DEVELOPMENT OF HEALTH CRITERIA FOR SCHOOL SITE RISK ASSESSMENT PURSUANT TO HEALTH AND SAFETY CODE SECTION 901(g):

CHILD-SPECIFIC REFERENCE DOSE (chRD) FOR SCHOOL SITE RISK ASSESSMENT- Chlorpyrifos

Final
June 2010

Integrated Risk Assessment Branch
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

FINAL

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Executive Summary

A child-specific reference dose (chRD) at 0.0001 mg/kg-day for chlorpyrifos has been established in this document pursuant to Health and Safety Code Section 901(g). Health and Safety Code Section 901(g) requires the Office of Environmental Health Hazard Assessment (OEHHA) to identify chemical contaminants commonly found at school sites to be of greatest concern based on child-specific physiological sensitivities, and to develop numerical health guidance values (HGVs) for these chemical contaminants for use in the assessment of risk at proposed or existing California school sites.

Chlorpyrifos, O,O-diethyl-O-(3, 5, 6-trichloro-2-pyridinyl)-phosphorothioate, is a broad-spectrum organophosphate insecticide. Despite the cancellation of its registration for most home, lawn and garden use by U.S. EPA since 2000, chlorpyrifos continues to be one of the most commonly used pesticides, and the potential risks to children are still of concern to OEHHA.

Inhibition of cholinesterase by its active metabolite chlorpyrifos oxon was once considered the lone mechanism of chlorpyrifos neurotoxicity. However, there is now evidence that chlorpyrifos directly targets events that are specific to the developing brain and that are not related to the inhibition of cholinesterase, including: inhibition of DNA synthesis, impairment of cell acquisition and differentiation, interactions with neurotrophic factors, interruption of cell signaling cascades, and alteration in synaptic function.

Based on our review of the existing literature, OEHHA concluded that there are age-related differences in the susceptibility to chlorpyrifos. Young animals are more sensitive to chlorpyrifos than adults. OEHHA also concluded that both cholinesterase and non-cholinesterase-related mechanisms contribute to the differential susceptibility between young and adults. The deficits may be manifested immediately after the exposure, or appear later in life.

OEHHA establishes a chRD of 0.0001 mg/kg-day for chlorpyrifos based on both cholinesterase inhibitions in dogs and rats and supporting information on cognitive deficiencies in rats.

Introduction

Developing a chRD or chRC

Health and Safety Code (HSC) Section 901(g) requires the Office of Environmental Health Hazard Assessment (OEHHA), in consultation with the appropriate entities within the California Environmental Protection Agency, to identify those chemical contaminants commonly found at school sites and determined by OEHHA to be of greatest concern based on child-specific physiological sensitivities. HSC Section 901(g) also requires OEHHA to annually evaluate and publish, as appropriate, numerical health guidance values (HGVs) for five of those chemical contaminants until the contaminants identified have been exhausted. HGVs established by this mandate are intended for use in the assessment of risk at proposed or existing California school sites. At this time, OEHHA focuses its evaluation on non-cancer effects of the identified chemicals, pending the completion of a new method for developing HGVs based on child-specific carcinogenic effects. Accordingly, current HGVs are in the form of a child-specific reference dose (chRD) or child-specific reference concentration (chRC).

This chapter serves as a background for the technical chRD or chRC reports. For those that are not familiar with this OEHHA program, it is advisable to review this chapter prior to analyzing the individual chRD reports.

Challenge

The use of appropriate HGVs and exposure parameters is essential to provide an unbiased assessment of the health risk at an existing or a proposed school site. Since schoolchildren have higher air, food and water intake relative to their body weight compared to adults; and have activity or behavioral patterns that may lead to higher exposure to environmental contaminants than adults, these higher intakes and unique activity patterns need to be considered in developing a set of child-specific exposure parameters for use in the risk assessment. OEHHA has analyzed these exposure parameters in issuing the report, Guidance for Assessing Exposures and Health Risks at Existing and Proposed School Sites (OEHHA, 2004) (http://www.oehha.ca.gov/public_info/public/kids/pdf/SchoolscreenFinal.pdf).

With respect to evaluating non-cancer risk by comparing the potential chemical exposure against the corresponding health criteria in the school setting, HGVs in the form of child-specific reference doses or concentrations should be used. Until the inception of the HSC Section 901(g) program, these child-specific HGVs were not available. For the most part, existing reference doses or concentrations for non-cancer endpoints, which were based on adult human or animal data, were used. However, a question has been raised that the intraspecies uncertainty factor of 10, the default factor, would not adequately protect children because it was mainly designed to account for genetic variability such as metabolizing isoenzyme variations. The Food Quality Protection Act of 1996 (http://www.epa.gov/opppsps1/fqpa/) was an attempt to address the issue of children's

sensitivity and susceptibility. It mandated an extra safety factor of 10 for pesticide tolerances in foods unless data existed to indicate that children were not more sensitive or susceptible than adults.

A case can be made for the development and application of child-specific HGVs based on studies in young animals or epidemiological analysis of pertinent data rather than relying solely on a safety factor or uncertainty factor. While locating the appropriate data is a challenge, OEHHA has strived to do so because children can be more (or less) susceptible to chemical effects due to pharmacokinetic and pharmacodynamic differences between them and adults, and thus empirical data in the young would be preferable. Vulnerability often depends on the organ system in question and its developmental stage. There are critical periods of structural and functional development during both prenatal and postnatal life, including adolescence, during which a particular structure or function may be more sensitive to disruption due to the action of a toxicant. Damage may not be evident until a later stage of development (DeRosa et al., 1998; Bigsby et al, 1999). The brain, for example, is an organ with distinct neurodevelopmental stages that occur in temporally distinct time frames across different regions, so the specific chemical, dose, and time of exposure during development will determine if a specific function in the brain will be altered (Faustman et al, 2000).

Differences also exist between children and adults with respect to their absorption, distribution, metabolism, and elimination of chemical contaminants. For example, absorption may be different in neonates because of the immaturity of their gastrointestinal tract and their larger skin surface area in proportion to body weight (Morselli et al. 1980; NRC, 1993); the gastrointestinal absorption of lead is greatest in infants and young children (Ziegler et al. 1978). Distribution of xenobiotics may be different; for example, infants have a larger proportion of their bodies as extracellular water, and their brains and livers are proportionately larger (Altman PL, 1974; Fomon, 1966; Fomon *et al*. 1982; Owen G.M., 1966; Widdowson E.M., 1964). The infant also has an immature blood-brain barrier (Adinolfi, 1985) (Johanson, 1980) and probably an immature blood-testis barrier (Setchell B.P., 1975). Many xenobiotic metabolizing enzymes have distinctive developmental patterns. At various stages of growth and development, levels of particular enzymes may be higher or lower than those of adults, and sometimes unique enzymes may exist at particular developmental stages (Komori *et al*. 1990; Leeder and Kearns, 1997; NRC, 1993; Vieira *et al*. 1996). Whether differences in xenobiotic metabolism make the child more or less susceptible also depends on whether the relevant enzymes are involved in activation of the parent compound to its toxic form or in detoxification. There may also be differences in excretion, particularly in newborns, who all have a low glomerular filtration rate and have not developed efficient tubular secretion and resorption capacities (Altman PL, 1974; NRC, 1993; West J.R., 1948). Children and adults may differ in their capacity to repair damage from chemical insults.

