that *in vivo* metabolism of chlorpyrifos is rapid.

This study is considered acceptable (nonguideline). It may partially fulfill guideline requirements in other areas.

In addition, the cholinesterase and metabolite determination study in rats (MRID 44648102), which is the companion study to the developmental neurotoxicity study and was discussed previously in Section h, also evaluates the pharmacokinetics of chlorpyrifos in pregnant dams and pups.

k. Oral/Dermal Absorption

As discussed previously, the oral and dermal absorption of chlorpyrifos were evaluated in a human pharmacokinetic study (Nolan et al. 1982, Accession No. 249203). In this study, chlorpyrifos was administered by a single oral dose of 0.5 mg/kg to 6 human male subjects and dermally at 0.5 or 5.0 mg/kg to 5 of these 6 subjects. Based on the urinary excretion of the 3,5,6-TCP metabolite, the minimum absorption orally was approximately 70% and dermally, it was approximately 1-3%. After oral administration, the maximum plasma concentration of metabolite was 0.979 µg/ml 6 hours post-dosing, and dermally, 0.052 µg/ml 24 hours post-dosing (this study does not fulfill a guideline).

l. Sensitivity/Susceptibility of the Young.

A number of studies published in the scientific literature have also been considered by the Agency and are discussed in the Hazard Identification and Assessment Review Committee (HIARC) April 6, 2000 report (HED No. 014088), February 2, 1998 report (HED No. 012471) and December 7, 1998 report (HED No. 013004). Summaries of several of these studies are presented in the attached report "Chlorpyrifos Children's Hazard: Sensitivity and Susceptibility" March 28, 2000, HED No. 014074 (which is an appendix to the April 6, 2000 HIARC report). The Weight of Evidence Characterization and Conclusions of the "Chlorpyrifos Children's Hazard: Sensitivity and Susceptibility" document (March 28, 2000, HED No. 014074) are presented below.

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 cholinesterase inhibition (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 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 cholinesterasse 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, 1998a,b), 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 of the high dose included, 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 (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 increase 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.

m. Scientific Literature

The Hazard Identification and Assessment Review Committee (HIARC) evaluated a number of neurotoxic studies in the published literature, or in preparation for publication at the time the committee met (December 1997), which are presented in the February 2, 1998 committee report (HED No. 012471). Summaries of these studies, as presented in the HIARC report, are presented below. Additional studies also considered by the Agency are cited in the reference list.
Data Summaries of Neurotoxicity Studies

In addition to the toxicology data base available to the Agency, the RfD Committee searched the open literature in order to better address the issue of plasma and brain cholinesterase correlation. Open literature information reviewed by the Committee demonstrated that, in the rat, plasma cholinesterase inhibition has been observed to correlate very well with brain cholinesterase inhibition following the administration of single doses of certain organophosphorus pesticides. The following are excerpts taken from a review by Dr. Brian Dementi, HED, OPP for three open literature studies used by the RfD Peer Review Committee in partial support of its current position on issues raised by the registrant.

1 Pope and Chakraborti (1992) evaluated the effects of three organophosphorus pesticides; parathion, methyl parathion and Chlorpyrifos administered subcutaneously, in both adults and neonates SD rats. Good correlations were

observed between ED_{50} values (dose inhibiting the enzyme 50%) and the Maximum Tolerated Dose (MTD), used as an indication of toxicity. In this publication the authors stated (P.41): "For example, when brain cholinesterase ED_{50} values were correlated with MTDs for both age groups, a correlation (r) value of 0.932 was obtained, indicating a good correlation between brain cholinesterase inhibitory potency and acute toxicity among the inhibitors. An even higher correlation (r=0.992) was noted, however, between plasma cholinesterase ED_{50} values and MTDs. In addition, there were no significant differences in the ED_{50} values of brain cholinesterase relative to plasma cholinesterase with either of the OP treatments in either age group." The authors indicated that, while plasma cholinesterase levels, under defined experimental conditions, may provide a quantitative estimate of the extent of cholinesterase inhibition in the central nervous system following organophosphate exposure, factors such as route of exposure and time after treatment when cholinesterase is assayed could influence the degree of correlation.

2. Pope et al (1992) investigated the effects of Chlorpyrifos in Sprague-Dawley rats following subcutaneous administration at the MTD, on a number of parameters, including plasma and brain cholinesterase inhibition SD rats. Following administration of the test material, cholinesterase activity was assessed periodically over a 12-week period. The following is a quotation from the study report (P.253): "Cholinesterase inhibition in plasma was not as extensive as in either cortex or striatum at any time point during the observation period, but roughly equivalent rates of recovery of enzyme activity were noted between plasma and the brain regions." Inhibition in the striatum and cortex were essentially identical. Inhibition for these brain regions were 94-96%, 82-83%, 58-60% and approximately 20% at weeks 2, 4, 6 and 12, respectively. By comparison, plasma cholinesterase was inhibited at the same respective time points by about 90%, 55%, 30% and 0%. The authors advise that cholinesterase activities were not significantly different between treatment groups at the 12-week time point.