U.S. EPA and the March of Dimes sponsored a workshop -- Identifying Critical Windows of Exposure for Children's Health -- in September 1999 to systematically review the state of knowledge on prenatal and postnatal exposures and subsequent outcomes (Selevan *et al.* 2000). The workshop focused on the nervous, immune,

respiratory, reproductive, and endocrine systems—organ systems that are still undergoing development and maturation in children and thus deemed to be highly vulnerable to chemical insults. Workshop participants noted that data pertaining to children's sensitivities to environmental contaminants during various critical developmental periods are limited. In particular, little attention has been given to studying peripubertal and adolescent exposures or adult consequences from childhood exposure. Thus, the state of scientific knowledge pertaining to chemical effects on children is and continues to be a limiting factor in OEHHA's ability to develop child-specific HGVs for these contaminants.

In summary, with rare exceptions the use of a study in children or young animals as the basis for a child-specific HGV is preferred, even when studies in adult humans or animals encompassing a greater dose range or a larger experimental population exist and a biological mechanism of action can be established from corroborating studies. If a study in the young does not exist, the challenge is to integrate studies supporting a biological mechanism for greater sensitivity in the young with studies on adults to justify the application of appropriate safety factors.

Process

In June 2002, OEHHA issued a report, "Development of Health Criteria for School Site Risk Assessment Pursuant to Health and Safety Code, Section 901(g): Identification of Potential Chemical Contaminants of Concern at California School Sites," documenting the process by which OEHHA identifies chemicals and presenting a compilation of seventy-eight chemicals. The report can be found at http://www.oehha.ca.gov/public_info/public/kids/schoolsrisk.html. The compilation, whose sole purpose is to provide OEHHA staff with a manageable list of chemicals to work from, has no regulatory status and is a living document – chemicals may be added or removed as new information becomes available.

The chRD development process begins with the prioritization of chemicals from the compilation described in the June 2002 report. OEHHA has employed the following criteria, recognizing that often the availability of health effect data may be the overriding consideration in the selection of chemicals for evaluation.

1. Chemicals having a strong indication of their presence at school sites according to monitoring studies or other reliable sources.

2. Chemicals cited to have possible adverse effects in three or more of the systems that are undergoing critical development during childhood: the nervous, immune, respiratory, reproductive, or endocrine systems.

3. Chemicals that other OEHHA programs have identified as a concern.

The Public Health Library at the University of California at Berkeley assisted in literature search. OEHHA, in turn, reviewed the citations and abstracts; and evaluated relevant

qualitative papers and quantitative studies. In developing health guidance values for children as mandated by Health & Safety Code 901(g) OEHHA has adopted the following: First, in order to protect children from infancy through the time they leave school, HGVs must consider school-aged children up to age 18, and infants and toddlers in daycare facilities located at school sites. Second, OEHHA considers the most sensitive species and endpoints in our evaluations. When evaluating various studies that use different test parameters to measure the same endpoint such as the nervous system, the lowest LOAEL or NOAEL from these studies would be selected. Third, the paucity of data has underscored the reality that the databases for sensitive endpoints may be incomplete. An uncertainty factor for database deficiency will be considered as appropriate. Fourth, because quantifying differences in susceptibility between a developing organ system and a mature one are hampered by the availability of studies that intentionally compare an effect in young animals with one in adult animals and available data are mainly from developmental toxicity studies that limit dosing to the mother during pregnancy, OEHHA staff have decided that these studies can be used for development of a child-specific health guidance value (chRD or chRC) if it is reasonable to assume that the effect of the chemical on the target organ in the offspring animal is likely to occur on the same target organ undergoing development after birth in humans. If studies that include gestational dosing of the mother and lactational dosing of the offspring (a protocol of the U.S. EPA Developmental Neurotoxicity Health Effects Test) are available, OEHHA will also consider these studies acceptable for establishing a chRD or chRC if the development of the critical organ system continues during childhood.

Finally, these prenatal and perinatal studies are frequently part of a series of studies to elucidate a “mechanism of toxicity”. These studies may not have used a large number of animals or dose ranges. However, due to the critical windows in which cell proliferation and differentiation are occurring in specific organ systems during childhood, a study in young animals is usually preferred over one in adults, even adult humans. With corroborating studies showing a mechanism of action and biological plausibility, OEHHA will consider using these studies as appropriate. However, in rare cases, data from adult animals may be used, if they are from high quality studies and if there are data to provide a means of inference to critical windows of development in young animals so that an appropriate uncertainty or safety factor can be applied.

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Chlorpyrifos

What is Chlorpyrifos?

Chlorpyrifos, O,O-diethyl-O-(3, 5, 6-trichloro-2-pyridinyl)-phosphorothioate, is a broad-spectrum organophosphate insecticide. Also known as Dursban, Lorsban, and other trade names, chlorpyrifos was first introduced in 1965 for control of a wide variety of insects on food and feed crops. Chlorpyrifos is one of the most widely used organophosphate insecticides in the U.S. and is the most effective product available for the control of California red scale, a common insect pest of citrus grown in California. On June 8, 2000, the U.S. Environmental Protection Agency (EPA) announced a cancellation of registration for most home, lawn and garden use products containing chlorpyrifos based on human health risks (U.S. EPA, 2000a). Currently, chlorpyrifos is registered for use in orchards, row crops, golf course turf, non-structural wood treatments, greenhouses, industrial plant sites, and to control mosquitoes and fire ants. Although lower application rates and lower frequencies of treatment are occurring for some agricultural uses of chlorpyrifos since 2001, chlorpyrifos is still being widely used in agriculture (Table 1).

Table 1. Chlorpyrifos Use Trend in California
(Data from California Department of Pesticide Regulation's Pesticide Use Reports)

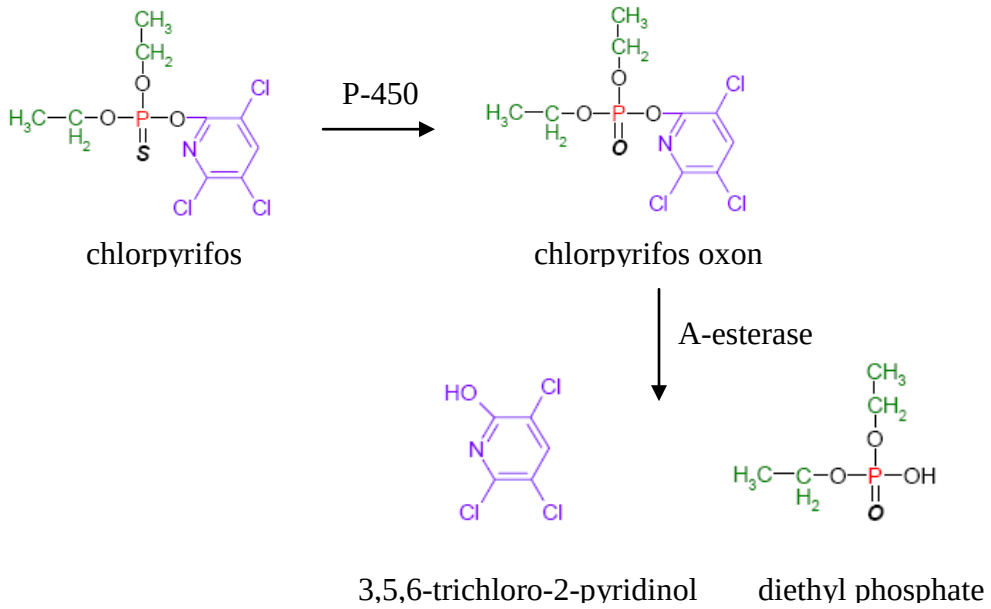
TOTAL CHLORPYRIFOS APPLIED IN THOUSAND POUNDS (Use includes both agricultural and reportable non-agricultural applications)										
1997	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**
3,152	2,355	2,257	2,093	1,674	1,419	1,546	1,775	1,993	1,919	1,430