   Although erythrocyte cholinesterase was not assayed in either of the above referenced publications, the data indicate that plasma cholinesterase inhibition correlated well with brain cholinesterase inhibition, and toxicity under certain conditions of each study. While in view of the author's discussion, this correlation may not hold to be true under all exposure scenarios, the correlation should be considered as substantive.

3. Padilla *et al*. (1994) correlations between plasma, whole blood and erythrocyte cholinesterase inhibition and brain cholinesterase inhibition were determined over a 35 day period following a single subcutaneous dose of Chlorpyrifos to Long Evans rats. The study revealed high correlation coefficients between inhibition of all three blood components enzymes and that of the frontal cortex during days 4-21

post-dosing. At the 35 day time point, plasma cholinesterase activity was less well correlated than was whole blood or erythrocyte cholinesterase activity with brain cholinesterase inhibition.

These three studies, collectively, reveal a good correlation between plasma and brain cholinesterase inhibition. In rats, at least in the Padilla study, erythrocyte cholinesterase appears to be more remarkably inhibited by Chlorpyrifos than either the plasma or brain enzyme activity. It is clear from the above discussion of the three animal studies that plasma cholinesterase inhibition has predictive value for brain cholinesterase inhibition in the case of Chlorpyrifos.

Data Summaries from the Open Literature

In a review of the open literature, studies were identified which specifically addressed the differences between the response of adult rats versus fetal, neonatal, or weanling rats following either acute or subacute doses of Chlorpyrifos. In all of these studies, the Chlorpyrifos was administered by subcutaneous injection which does not approximate any exact scenario of potential human exposure, although it could be argued that it may mimic some aspects of direct dermal exposure to Chlorpyrifos. Nevertheless, the conclusions of these studies support the findings of the guideline studies, as described above.

A. Studies that address the neurochemical and neurobehavioral effects of maternal versus fetal or neonatal rats following *in utero* exposure to Chlorpyrifos are described below:

1. In a study by Chanda *et al*. (1995), which expanded findings reported by Chanda et al. in 1993, a single dose of 200 mg/kg of Chlorpyrifos was administered by subcutaneous injection to Sprague-Dawley rats on gestation day 12. Any dams with moderate to severe signs of cholinergic toxicity at 2-3 days after dosing were eliminated from study. The dams that remained on study and their offspring were killed on gestation day 16 or 20 or postnatal day 3 for tissue collection and analysis. Extensive AChE inhibition (82-88%) was noted in maternal brain at all three time points following acute exposure. At gestation days 16 and 20, fetal brain AChE activity was inhibited 42-44%. While some degree of recovery was observed in pup brain by postnatal day 3, AChE activity was still inhibited 30% in treated pups cross-fostered to control dams. In vitro inhibition of maternal and fetal (gestation day 20) brain AChE activity by the active metabolite, Chlorpyrifos oxon, suggested that the prenatal brain AChE activity was somewhat more sensitive (IC50 at 37.0^{o}C, 20 min. dam, 26.6 $\pm$ 1.8 x 10^{-9} M; fetus, 6.7 $\pm$ 0.4 x 10^{-9} M). Maternal brain muscarinic receptor binding was more extensively reduced (30-32%) at gestation day 20 and postnatal day 3 as compared to the developing brain at gestation

day 20 (16%) and postnatal day 3 (11%). A simple postnatal reflex test, righting reflex, was transiently altered by Chlorpyrifos. The study authors concluded that acute Chlorpyrifos exposure (subcutaneously injected) to dams during gestation produces more extensive neurological effects in the dam relative to the developing fetus.

2. Chanda and Pope (1996) examined the relative neurotoxicity of repeated, lower-level exposures to Chlorpyrifos during gestation in Sprague-Dawley rats. Doses of 6.25, 12.5, or 25 mg/kg/day of Chlorpyrifos were injected subcutaneously on gestation days 12-19. The dams and offspring were killed on gestation day 16 or 20 or postnatal day 3. No clinical signs of maternal toxicity were observed at any dose level; maternal body weight gain values were similar to control for all treated groups. Fetal body weight was similar to control for all treated groups, but a significant decrease in fetal body weight was observed on postnatal day 1 in the 25 mg/kg/day dose group. A significant dose-related inhibition of acetylcholinesterase was observed following the three dosing regimens at gestation day 20. In each case, maternal brain AChE inhibition was greater than the fetal brain AChE inhibition with all three doses. AChE inhibition (83-90%) was noted in maternal brain at all three collection times following repeated exposures at 25 mg/kg/day. Higher AChE inhibition (58%) was noted in fetal brain at gestation day 20 compared to 19-25% on postnatal day 3 in treated pups cross-fostered to control dams and in control pups cross-fostered to treated dams following repeated exposures at 25 mg/kg/day. Although similar reductions in brain muscarinic receptor binding were observed at gestation day 20 and postnatal day 3 in dams and developing brain between acute and repeated dosing regimens, greater changes in [^{3}H]cis-methyl dioxolane and [^{3}H]cytisine binding were observed following repeated exposures. Righting reflex and cliff avoidance tests were markedly altered following repeated exposures. The study authors concluded that the lower-level repeated exposures to Chlorpyrifos caused extensive neurochemical and neurobehavioral changes in developing rats in the absence of maternal toxicity (signs of clinical toxicity and body weight gain data). An additional conclusion that can be drawn from this study is that repeated dosing of Chlorpyrifos during gestation resulted in AChE inhibition in both dams and fetuses at dose levels as low as 6.25 mg/kg/day, and that the maternal response, as measured by brain cholinesterase inhibition on gestation day 20, was more severe than the fetal response.