Chlorpyrifos can be absorbed from the gastrointestinal tract and to a lesser extent through skin or by inhalation. The metabolism of chlorpyrifos is similar in both humans and other mammals. Chlorpyrifos is bioactivated to chlorpyrifos oxon in the liver through cytochrome P450 mediated desulfuration. Chlorpyrifos oxon is subsequently hydrolyzed by A-esterase to diethylphosphate and 3,5,6-trichloro-2-pyridinol (TCP), which is the major biological metabolite and environmental breakdown product of chlorpyrifos (Figure 1). The biological half-life of chlorpyrifos is relatively short, about 18 hours in plasma and 62 hours in fat. Chlorpyrifos metabolites are excreted primarily through the kidneys in the urine. Chlorpyrifos oxon is the active metabolite of chlorpyrifos, mediating the toxic effects of chlorpyrifos by binding irreversibly with acetylcholinesterase, eliciting cholinergic hyperstimulation in the nervous system and in neuro-muscular junctions.

Chlorpyrifos is a category II pesticide with an oral LD_{50} in rats ranging from 82 to 270 mg/kg. Clinical signs of acute poisoning associated with cholinergic hyperstimulation may include dizziness, vomiting, nausea, diarrhea, headache, blurred vision, salivation, sweating, slurred

speech, anxiety, respiratory failure and cardiac arrest. The major effects of chronic exposure are cholinergic signs and decrease in plasma, red blood cell (RBC), and brain cholinesterase activity. Developmental toxicity observed in animal studies may include decreased fetal length and weight, skeletal variations, alterations in brain development and behavioral abnormalities in offspring of exposed mothers. Some studies suggest that chlorpyrifos may be genotoxic, while no chronic studies have indicated chlorpyrifos is carcinogenic to this point. Chlorpyrifos is moderately persistent in the environment. The soil half-life of chlorpyrifos is usually between 30 and 120 days. The half-life of chlorpyrifos in water is relatively short, from a few days to two weeks.

Figure 1. The Metabolism of Chlorpyrifos



Modified from Biomarkers of Exposure: Organophosphates (National Pesticide Information Center)

What characteristics make chlorpyrifos of concern pursuant to Health & Safety Code Section 901 (g)?

Although the June 2000 Memorandum of Agreement between the US EPA and the technical registrants prohibited all the domestic use of chlorpyrifos, it continues to be one of the most commonly used organophosphate pesticides for agricultural and commercial operations, and the risks to children are still of concern to OEHHA. Because of its extensive use, the metabolites of chlorpyrifos are frequently found in human tissue. The chlorpyrifos metabolite TCP has been found in the urine of 82 percent of adults sampled from all regions of the country (CDC 2001). A second report released two years later showed similar levels of TCP in urine samples (CDC 2003). In California, a joint study conducted by the California Air Resource Board and the California Department of Health Services between 2001 and 2002 showed that chlorpyrifos residue was present in 80 percent of all floor dust samples in California's portable classrooms.

The half-life of chlorpyrifos indoors is estimated to be 30 days, but some studies show chlorpyrifos present in ambient air up to eight years post-application. The half-life of chlorpyrifos in water is relatively short, from a few days to two weeks. However, a study done in Chesapeake Bay showed that the hydrolysis half-lives of chlorpyrifos varied from 24 days in the Patuxent River to 126 days in the Susquehanna River, and the author indicates that there might be a potentially long environmental half-life for this chemical (Liu et al, 2001). The soil half-life of chlorpyrifos is usually between 30 and 120 days, but can vary from 2 weeks to over one year, depending on the climate, soil type and other conditions. Reports from the USDA Forest Service showed that the termiticide formulation of chlorpyrifos can be effective against termites for more than 15 years (Wagner, 2003).

Existing Health Criteria for Chlorpyrifos

U.S. EPA (IRIS) Reference Dose (RfD). U.S. EPA's Integrated Risk Information System has established an RfD of 0.003 mg/kg-day for chronic oral exposure of chlorpyrifos (U.S. EPA, 1988). The RfD is based on a 1972 human study conducted by Dow Chemical Company (Coulson *et al*, 1972). Sixteen healthy adult male volunteers were separated into four experimental groups and treated (4 per dose group) with 0, 0.014, 0.03, or 0.1 mg/kg-day of chlorpyrifos by tablet for 20 days. The 0.10 mg/kg-day treatment was terminated after 9 days because of the runny nose and blurred vision in one of the subjects. The plasma cholinesterase in this group was reduced by about 65 percent compared to the controls. No reduction in plasma cholinesterase was seen at the lower doses. The RBC cholinesterase activity was unaffected at any dose examined. Based on the decreased plasma cholinesterase activity at 0.10 mg/kg-day, the NOEL for plasma cholinesterase inhibition is 0.03 mg/kg-day. The RfD of 0.003 mg/kg-day was calculated based on the NOEL of 0.03 mg/kg-day and an uncertainty factor of 10 (human variability). The U.S. EPA's RfD was established in 1988 based on the 20-day human study, which did not measure chronic chlorpyrifos toxicity because of the insufficient exposure duration. The human study is also limited because it only included 4 test subjects in each treatment group.

ATSDR Minimal Risk Level (MRL). The Agency of Toxic Substances and Disease Registry (ATSDR) has established a MRL of 0.001 mg/kg-day for chronic oral exposure of chlorpyrifos (ATSDR, 1997). The MRL is based on a 2-year rat study conducted by Dow Chemical Company (McCollister et al, 1974). Sherman rats (25 rats/sex/dose) were treated with chlorpyrifos at 0, 0.01, 0.03, 0.1, 1, or 3 mg/kg-day for 2 years starting at 7 weeks of age. Supplementary groups (5-7 rats/sex/dose) were included in the study to provide interim pathological examination and cholinesterase (ChE) determinations. Brain ChE was inhibited by 56 percent in the 3 mg/kg/day treatment group during the 2-year study. No reduction in brain ChE was seen at the lower doses. Plasma and RBC ChE activity were reduced at 1 and 3 mg/kg-day. Neither plasma nor RBC cholinesterase was affected by the treatment at 0.1 mg/kg-day or below. A NOEL of 0.1 mg/kg-day was established based on the reduced plasma and RBC ChE activity. ATSDR applied an uncertainty factor of 100 (10 for intra-species variation, and 10 for extrapolation from animals to human) to the NOEL and a MRL of 0.001 mg/kg-day was derived.