B. Studies that address the comparison of the neurotoxic response of adults and neonatal or weanling animals include the following:

1. Pope et al. (1991) compared the time course of cholinesterase inhibition and recovery in whole brain between neonatal (postnatal day 7) and adult (80-100 days of age) Sprague-Dawley rats after acute treatment (by subcutaneous injection) with maximum tolerated doses of Chlorpyrifos and other organophosphate pesticides. The neonates were more sensitive clinically than adults to Chlorpyrifos exposure: the MTD for neonates was 45 mg/kg s.c., while for adults the MTD was 279 mg/kg s.c. In general, maximal brain ChE inhibition was similar (>78%) in both age groups, but ChE activity recovered faster in neonates. Plasma and RBC ChE activities correlated relatively well with brain ChE activity in neonatal rats at all time points between 4 hours and 7 days posttreatment, but similar correlations between circulating and brain ChE activities in adults were more variable. The study authors concluded that neonatal rats are more sensitive to acute lethality from Chlorpyrifos (and other OP) exposure than are adults, and that MTD exposures produced extensive brain ChE inhibition in both age groups. Following OP exposures, however, significant compound-related and age-related differences in the duration of ChE inhibition can occur.

2. In a paper published in 1992, Pope and Chakraborti described a study in which they examined dose-related inhibition of both brain and plasma cholinesterase activity in neonatal and adult rats exposed to Chlorpyrifos and other organophosphate pesticides. It was found that ED_{50} estimates for both brain and plasma cholinesterase correlated highly with previously derived MTD values. The correlation between the extent of brain and plasma cholinesterase inhibition across dose in neonatal rats was high but lower in adults. The study authors concluded that in vivo inhibitory potency of Chlorpyrifos and the other organophosphate pesticides tested towards either brain or plasma ChE activity is highly correlated with sensitivity to acute toxicity in both neonatal and adult rats.

3. Chakraborti *et al*. (1993) further pursued the premise that neonatal rat (7 days of age) are markedly more sensitive to acute toxic effects of Chlorpyrifos exposure than are adult rats (3 months of age), and compared subacute exposures in the same age groups. Repeated doses of Chlorpyrifos (40 mg/kg by subcutaneous injection, every 4 days for a total of 4 doses) resulted in extensive inhibition of cortical hippocampal, and striatal cholinesterase activity in adult Sprague-Dawley rats at 4 (90-92%) and 14 (71-78%) days after the last treatment. Young rats treated in the same manner, beginning of postnatal day 7, showed a much lower degree of ChE inhibition (21-60%) at these time points. Muscarinic receptor ([^{3}H]quinuclidinyl benzilate, QNB) binding in cortex, hippocampus, and striatum was reduced in adult brain at 4 (30-43%) and 14 (22-32%) days after the final treatment, whereas receptor densities were only marginally

affected in young rats (5-11% reduction). Basal motor activity levels were not affected in either young or adult rats as a function of Chlorpyrifos exposure. After challenge with scopolamine (1 mg/kg by intraperitoneal injection) higher learning activity levels were observed in adult rats at 2, 4, 6, and 8 weeks after treatment; there was no similar increase in activity levels in treated neonatal rats. According to the study authors, these data suggested that although neonatal rats are more sensitive to acute lethal effects from high doses of Chlorpyrifos, adult rats exhibit more persistent neurochemical and neurobehavioral alterations following repeated, lower-level exposures.

4. In a study by Stanton et al. (1994), a single subcutaneous injection of Chlorpyrifos was administered to Long-Evans rat weanlings (21 days of age) at dose levels of 90, 120, or 240 mg/kg; T-maze delayed alternation was tested on postnatal days 23 or 26. Acetylcholinesterase activity and muscarinic receptor density (QNB binding) were determined in hippocampus and cortex of brains taken from pups 15 hours after the end of behavioral testing (the morning of postnatal days 24 and 27) Pups at 240 mg/kg showed signs of overt toxicity that precluded behavioral testing. Exposure to 120 mg/kg produced a transient selective memory impairment (a deficit in delayed alternation but not position discrimination) relative to the 90 mg/kg and vehicle groups. Exposure to Chlorpyrifos on postnatal day 21 produced dose-related inhibition and recovery of brain AChE over the postnatal day 24-27 age range. A similar pattern was observed in hippocampus. Binding of [^{3}H]QNB was reduced in frontal cortex on postnatal day 27 only at the 240 mg/kg dose. No significant effects were observed in the hippocampus. These results suggested to the study authors that the neurochemical effects of acute Chlorpyrifos administration are more transient and the behavioral effects are smaller and shorter-lived than what has previously been reported in adult rats.