The OEHHA analysis, discussed below, also relies in part on the McCollister rat study and the cholinesterase inhibitory effects of chlorpyrifos.

U.S. EPA Reference Dose (RfD) and Population Adjusted Dose (PAD). The Office of Pesticide Programs (OPP) at U.S. EPA has established two health guidance values for chronic dietary assessment in support of the reregistration eligibility decision for chlorpyrifos (U.S. EPA, 1999; U.S. EPA, 2000b; U.S. EPA, 2002). These health guidance values are based on 5 animal studies: a 2-year dog study (McCollister et al, 1974), a 90-day dog study (Barker, 1989), a 90-day rat study (Crown, et al, 1985), a 2-year rat study (Crown et al, 1990) and a developmental neurotoxicity study in rats (Hoberman et al, 1998a, b). McCollister's 2-year dog study is a key study from which a NOEL was derived. This study was conducted in two separate phases. In phase A, 11-month old dogs (3 males and 3 females per group) were treated with chlorpyrifos at 0, 0.01, 0.03, 0.1, 1.0, and 3.0 mg/kg-day by diets for 1 year. In phase B, 10-month old dogs (4 males and 4 females per group) were treated with chlorpyrifos at 0, 0.01, 0.03, 0.1, 1.0, and 3.0 mg/kg-day by diets for 2 years. The cholinesterase activity was decreased at 0.03, 0.1, 1.0 and 3.0 mg/kg-day in plasma, 0.1, 1.0 and 3.0 mg/kg-day in red blood cells, and 3.0 mg/kg-day in brain. A NOEL of 0.03 mg/kg-day was established based on reduced plasma and RBC ChE activity. McCollister's 2-year dog study and the cholinesterase inhibitory effects of chlorpyrifos has also been used by OEHHA as part of the basis for deriving a chRD, and it is further discussed below.

OPP also considered the qualitative differences between F_0 and F_1 females in a developmental neurotoxicity study as part of the basis to retain the 10X FQPA safety factor. Hoberman et al. (1998a, b) observed a qualitative difference in response to chlorpyrifos between the F_0 and F_1 female rats (cholinesterase inhibition in F_0 female rats vs. morphologic alterations in the brain of F_1 females).

Based on the weight of evidence consideration from the 5 studies in dogs and rats, OPP used the NOEL of 0.03 mg/kg-day as the basis for the chronic RfD. They applied an uncertainty factor of 100 (10 for intra-species variation, and 10 for extrapolation from animals to human) to the NOEL to derive a RfD of 0.0003 mg/kg-day. OPP includes an additional FQPA safety factor of 10 for children and women 13-50 due to 1) age-related difference in cholinesterase inhibition (Zheng et al, 2000; Moser and Padilla 1998), 2) qualitative difference between dams and adult offspring in the developmental neurotoxicity study (Hoberman et al, 1998a,b), and 3) uncertainties regarding the potential non-cholinergic adverse effects of chlorpyrifos, which are further discussed below. This additional FQPA Safety Factor results in a Population Adjusted Dose (PAD) of 0.00003 mg/kg-day for children and women ages 13 to 50. The U.S. EPA's RfD and Population Adjusted Dose are the most current and health-protective among existing health criteria for chlorpyrifos.

Current Evaluation Results

Human epidemiological studies have indicated that chlorpyrifos exposure early during development is associated with the deficits developed in infants. Reduction in birth weight, decrease in birth length, and birth defects were observed in infants exposed to chlorpyrifos

during pregnancy (Perera et al, 2003; Sherman, 1995; Whyatt et al, 2005; Rull et al, 2004; Rauh et al, 2004). A definitive evaluation with a focus on the age-related difference in chlorpyrifos toxicity is thus becoming necessary and important.

I. Age-Related Differences in the Detoxification of Chlorpyrifos.

A-esterase (e.g., chlorpyrifos oxonase and paraoxonase) and carboxylesterase are known to play an important role in the detoxification of chlorpyrifos. Berkowitz et al. (2004) studied the correlation between the paraoxonase activity and chlorpyrifos neurotoxicity. A total of 404 mothers and their infants were studied. The level of pesticide exposure was evaluated by a combined approach of a prenatal pesticide exposure questionnaire introduced to the mothers and the measurement of maternal urine pesticide metabolite levels. The maternal paraoxonase-1 (PON1) activity was also measured at the same time. Infant head circumference, which is an indicator for neurodevelopmental disorders, remained unchanged between the two groups with chlorpyrifos urine metabolite TCP above the limit of detection and below the limit of detection. However, when maternal PON1 activity was taken into consideration, infants born to mothers with low paraoxonase-1 (PON1) activity and with urine TCP level above the limit of detection showed significant reduction in head circumference. The results thus indicated that chlorpyrifos may have adverse effects on infants born to mothers with a low level of PON1 activity (Berkowitz et al, 2004).

Animal studies indicated that paraoxonase pretreatment provides protection in rats challenged with chlorpyrifos oxon. Serum paraoxonase activity was 7-folder higher in rabbits compared to rats. Rats injected with purified rabbit serum paraoxonase, and exposed to chlorpyrifos oxon or paraoxon subsequently, showed less cholinesterase inhibition compared to the control group (Costa et al, 1990). In a separate animal study, paraoxonase-1 knockout mice were created by gene targeting. PON1 null mice showed a high degree of susceptibility to chlorpyrifos or chlorpyrifos oxon-induced acetylcholinesterase inhibition. In addition, PON1 null mice were more susceptible to lipoprotein oxidation and atherosclerosis. The results thus indicated that PON1 knockout mice are more susceptible to chlorpyrifos and its metabolite chlorpyrifos oxon (Shih et al, 1998).

Human studies showed that young children have less serum paraoxonase activity than adults. A-esterase activity is 3-fold lower in infants than adults (Augustinsson and Brody, 1962; Ecobichon and Stephens, 1973). Paraoxonase activity in newborn cord blood is 2.4-fold lower than in adults, suggesting that its activity is not fully developed at birth (Mueller et al, 1983). A multiethnic cohort study including both adults and neonates at Mount Sinai Hospital in New York City demonstrated that neonates have lower paraoxonase-1 (PON1) activity than adults. The differences are 2.6, 3.6, and 4.6 times for African Americans, Caribbean Hispanics, and Caucasians, respectively. In addition, the differences in the activity between different PON1 genotypes are also greater in neonates compared to adults (Chen et al, 2003).