C. One study further examined specific aspects of neurological toxicity in rats that were exposed postnatally:

1. Whitney et al. (1995) administered Chlorpyrifos by subcutaneous injection to neonatal rats in apparently subtoxic doses that cause no mortality and little or no weight deficits. Developing brain regions (cerebellum, forebrain, and brainstem) were examined. One-day old rats showed significant inhibition of DNA and protein synthesis in all brain regions within 4 hours of treatment with 2 mg/kg. In comparison, when 0.6 µg Chlorpyrifos was administered directly to the brain via intracisternal injection, equivalent results were observed; this indicates that the inhibition in DNA synthesis was not secondary to systemic toxicity and also suggests

that the Chlorpyrifos does not need to be metabolically activated to the oxon in order to produce neurological effects in neonates. At 8 days of age, inhibition of DNA synthesis was also seen; however, there was regional selectivity, with sparing of the cerebellum. It was also determined that the effects of Chlorpyrifos on DNA and protein synthesis were not secondary to generalized cell damage or suppression of cell metabolism, since ornithine decarboxylase activities were normal. 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.

Additional Studies which were in Preparation for Publication as of December 1997

Additional studies have been identified which have not been published in the peer-reviewed literature, but which are in process of preparation for publication. In these studies, the issues of differential sensitivities between adults and young animals following *in utero* and/or postnatal exposure to Chlorpyrifos were further addressed. A major difference between these and previous studies was that exposure was by the oral route, rather than by subcutaneous injection.

A. Studies that address the neurochemical and neurobehavioral effects of maternal versus fetal or neonatal rats following *in utero* exposure to Chlorpyrifos are described below:

1. Lassiter *et al*. conducted a study to compare the degree and define the time course of ChE inhibition in the dam, placenta, and fetus following late gestational exposure to Chlorpyrifos. The Chlorpyrifos was administered to Long Evans rats by gavage in corn oil at doses of 0 or 7 mg/kg/day on gestation days 14-18; animals were killed at 2, 5, 10, 24, 48, and 120 hours after the last dose. Body weight gain was not affected in treated dams, but maternal blood and brain ChE activity was inhibited (80-90%), reaching a nadir at 5 hours after the last dose. By 120 hours AChE activity had recovered to 30-45% inhibition. Fetal brain ChE inhibition was maximal at 25%, also at 5 hours after last dose, and recovered to control levels by 48 hours. To test the hypothesis that the placenta may be protecting the fetus from CPF-oxon, the placental tissue was analyzed. It was found that two of the enzymes known to detoxify CPF-oxon were either not enriched (Chlorpyrifos-oxonase) or only slightly enriched (carboxylesterase; $\leq$35%) in placental tissue as compared to maternal blood, indicating that the placenta may not be a site for preferential Chlorpyrifos-oxon metabolism which would protect the fetus.

2. In a study by Barone *et al*., Chlorpyrifos was administered by gavage to Long Evans rats (16/group) on gestation days 14-18 at dose levels of 0, 3,

or 5 mg/kg/day. A subset of animals was killed on gestation day 19; maternal blood and brain and fetal brain ChE activity was assayed. Remaining litters were allowed to deliver. Animals were killed on postnatal day 1, 4, 7, 12, 17, 21, and 92, and brains were dissected into 7 distinct regions for analysis of ChE activity, DNA and protein content, and serum thyroid hormone levels. Other developmental landmarks examined were eye opening (PND14-17), vaginal opening (PND32-45) preputial separation (PND 40-50, estrus cyclicity (PND 50-85), and testis weights (PND 92). Maternal weight gain, litter size, sex ratio, postnatal survival, and pup brain and body weights were not affected by late gestation Chlorpyrifos exposure. ChE activity was inhibited in both the maternal blood (60-80%) and brain (4-75%) on gestation day 19, whereas fetal brain ChE inhibition was $\leq$10%. There was no effect on the ontogeny of circulating thyroid hormones (serum T3 and T4), regional brain DNA or protein levels. Trends emerged in a dose-related fashion for eye opening, vaginal opening, and preputial separation. The study authors concluded that, in general, following late gestational exposure to Chlorpyrifos, the dam appears to protect the fetus from cholinesterase inhibition and from long-term adverse consequences.

3. Phillips *et al*. studied behavioral effects following exposure of Long-Evans rats to Chlorpyrifos (0, 3, or 5 mg/kg/day by gavage) on gestation days 14-18. Maternal effects were evaluated in the dams; there was a trend toward lower open field activity at 5 mg/kg/day. Offspring were evaluated for righting reflex on postnatal day 2-7, and 10 pups/dose/sex were tested for a range of neurobehavioral endpoints using a functional observation battery and motor activity assessments on postnatal days 17, 24, 65, and 92. On postnatal day 2, a trend towards slower righting reflex was evident in offspring of high-dose dams, but by postnatal day 7, all rats were righting normally. Few significant behavioral changes were detected at later time points. Male rats at 5 mg/kg/day showed decreased handling reactivity on postnatal day 24, and decreased activity and rearing in the open field testing throughout the course of testing. Female rats showed increased reactivity before weaning in the high-dose group and increased open field activity thereafter in the low-dose group. These data suggested to the study authors that there were qualitative sex-related differences associated with Chlorpyrifos exposure, but the effects were small. It was concluded that there were few persistent neurobehavioral consequences of Chlorpyrifos following late gestational exposure.