Some animal studies also showed that levels of A-esterase and carboxylesterase were much lower in newborn and juvenile rats than in adults. A study in Long-Evans rats showed liver and plasma carboxylesterases are 6-fold lower in newborn compared to adults, and 2-fold lower in juvenile than in adults. Chlorpyrifos oxonase, the A-esterase that hydrolyzes chlorpyrifos oxon,

showed a 30-fold difference between newborns and adults (Moser et al, 1998). A separate study in Long-Evans rats showed an 11-fold difference between rats at 4 days of age and adult rats in plasma chlorpyrifos oxonase activity and a 2-fold difference in liver chlorpyrifos oxonase (Mortensen et al, 1996). A study in Sprague-Dawley rats also showed lower levels of carboxylesterase activity in young rats compared to adults. The enzyme activity in plasma, liver and lung were 5-, 11- and 4-fold lower at 7 days of age compared to adults. The differences were 2.7-, 2- and 1.7- fold respectively at 21 days of age compared to adults (Karanth and Pope, 2000). Another study in Sprague-Dawley rats showed a 4-fold difference between 1-day-old and 80-day-old for liver carboxylesterase activity (Atterberry et al, 1997). The levels of liver microsomal carboxylesterases were also low in young rats. Comparing to the adults, the levels were 6-fold and 2-fold lower in one-week-old and four-week-old rats respectively (Morgan et al, 1994). The lack of enzymes to detoxify chlorpyrifos in young vs. adults would make children more sensitive to chlorpyrifos toxicity compared to adults.

II. Age-Related Differences in Chlorpyrifos-Induced Cholinesterase Inhibition.

Studies have shown that immature organisms are more sensitive to chlorpyrifos-induced cholinesterase inhibition following acute high dose exposure. Chlorpyrifos, given by oral gavage to young rats (17 days of age) at 15 mg/kg, produced cholinesterase inhibition and behavioral changes similar to those in adult rats (70 days of age) at 80 mg/kg. The same degree of cholinesterase inhibition can be achieved in postnatal day 17 rats at a 5-fold lower dose compared to adults (Moser and Padilla 1998). The maximum tolerated dose (MTD) of chlorpyrifos following subcutaneous injections was 45 mg/kg in neonatal rats at 7 days of age compared to 279 mg/kg in adult rats at 80-100 days of age (Pope et al, 1991). Pope and Chakraborti (1992) also studied the dose that would cause 50 percent inhibition of cholinesterase activity (ED50) following subcutaneous injections. The ED50 for brain cholinesterase inhibition was 19.8 mg/kg in neonatal rats at 7 days of age compared to 44 mg/kg in adult rats at 3 months of age (Pope and Chakraborti 1992).

Zheng et al. (2000) compared chlorpyrifos-induced cholinesterase inhibition in neonatal and adult rats following single or repeated oral exposure at non-lethal doses (0.15-15 mg/kg-day). Despite the fact that immature rats still show greater sensitivity to single oral exposure (NOELs for cholinesterase inhibition in plasma, RBC and brain were 0.15-1.5 in neonates vs. 1.5-15 mg/kg-day in adults), no apparent age-related differences were seen following repeated exposure for 14 days (NOELs for cholinesterase inhibition in plasma, RBC and brain were 0.75 in neonates vs. 0.15-1.5 mg/kg-day in adults) (Zheng et al, 2000). Zheng's repeated exposure results were consistent with other studies showing that while young animals are more sensitive to the acute toxicity of chlorpyrifos than adults, the difference is not evident following repeated exposure (Zheng et al, 2000; Liu et al, 1999; Chakraborti et al, 1993). It is uncertain whether there is likely to be a similar age-related difference in sensitivity to chronic low dose exposure to chlorpyrifos, which is more relevant to the environmental chlorpyrifos exposure of the general human population.

III. Non-Cholinesterase Mechanisms of Chlorpyrifos Neurotoxicity.

Inhibition of cholinesterase by its active metabolite chlorpyrifos oxon was once considered the lone mechanism of chlorpyrifos neurotoxicity. Studies from the past decade led us to a better understanding of the mechanism of chlorpyrifos neurotoxicity. There is now evidence that chlorpyrifos directly targets events that are specific to the developing brain and that are not necessarily related to the inhibition of cholinesterase (Qiao et al, 2001; Qiao et al, 2003b; Qiao et al, 2004; Qiao et al, 2005; Whitney et al, 1995; Dam et al, 1998; Song et al, 1997). Indeed, the greater toxicity of chlorpyrifos in juvenile animals cannot be explained solely by developmental differences in cholinesterase-mediated events, nor do age-related increments in chlorpyrifos metabolism account for differential toxicity. Immature animals actually recover more rapidly from cholinesterase inhibition, so measurements of cholinesterase activity alone may not be sufficient for the assessment of adverse effects. Chlorpyrifos-induced neurochemical and neurobehavioral changes unrelated to ChE inhibition, such as those listed below, are of equal concern for human health risk assessment:

1. Chlorpyrifos affects the developing brain during cell division. Chlorpyrifos exerts antimitotic actions on developing neural cells independently of cholinesterase inhibition (Qiao et al, 2001; Qiao et al, 2003a; Dam et al, 1998; Whitney et al, 1995, Campbell et al, 1997). Administration of chlorpyrifos by subcutaneous injections to neonatal rats at doses that were devoid of any overt toxicity showed significant inhibition of DNA synthesis and subsequent cell loss in brain regions examined. For example, single dose (2 mg/kg) subcutaneous administration of chlorpyrifos on postnatal day 1 and day 8 showed acute inhibition of DNA synthesis in rat brain. Repeated chlorpyrifos administration on postnatal day 1 through day 4 at 1 mg/kg-day showed persistent inhibition of DNA synthesis. Chlorpyrifos treatment on postnatal day 11 through day 14 at 1 or 5 mg/kg-day led to deficits in cell number in forebrain, which were seen between 15 and 20 days of age rather than during the chlorpyrifos treatment. The results thus indicate that, with postnatal exposure, cell loss and deterioration of cell function continue well after the end of the exposure period and after cholinesterase activity returns to normal. Additional experiments also demonstrated that the effects are not cholinesterase-related. For example, Qiao et al (2001) showed that chlorpyrifos can inhibit DNA synthesis in cultured neural cell lines to a much greater extent than the oxon despite the fact that chlorpyrifos is a weaker cholinesterase inhibitor. The results therefore indicate that the effects of chlorpyrifos on DNA synthesis may not be mediated through cholinesterase inhibition by chlorpyrifos oxon.

2. Chlorpyrifos interferes with RNA synthesis during differentiation. Neonatal rats treated with chlorpyrifos on postnatal days 1 through 4 (1 mg/kg-day) and postnatal days 11 through 14 (5 mg/kg-day) showed a significant reduction in total cellular RNA in the brain as one of the earliest detectable events (Johnson et al, 1998). Alterations in RNA concentration and content were seen in the developing brain when tested 1 or 6 days after chlorpyrifos exposure. The results indicate that chlorpyrifos targets pivotal macromolecules that control cell differentiation during brain cell development. The lower threshold for these subcellular effects compared to that for systemic toxicity demonstrates that the developing brain is a selective target for chlorpyrifos, a factor that should be fully considered in the risk assessment process.