4. The effects of gestational exposure to Chlorpyrifos on the developmental profiles of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity in the rat brain was studied by Lassiter et al. It has been suggested that AChE has a role in the coordinated spatiotemporal development of the nervous system, and it was hypothesized that BuChE could also have a developmental function. Profiles of AChE and BuChE activity were not

used as an index of Chlorpyrifos-induced inhibition, but rather as molecular markers of normal brain development. In this study, Long Evans rats were administered Chlorpyrifos at 0, 3, or 5 mg/kg/day on gestation days 14-18. Brain regions were collected on postnatal days 1, 4, 7, 12, 17, 21, and 92. Cortical and cerebellar developmental profiles were studied because they represent early and late-developing regions, respectively. The developmental pattern of AChE and BuChE activity varied with respect to brain region and age, but gestational Chlorpyrifos exposure appeared to have no effect on the region-specific profiles of either AChE or BuChE activity.

B. Studies that address the comparison of the neurotoxic response of adults and neonatal or weanling animals including possible mechanisms of the differences observed, are described below:

1. Moser and Padilla (1998) compared the effects of acute oral Chlorpyrifos exposure in adult (70 days of age) and young (postnatal day 17) rats. They verified the findings of Pope, that neonatal rats (10-27 days of age) were between 5-7 times more sensitive than adults to acute doses of Chlorpyrifos at the maximum tolerated dose, with greater sensitivity identified in the youngest neonates. Then the time-course of the effects of an acute dose of Chlorpyrifos was evaluated for adults and postnatal day 17 pups. Assessments included behavioral evaluations (functional observation battery and motor activity), cholinesterase activity measurements, and muscarinic receptor assays. Doses were administered by gavage at levels that were selected to produce similar effects in young and adult rats; adults received 80 mg/kg and pups received 15 mg/kg. Following testing, tissues were taken at 1, 2, 3.5, 6.5, 24, 72, 168, or 336 hours posttreatment. In adult rats, behavioral changes and brain and blood ChE inhibition followed the same temporal pattern. Peak effect occurred in male rats about 3.5 hours postdose. The onset of changes was quicker in females, but the time-course was more protracted and recovery was slower. In pups, maximal behavioral effects occurred 6.5 hours after dosing, without gender differences. Partial to full recovery of behavioral changes was observed at 24 and 72 hours, similar to adults. Blood and brain ChE inhibition in young rats had nearly recovered by 1 week postdose, but adult brain ChE had not fully recovered at 2 weeks. Muscarinic receptor binding assays showed apparent down-regulation in some brain areas at 24 and 72 hours. The study authors concluded that: 1) young rats show similar behavioral changes, although at a 5-fold lower dose; 2) the onset of maximal effects is somewhat delayed in the young rats, 3) ChE activity tends to recover more quickly in young rats, but; 4) the young rats appear to have more extensive muscarinic receptor down-regulation; and 5) young rats show no gender-related differences.

2. Chanda *et al*. studied the developmental profiles of two organophosphate

detoxifying enzymes, carboxylesterase (CaE; which can bind to OPs and reduce the effective concentration at the target enzyme site) and A-esterase (which can hydrolyze OPs to form nontoxic metabolites). Liver and plasma CaE and A-esterase activities were measured in Long Evans rats on postnatal days 1, 4, 76, 12, 17, 21, and 90. At postnatal day 1, liver and plasma CaE activities were 8 times lower and A-esterase activities were 11 and 35 times lower than that of adults. In general, as the rats developed, A-esterase appeared to mature faster than CaE. Enzyme levels were compared against the sensitivity of young rats to acute Chlorpyrifos exposure at various ages; during development, an inverse relationship between the enzyme activities and sensitivity to Chlorpyrifos toxicity was observed. It was concluded that a lack of these detoxifying enzymes in young rats could at least partially explain their increased sensitivity to Chlorpyrifos.

3. Mortensen *et al*. tested the hypothesis that young rats have less Chlorpyrifos-oxonase (CPFOase) activity than adults. CPFOase activity was measured in the brain, plasma, and liver of male postnatal day 4 and adult Long Evans rats. No brain CPFOase activity was measured at either age. Plasma and liver CPFOase activities were markedly lower (1/11 and ½, respectively) at postnatal day 4 compared to adult. To determine if the CPFOase activity could hydrolyze physiologically relevant concentrations of CPFO, the shifts in tissue AChE IC_{50} for CPFO in the presence or absence of CPFOase activity were compared. An increase in the "apparent" IC_{50} would be expected if CFPOase hydrolyzed substantial amounts of CPFO during the preincubation with CPFO. In the adult, both plasma and liver AChE "apparent" IC_{50} values were higher in the presence of CPFOase activity, suggesting that the CPFOase in those tissues was capable of hydrolyzing physiologically relevant concentrations of CPFO within 30 minutes. In young animals, however, there was less of a shift in the IC_{50} curves compared to the adult, confirming that the young animal has less capacity than the adult to detoxify physiologically relevant concentrations of CPFO via CPFOase.

4. In a further study by Mortensen, Hooper, and Padilla, the developmental profiles, kinetic parameters, and intrinsic (i.e., *in vitro*) sensitivity of male rat brain acetylcholinesterase were compared. The brains of postnatal day 4, 11, 17, 27, 40, or adult (PND 90) Long-Evans rats were collected, homogenized, and diluted to obtain approximately the same AChE activity for each age. Brain homogenates were incubated with varying concentrations of inhibitor (Chlorpyrifos-oxon, aldicarb, carbaryl, or maloaoxon), and AChE activity was measured. It was found that young and adult brain differed primarily in their specific activity; their K_ms, substrate profiles, and *in vitro* sensitivity to the selected anticholinesterase insecticides were not different.

n. Paraoxonase.

Chlorpyrifos, and some other organophosphate (OP) compounds, are detoxified via a two-step pathway involving bioactivation of the parent compound to an oxon by the cytochrome P450 systems, and then hydrolysis of the resulting oxon compounds by esterases such as liver or serum paraoxonase (PON1) (located in the plasma) (Davies et al. 1996, Furlong et al. 1998, Shih et al. 1998). In the human population, serum PON1 activity is genetically determined (polymorphic) and individuals express widely different levels of this enzyme (Davies et al. 1996). Therefore, it is possible that some individuals may be more sensitive to chlorpyrifos toxicity based on genetic factors that regulate serum PON1 activity resulting in a reduced capacity to detoxify chlorpyrifos-oxon. Paraoxonase data were collected for individuals in a recent single dose human study (Kisicki et al. 1999). HED will evaluate these data once they are submitted to the Agency.

In animals, there is evidence that serum paraoxonase is protective against poisoning by OPs. Animals with low PON1 levels were more sensitive to specific OP compounds than animals with high enzyme levels. For example, birds, which have very low to undetectable PON1 activity are more sensitive than various mammals to the acute toxicity of oxons for other OPs (paraoxon, diazinon oxon and pirimiphos oxon). Further rabbits, which have a sevenfold higher serum PON1 activity than rats, are more resistant to the acute toxicity of chlorpyrifos (approximately 9 and 25 fold for acute oral and dermal toxicity, respectively). Rabbit paraoxonase hydrolyzes chlorpyrifos-oxon with a much higher turnover number than does rat paraoxonase (Costa et al. 1999, Li et al. 1993).

REFERENCES

Barone, S., C. Lau, V.C. Moser, P.M. Phillips, K.L. McDaniel, D. Hunter, R. Marshall, P. Kodavanti, F. Dern-Yellin, and S. Padilla. (1997) Developmental effects of gestational exposure to chlorpyrifos in the rat [abstract 1301]. *Toxicologist* 36(1):256.

Brzak, K.A., Harms, D.W., Bartels, M.J., and Nolan, R.J. 1998. Determination of Chlorpyrifos, Chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol in rat and human blood. J. Anal Toxicol. 22:203-230.

Capodicasa, E., Scapellato, M.L., Moretto, A., Caroldi S., and Lotti, M. 1991. Chlorpyrifos-induced delayed polyneuropathy. Arch Toxicol. 65:150-155.

Campbell, C.G., Seidler, F.J, and Slotkin, T.A. (1997). Chlorpyrifos interferes with cell development in rat brain regions (Brain Res. Bull 43(2):179-189.

Chakraborti, T.K., J.D. Farrar, and C.N. Pope. (1993) Comparative neurochemical and neurobehavioral effects of repeated chlorpyrifos exposures in young and adult rats. *Pharmacology Biochemistry and Behavior* 46:219-224.

Chanda, S.M., J. Chaudhuri, T. Chakraborti, and C. Pope. (1993) Persistent fetal brain cholinesterase inhibition induced by a single maternal dose of chlorpyrifos [abstract 257]. *Toxicologist* 13:88.

Chanda, S.M., P. Harp, J. Liu, and C.N. Pope. (1995) Comparative developmental and maternal neurotoxicity following acute gestational exposure to chlorpyrifos in rats. *Journal of Toxicology and Environmental Health* 44:189-202.

Chanda, S.M., S.R. Mortensen, S. Barone, V.C. Moser, and S. Padilla. (1997) Developmental profiles of two organophosphate detoxifying enzymes: carboxylesterase and A-esterase [abstract 1757]. *Toxicologist* 36(1):346.

Chanda S.M. and C.N. Pope. (1996) Neurochemical and neurobehavioral effects of repeated gestational exposure to chlorpyrifos in maternal and developing rats. *Pharmacology Biochemistry and Behavior* 53(4):771-776.

Costa LG, Li WF, Richter RJ, Shih DM, Lusis A, and Furlong CE. 1999. The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism. Chemico-Biological Interactions 119-120: 429-438

Dam K, Garcia SJ, Seidler FJ, Slotkin TA (1999a) Neonatal chlorpyrifos exposure alters synaptic development and neuronal activity in cholinergic and catecholaminergic pathways. Developmental Brain Res. 116:9-20.