3. Chlorpyrifos interrupts cell signaling. The adenylyl cyclase signaling transduction pathway is involved in cell replication and differentiation in virtually all prokaryotic and eukaryotic cells. Therefore interference with this pathway during development would be expected to have a significant impact on brain cell development. When the effects of otherwise subtoxic doses of chlorpyrifos on adenylyl cyclase activity were examined in the developing brain, profound effects were found (Song et al, 1997). Importantly, low doses (1 mg/kg-day) of chlorpyrifos given early in development (postnatal days 1-4), with minimal cholinesterase inhibition, had a much greater effect on the adenylyl cyclase pathway than did larger doses (5 mg/kg-day) administered later in development (postnatal days 11-14), even though the latter exposure produced a much greater inhibition of cholinesterase. The effects on adenylyl cyclase were not evident during the immediate period of chlorpyrifos treatment. The largest effects on signaling appeared after several days of delay, at a time point when cholinesterase activity had returned to normal values. The results demonstrated that non-cholinergic mechanisms play a key role in the adverse effects of chlorpyrifos on brain development. Thus, conversion of chlorpyrifos to its metabolite chlorpyrifos oxon, and the subsequent inhibition of cholinesterase, might not be the only factor in determining developmental neurotoxicity of this chemical.

4. Chlorpyrifos interferes with important nuclear transcription factors involved in cell differentiation. The ability of chlorpyrifos to affect nuclear transcription factors involved in cell replication and differentiation was also studied (Crumpton et al, 2000). Apparently subtoxic doses (e.g., 1 mg/kg daily by subcutaneous injection) of postnatal chlorpyrifos treatment (postnatal days 1-4 or postnatal days 11-14) interfered directly with the binding activity of AP-1 and SP-1 transcription factors, which are involved in activation of many genes required in differentiation. The changes were present in both forebrain and cerebellum. Unlike the forebrain, cerebellum is a brain region with sparse cholinergic innervation. Again, this study indicates the direct actions of chlorpyrifos on brain cell development, effects not related to cholinesterase inhibition.

5. Chlorpyrifos impairs cholinergic synaptic function during development. Effects of chlorpyrifos on cholinergic synaptic function were also studied. Choline acetyltransferase (ChAT), the enzyme responsible for the synthesis of acetylcholine, is a constitutive marker for cholinergic nerve terminals. Hemicholinium-3 (HC-3) binding to the presynaptic choline transporter, which is responsive to neuronal activity, is widely used as an index of nerve impulse activity. Choline acetyltransferase (ChAT) activity and hemicholinium-3 (HC-3) binding were thus studied as indices of synaptic proliferation and synaptic function. Low doses of chlorpyrifos (1 or 5 mg/kg by subcutaneous injection) at different postnatal stages caused reduction in both synaptic proliferation and synaptic activity; deficits appeared almost immediately after the exposure (Dam et al, 1999).

6. Chlorpyrifos affects the catecholamine system in the developing brain. Effects of chlorpyrifos were not limited to the cholinergic system. Catecholamine pathways were also involved (Dam et al, 1999). Postnatal chlorpyrifos (1 or 5 mg/kg) was shown to augment the release of both dopamine and norepinephrine within the central nervous system in experimental rats. Notably, the cerebellum, a region with sparse cholinergic innervation, was affected the most. The results also suggest that non-cholinergic mechanisms may play a key role in the adverse effects of chlorpyrifos on brain development.

7. Chlorpyrifos elicits oxidative stress in the developing brain. Reactive oxygen species are thought to be involved in the toxicity of many neurotoxicants. Investigators (Qiao et al, 2005; Bagchi et al, 1995) also evaluated the ability of chlorpyrifos to produce lipid peroxidation, an index of oxidative stress. Their results indicate that chlorpyrifos elicits oxidative damage as demonstrated by the increased lipid peroxidation after chlorpyrifos exposure to developing neural cells both in vitro (1 nmol/ml) and in vivo (41 mg/kg by oral route). Therefore the production of reactive oxygen species and resulting tissue damage may also contribute to the toxic manifestations of chlorpyrifos.

8. Chlorpyrifos interferes with gliogenesis and axonogenesis. Neurons are not the only target of chlorpyrifos in the CNS. Chlorpyrifos also targets glia during gliogenesis and axonogenesis. Both prenatal (1-40 mg/kg on gestational days 17-20) and postnatal (1 mg/kg on postnatal days 1-4, 5 mg/kg on postnatal days 11-14, 1.5 and 3 mg/kg on postnatal days 1-6) chlorpyrifos exposures by subcutaneous injection or oral administration cause alterations in neuroprotein markers for oligodendrocytes, neuronal cell bodies, and developing axons. The deficiencies occur both in the immediate post-treatment period and later during development (Garcia et al, 2002; Garcia et al, 2003; Betancourt et al, 2006). Morphological changes such as a decrease in the number of glial cells were also observed in juvenile rat brain after neonatal chlorpyrifos exposure (Roy et al, 2004). Gliogenesis and axonogenesis are late events in brain development. These findings thus indicate that chlorpyrifos targets developing organisms over a wide developmental period. Roy et al (2004) state that "the vulnerable period for adverse effects of chlorpyrifos is likely to extend into childhood or adolescence."

9. Chlorpyrifos alters levels of neurotrophins in the developing brain. Chlorpyrifos has been shown to alter neurotrophin levels in rodent studies (Betancourt et al, 2006; Betancourt et al, 2007). Young Sprague-Dawley rats were given chlorpyrifos by oral gavage at 4 or 6 mg/kg-day on postnatal days 10-20. Chlorpyrifos altered the expression of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) at both mRNA and protein levels in different brain regions. Significant changes were mainly seen in the high-dose groups (Betancourt et al, 2007). In a separate study, lower doses of chlorpyrifos were used at an earlier treatment window. Sprague-Dawley rats were given chlorpyrifos daily by oral gavage at 1.5 or 3.0 mg/kg-day on postnatal days 1-6; the expression of neurotrophin growth factors such as NGF and reelin mRNA was reduced in both treatment groups. Low-dose chlorpyrifos exposure during the early postnatal period may therefore alter the expression of neurotrophins that are involved in brain development (Betancourt et al, 2006). Alteration of neurotrophin levels during brain development is associated with behavioral deficits and may be involved in the pathogenesis of certain neurological disorders (Croll et al, 1999; Govindarajan et al, 2006; Tsai, 2005).

10. Behavioral abnormalities after chlorpyrifos exposure. There is evidence that chlorpyrifos may be especially damaging to the developing brain, targeting diverse events in neural development. Effects that are unique to the developing brain include inhibition of DNA synthesis, impaired cell acquisition and differentiation, interactions with neurotrophic factors, effects on cell signaling cascades involved in cell differentiation and alteration in synaptic function. To determine whether these biochemical changes elicit behavioral abnormalities, behavioral studies were also conducted in developing rats. The administration of chlorpyrifos to