Dam K; Seidler FJ; Slotkin TA (1999b) Chlorpyrifos releases norepinephrine from adult and neonatal rat brain synaptosomes. Brain Res Dev Brain Res, 118(1-2):129-33.

Das KP, Barone S (1999) Neuronal differentiation in PC 12 cells is inhibited by chlorpyrifos and its metabolites: Is acetylcholinesterase inhibition the site of action? Toxicol. Applied Pharmacol. 160:217-230.

Davies HG, Richter RJ, Keifer M, Broomfield CA, Sowalla J, and Furlong CE. 1996. The effect of the human serum paraoxonase polymorphism is reverse with diazoxon, soman and sarin. Nat Genet. Nov 14(3):334-6.

Dittenber, D.A 1997. Chlorpyrifos: Evaluation of Single Oral Doses on Cholinesterase and Neurotoxic Esterase Inhibition in F344 Rats. Toxicology Laboratory, Dow Chemical Co. Study No. 960036. March 13, 1997. MRID No. 44273901.

Furlong, CE., Li WF., Costa, LG., Richter RJ., Shih DM, and Lusis AJ. 1998 Genetically determined susceptibility to organophosphorus insecticides and nerve agents: developing a mouse model for the human PON1 polymorphism. Neurotoxicology. Aug-Oct: 19(4-5):645-60

Hoberman A.M. 1998a,b. Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD®BR VAF/Plus® presumed pregnant rats. Argus Research Laboratories, Inc., Horsham, Pennsylvania, laboratory study No. 304-001, sponsor study No. K-

044793-109, May 1, 1998: MRID 44556901, MRID 44661001.

Johnson, D.E., Seidler F.J., and Slotkin, T.A. 1998. Early Biochemical Detection of Delayed Neurotoxicity Resulting from Developmental Exposure to Chlorpyrifos. Brain Research Bulletin. 45(2):143-147.

Kisicki J.S., Seip, C.W., and Combs M.L. 1999. A Rising Dose Toxicology Study to Determine the No-Observable-Effect-Levels (NOEL) for Erythrocyte Acetylcholinesterase (AChE) Inhibition and Cholinergic Signs and Symptoms of Chlorpyrifos at Three Dose Levels. MDC Harris Laboratory, Lincoln Nebraska, Study No. 21438 (for the Harris Project) and DR K-0044793-284 (for Dow AgroSciences), April 19, 1999, MRID No. 44811002.

Lassiter, T.L., S. Padilla, and S. Barone. (1997) Effects of gestational exposure to chlorpyrifos on the developmental profiles of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity in the rat brain [abstract 1313]. *Toxicologist* 36(1):259.

Lassiter, T.L., D. Hunter, R. Marshall, S. Mortensen, S. Chanda, K. Das, and S. Padilla. (1997) The fetal brain appears to be protected from late gestational exposure to chlorpyrifos. Platform/Poster submission, Neurobehavioral Teratology Society Meeting.

Lassiter TL, Padilla S, Mortensen SR, Chanda SM, Moser VC, Barone S (1998) Gestational exposure to chlorpyrifos: Apparent protection of the fetus? Toxicol. Applied Pharmacol. 152: 56-65.

Li WF, Costa LG, Furlong CE. 1993. Serum paraoxonase status: a major factor in determining resistence to organophosphates. J Toxicol Environ Health. Oct-Nov: 40(2-3):337-46.

Mattsson J.L., Maurissen J.P., Spencer, P.J., Brzak K.A., and Zablotny C.L. 1998. Effects of Chlorpyrifos administered via gavage to CD rats during gestation and lactation on plasma, erythrocyte, heart and brain cholinesterase and analytical determination of chlorpyrifos and metabolites. Health and Environmental Research Laboratories, The Dow Chemical Co. for Dow AgroSciences, August 31, 1998. Unpublished Study. MRID 44648101.

Maurissen J.P., Shankar, M.R., Mattsson J.L. 1996. Chlorpyrifos: cognitive study in adult Long-Evans rats. The Toxicology Research Laboratory, Health and Environmental Studies, The Dow Chemical Co. Midland, MI. Laboratory Project Study ID K-044793-096. April 29, 1996. MRID No. 44020901. Unpublished.

Mendrala A.L., and Brzak K.A. 1998. Chlorpyrifos: Part A-concentration-time course of chlorpyrifos and chlorpyrifos-oxon in blood. Health and Environmental Research Laboratories. The Dow Chemical Co. Midland MI. Laboratory Project Study ID: 971187A. August 31, 1998. MRID No. 44648102. Unpublished.

Mortensen, S.R., Hooper M.J. S. Padilla. 1998. Rat brain acetylcholinesterase activity: developmental profile and maturational sensitivity to carbamate and organophosphorus inhibitors.

Toxicology. 125:13-19.

Mortensen, S.R., S.M. Chanda, M.J. Hooper, and S. Padilla. (1997, draft) Maturational differences in chlorpyrifos-oxonase activity may contribute to age-related sensitivity to chlorpyrifos.

Mortensen, S.R., M.J. Hooper, and S. Padilla. (1997, draft) Developmental profiles and maturational sensitivity of rat brain acetylcholinesterase activity.

Moser, V.C. and S. Padilla. 1998. Age- and gender-related differences in the time-course of behavioral and biochemical effects produced by oral chlorpyrifos in rats. Toxicology and Applied Pharmacology. 149:107-119.

Moser, V.C., Chanda, S.M., Mortensen S.R., and Padilla, S. 1998. Age- and Gender-Related Differences in Sensitivity to Chlorpyrifos in the Rat Reflect Developmental Profiles of Esterase Activities. Toxicological Sciences. 46:211-222.

Nolan R.J., Rick D.L., Freshour M.L., and Saunders J.H. 1982. Chlorpyrifos: Pharmacokinetics in human volunteers following single oral and dermal doses. The Dow Chemical Co. Biomedical Medical Research Lab. Toxicology Research Lab. Midland MI. Accession No. 249203.

Padilla S., Wilson, V.Z., and Bushnell, P.J. (1994). Studies on the correlation between blood cholinesterase inhibition and "Target Tissue" inhibition in pesticide-treated rats. Toxicology 92;11-25.

Phillips, P.M., K.L. McDaniel, T.L. Lassiter, S. Barone, and V.C. Moser. (1997) Behavioral effects of gestational exposure to chlorpyrifos in rats [abstract 1300]. *Toxicologist* 36(1):256.

Pope, C.N., T.K. Chakraborti, M.L. Chapman, J.D. Farrar and D. Arthun. (1991) Comparison of in vivo cholinesterase inhibition in neonatal and adult rats by three organophosphorothioate insecticides. *Toxicology* 68:51-61.

Pope, C.N. and T.K. Chakraborti. (1992) Dose-related inhibition of brain and plasma cholinesterase in neonatal and adult rats following sublethal organophosphate exposures. *Toxicology* 73:35-43.

Pope, C.N, Chakraborti, T.K., Chapman, M.L and Farrar, J.D. (1992). Long-Term neurobehavioral and behavioral effects induced by acute chlorpyrifos treatment (1992) Pharm.Biochem.Behav. 42:251-256

Pope, C.N and Liu, J (1997). Age-Related Differences in Sensitivity to Organophosphorous Pesticides. *Environmental Toxicol. And Pharmacol.* 4;309-314.

Richardson R.J., Moore T.B., Kayyali, U.S., and Randall J.C. 1993. Chlorpyrifos: Assessment of Potential for Delayed Neurotoxicity by Repeated Dosing in Adult Hens with Monitoring of Brain Acetylcholinesterase, Brain and Lymphocyte Neurotoxic Esterase, and Plasma

Butyrylcholinesterase Activities. Fund. Applied. Toxicology. 21:89-96.

Roy TS, Andrews JE, Seidler FJ, Slotkin TA (1998) Chlorpyrifos elicits mitotic abnormalities and apoptosis in neuroepithelium of cultured rat embryos. Teratology 58:62-68.

Shih DM, Gu L, Xia YR, Navab M, Li WF, Hama S, Castellani LW, Furlong CE, Costa LG, Fogelman AM and Lusis AJ. 1998. Mice Lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. Nature. Jul 16: 394 (6690):284-7

Slokin T.A. 1999. Developmental Cholinotoxicants: Nicotine and Chlorpyrifos. Environmental Health Perspectives. 107, Supplement 1, 71-80.

Song, X., Seidler, F.J., Saleh, J.L., Zhang, J. Padilla, S., Slotkin T.A. 1997. Cellular mechanisms for developmental toxicity of chlorpyrifos: targeting the adenylyl cyclase signaling cascade. Toxciol Appl. Pharmacol. 145:158-174.

Stanton, M.E., W.R. Mundy, T. Ward, V. Dulchinos, and C.C. Barry. (1994) Time-dependent effects of acute chlorpyrifos administration on spatial delayed alternation and cholinergic neurochemistry in weanling rats. *NeuroToxicology* 15(1):201-208.

Tang J, Carr RL, Chambers JE (1999) Changes in rat brain cholinesterase activity and muscarinic receptor density during and after repeated oral exposure to chlorpyrifos in early postnatal development. Toxicological Sciences 51:265-272.

Whitney, K.D., Seidler, F.J., and Slotkin, T.A (1995). Developmental Neurotoxicity of Chlorpyrifos Cellular Mechanism.*Toxicol. And Pharmacol*134:53-62

Zheng, Q., Olivier K., Won Y., and Pope C. 1999. Comparative Cholinergic Neurotoxicity of Oral Chlorpyrifos Exposures in Neonatal and Adult Rats. Abstract and Poster Presentation. presented at the 38th Annual Society of Toxicology Meeting in New Orleans, March 14-18. The Toxicologist Vol 48, No.1-S, #874. March 1999.

Zheng Q, Olivier K, Won YK, Pope CN (2000) Comparative cholinergic neurotoxicity of oral chlorpyrifos exposures in preweanling and adult rats. Accepted for Publication