pregnant CD-1 mice by oral gavage at 6 mg/kg-day daily on gestational days 14-17 elicited behavioral alterations in pups when studied during the first two weeks after birth. The behavioral changes include reduced motor activity and decreased response to a distress condition (Venerosi et al, 2009). Rats administered chlorpyrifos at 1.0 mg/kg by subcutaneous injection on postnatal days 1-4 exhibited decreased locomotor activity and deficits in coordination skills (Dam et al, 2000). The deficits occurred both during chlorpyrifos exposure and for days after the treatment, indicating both immediate and delayed behavioral abnormalities induced by chlorpyrifos. Another study further confirmed chlorpyrifos-induced behavior alterations (Jett et al, 2001). Two groups of rats were given chlorpyrifos by subcutaneous injection at different developmental stages. The early treatment group was given chlorpyrifos on postnatal days 7, 11 and 15 at 0, 0.3, or 7 mg/kg (17-20 rats per dose group). The late treatment group was treated on postnatal days 22 and 26 at 0, 0.3, or 7 mg/kg (7-8 rats per dose group). The two treatments covered key periods during development, from postnatal day 7 through postnatal day 26 including both preweaning and postweaning stages. Behavior tests were conducted from postnatal day 24 through day 28 for rats from both groups. Rats treated with 7 mg/kg in the early group and 0.3 or 7 mg/kg in the late group showed chlorpyrifos-induced alteration in cognitive function as measured in the Morris swim test. These effects did not appear to be related to cholinesterase inhibition, as there were no cholinergic signs, brain cholinesterase inhibition, or growth impairment in any treatment group. The authors indicate that a deficit in cognitive function in juvenile rats is thus an important functional correlate of the molecular and biochemical effects of chlorpyrifos in the immature brain. OEHHA has used this study and the neurobehavioral effects of chlorpyrifos as supporting information for deriving a chRD, as further discussed below.

11. **Other effects of chlorpyrifos**. Adult male rats of Wistar strain were given chlorpyrifos orally at 7.5, 12.5, or 17.5 mg/kg-day for 30 days. Chlorpyrifos altered testicular function in treated animals; changes include reduced testis weight and sperm counts, decrease in serum testosterone level, degenerative changes in seminiferous tubules, and infertility (Joshi et al, 2007).

Significant weight gain was seen in chlorpyrifos-treated animals (Lassiter and Brimijoin, 2008; Meggs and Brewer, 2007). Pregnant Long-Evans rats were given chlorpyrifos daily by oral gavage at 2.5 mg/kg-day from gestational day 7 to postnatal day 21. Excess weight gain was observed in male offspring when they reached young adulthood. Although no direct link has been established between chlorpyrifos exposure and obesity, the limited animal data raised concern considering the widespread chlorpyrifos exposure among the general population and the high percentage of obesity in the United States (Lassiter and Brimijoin, 2008).

A human study conducted at a Massachusetts infertility clinic showed an inverse relationship between serum estradiol concentration and the level of urine 3,5,6-trichloro-2-pyridinol (TCP), the major urine metabolite of chlorpyrifos and chlorpyrifos-methyl. A reduction in estradiol concentration is associated with an increase in urine TCP level in adult men (Meeker et al, 2008).

IV. Late Arising Deficits in Young Animals After Brief Subtoxic Exposure to Chlorpyrifos During Development.

As discussed above, it is increasingly evident that the developmental neurotoxicity of chlorpyrifos may depend on a variety of mechanisms, rather than reflecting simply the inhibition of cholinesterase. Accordingly, their impact is evident over a wide developmental period. It must be noted that, with postnatal chlorpyrifos exposure, many of the neurotoxic effects appear after a delay. Therefore, there is increasing concern over the long-term neurobehavioral consequences of fetal and neonatal exposure to chlorpyrifos, since the damage may not be evident until a later stage of development. Accordingly, a definitive evaluation of the consequences of fetal and postnatal exposure will require a longitudinal study from early development through adulthood. Some recent studies discussed below addressed this concern.

1. Late arising deficits after postnatal chlorpyrifos exposure. Animals exposed to chlorpyrifos postnatally were examined in the early postnatal period and into adolescence and adulthood. They showed later-emerging, persistent deficits in cholinergic synaptic function and related cognitive behavioral performance. Defects emerged in adolescence or adulthood even in situations where normative values were initially restored in the immediate post-exposure period. For example, chlorpyrifos was given to newborn rats by subcutaneous injection at 1 mg/kg-day on postnatal days 1-4 or at 5 mg/kg-day on postnatal days 11-14, treatments that were devoid of overt toxicity. Spontaneous alternation in the T-maze, locomotor activity in the Figure-8 apparatus and learning in the 16-arm radial maze were tested throughout adolescence and adulthood. Both early and late postnatal chlorpyrifos exposure caused long-term changes in cognitive performance (Levin et al, 2001). In a separate study, chlorpyrifos was given to Sprague-Dawley rats on postnatal days 1-21 by oral gavage at three dose levels: low (1 mg/kg-day on PN1-5, 1 mg/kg-day on PN6-13, and 1 mg/kg-day on PN14-20), medium (1 mg/kg-day on PN1-5, 2 mg/kg-day on PN6-13, and 4 mg/kg-day on PN14-20), and high (1.5 mg/kg-day on PN1-5, 3 mg/kg-day on PN6-13, and 6 mg/kg-day on PN14-20). Working and reference memory was studied for 4 weeks beginning on postnatal day 36. Chlorpyrifos exposure during development elicited long-lasting alterations in working and reference memory in the medium and high dose groups; the deficits persisted into adolescence and adulthood, long after the termination of exposure (Johnson et al, 2009).

The late-arising behavioral deficits in animals exposed to chlorpyrifos postnatally were accompanied by delayed neurotoxic changes in neurochemical indices of cholinergic synaptic activity and in other neurotransmitter systems regulated by cholinergic input (Slotkin et al, 2001, 2002). Animals exposed during the same postnatal stages showed deficits in cholinergic synaptic function as reflected by the changes on choline acetyltransferase (ChAT) activity and hemicholinium-3 (HC-3) binding. The deficits in cholinergic synaptic function persisted into adolescence and adulthood, long after the termination of exposure and well after the restoration of cholinesterase activity. The same postnatal chlorpyrifos exposure also elicited widespread alterations in the catecholaminergic system that continued into adulthood. The content and utilization rates of both dopamine and norepinephrine were altered in multiple brain regions examined.

Developmental exposure to chlorpyrifos also caused long-lasting changes in the serotonergic (5HT) system (Aldridge et al, 2004, 2005a, 2005b). Young rats briefly exposed to chlorpyrifos at an early postnatal stage (postnatal days 1-4, 1 mg/kg-day by subcutaneous injection) showed anhedonia and decreased anxiety in adulthood as evidenced by alterations both in the elevated plus maze test and the anhedonia test. These effects involve serotonergic mechanisms and resemble animal models of depression. The long-term alterations in behaviors were accompanied by alterations in 5HT function, as early postnatal exposure to chlorpyrifos triggered long-term increases in 5HT turnover across multiple brain regions in adulthood. Chlorpyrifos exposure during different developmental stages also elicited long-lasting alterations in 5HT receptors, the presynaptic 5HT transporter and 5-HT mediated signaling pathway. Exposures to chlorpyrifos during development that were not overtly toxic thus elicited lasting alterations of the 5HT system in association with 5HT-related behavioral changes.

As discussed above, alterations in adenylyl cyclase signaling were observed in the immediate post-treatment period of chlorpyrifos. Animals exposed during different prenatal or postnatal periods also showed impairment of adenylyl cyclase signaling in adulthood; significant changes in adenylyl cyclase signaling can be seen in a wide variety of brain regions studied (Meyer et al, 2004).

2. Late arising deficits after prenatal chlorpyrifos exposure. The same dose of chlorpyrifos given prenatally did not produce the same deficiencies in cholinergic synapses as we have seen following postnatal treatment. However, despite the initial sparing, animals exposed prenatally still developed behavioral deficits in adolescence and adulthood, associated with impaired cholinergic function (Qiao et al, 2002, 2003b, 2004; Levin et al, 2002; Icenogle et al, 2004).

Using treatment regimens that lie below the threshold for fetal growth impairment, Qiao et al (2002, 2003b, 2004) identified postnatal deficits in cholinergic activity that persisted into adulthood. Chlorpyrifos was given to pregnant rats on gestational days 9-12 or gestational days 17-20 at 1, 2 or 5 mg/kg-day. Subsequent development of acetylcholine systems was examined and the effects were compared to those on general biomarkers of cell development. Hemicholinium-3 (HC-3) binding to the presynaptic choline transporter, which is responsive to neuronal activity, was markedly impaired. Deficits were again apparent in adolescence and adulthood. Chlorpyrifos also caused late-emerging abnormalities of neural cell packing density, cell number, cell size and neuritic extensions that may represent a contributory factor for cholinergic synaptic dysfunction. Accordingly, the major change elicited by prenatal chlorpyrifos administration appears to be a reduction in cholinergic synaptic function, effects that were demonstrable even at exposure to 1 mg/kg-day, a dose that lies below the threshold for maternal and fetal growth impairment and for inhibition of fetal brain cholinesterase.

Prenatal chlorpyrifos exposure also impaired working and reference memory in adolescence and adulthood (Levin et al, 2002; Icenogle et al, 2004). Although chlorpyrifos has no effects on growth and viability, offspring showed behavioral impairment when tested in adolescence and adulthood. For example, locomotor hyperactivity was discovered in early T-maze and in the elevated plus-maze trials. Changes in the rate of habituation were identified. Impairment in learning and working memory was also demonstrated with the 16-arm radial maze. Scopolamine, a muscarinic antagonist, was used to challenge the animals to determine if

behavioral alterations are related to impaired cholinergic function, the results indicate that otherwise nontoxic prenatal exposures to chlorpyrifos elicit deficits in cholinergic function that influence cognitive performance in adolescence and adulthood.

These findings indicate that the developing brain is adversely affected by chlorpyrifos regardless of whether exposure occurs early or late in brain development, and that defects emerge in adolescence or adulthood even in situations where normative values are initially restored in the immediate post-exposure period. Accordingly, developmental neurotoxicity consequent to fetal or childhood chlorpyrifos exposure may occur in settings in which immediate symptoms of intoxication are absent.

V. Potential Adverse Effect of TCP in Developing Brain.

Trichloropyridinol (TCP), the major catabolic product of chlorpyrifos, was once considered the inactive metabolite of chlorpyrifos. However, Qiao et al, 2001 showed that TCP inhibits DNA synthesis in vitro. The effect of TCP was seen in both neuronotypic PC12 cells and gliotypic C6 cells, indicating that TCP may affect both neurons and glia. TCP has also been shown to inhibit neurite outgrowth, a morphological marker of neural cell differentiation (Das et al, 1999). Most importantly, TCP accumulates in high concentrations in the fetal brain after maternal chlorpyrifos administration and is also found as the major chlorpyrifos residue in children. TCP concentration is about 3-fold higher in the fetal brain compared to adults (Hunter et al, 1999). Thus, additional effects may be contributed by the supposedly “inactive” metabolite TCP to the age-related differences in the toxicity of chlorpyrifos, and considering the higher concentration and longer half-life of TCP compared to chlorpyrifos and chlorpyrifos oxon, even a relatively small effect of TCP in vivo may be dangerous to the developing organisms.

Recommendation of Child-Specific Reference Dose (chRD) for Chlorpyrifos

Based on our review of the existing literature, OEHHA concluded that there are age-related differences in the susceptibility to chlorpyrifos. OEHHA also concluded that both cholinesterase and non-cholinesterase related mechanisms contributed to the differential susceptibility between young and adults. The deficits may be manifested immediately after the exposure, or appear later in life. Young animals are more sensitive to chlorpyrifos compared to adults based on the following findings:

- Quantitative differences in the detoxification of both chlorpyrifos and its metabolite chlorpyrifos oxon between young and adults. The slower removal of the toxic forms of chlorpyrifos in young vs. adults would make children more vulnerable, a factor that should be fully considered in the risk assessment process.
- Chlorpyrifos targets diverse events that are specific to the developing brain. The developing brain is a selective target for chlorpyrifos, a factor that should be fully considered in the risk assessment process. Although it is difficult to quantify neurodevelopmental impairment, numerous research articles provided clear evidence of increased susceptibility of neonates to chlorpyrifos.

- Brief exposure to a subtoxic dose of chlorpyrifos early during development elicits long-term deficits later in life. The low threshold for the adverse effects, the lack of immediate symptoms of intoxication and the long lasting damage make childhood exposure even more dangerous, a factor that should also be fully considered.

I. Neurobehavioral Endpoint

As indicated above, the many neurochemical or neurobehavioral studies have limited dose selections and small sample size. It is difficult to identify a LOAEL or NOAEL from these studies. The routes of exposure in some studies also create uncertainty.

One example is the Jett et al (2001) neurobehavioral study, as described on page 17. Jett et al (2001) studied cognitive impairment in rats that were given subcutaneous doses of chlorpyrifos at two key periods during development, preweaning or postweaning stages. Chlorpyrifos-induced alteration in cognitive function was seen at the dose level of 0.3 mg/kg.

Jett's neurobehavioral study in developing rats covers the vulnerable developmental windows and fits the purpose of school site risk assessment, making it useful for qualitatively identifying an effect of concern. However, the uncertainty associated with the study limits its use for the quantitative calculation of the proposed chRD – extrapolation between routes of administration of chlorpyrifos adds significant uncertainty. Chlorpyrifos is rapidly and well absorbed after oral administration both in humans (70 to 93 percent; Nolan et al, 1984; Griffin et al, 1999) and in rats (90 percent; Bakke et al, 1976). However, orally administered chlorpyrifos will undergo first-pass metabolism prior to reaching the systemic circulation. This is not the case with subcutaneous injection. The quantity and duration of the parent chlorpyrifos, chlorpyrifos oxon, and TCP reaching the systemic circulation by these two routes are not sufficiently clear to make this extrapolation. However, despite its inherent uncertainty with regard to dose, the Jett study still provides valuable supporting information on the developmental neurotoxicity of chlorpyrifos. The developmental neurobehavioral endpoint should be considered in the future when there are more studies available.

II. Calculation of the chRD: Cholinesterase Inhibition Endpoint

Plasma and RBC cholinesterase inhibition are not considered to be adverse effects but are widely used as a surrogate for the toxicity of organophosphate chemicals; they are used by agencies such as U.S. EPA and ATSDR to develop their reference dose for chlorpyrifos.

The 2-year dog study conducted by Dow Chemical Company (McCollister et al, 1974) has good dose selection and data quality. It is accepted by agencies such as California Department of Pesticide Regulation (CDPR) and is a critical study used by U.S. EPA's Office of Pesticide Program to develop their RfD and Population Adjusted Dose, described above. A NOEL of 0.03 mg/kg-day was established based on reduced plasma and RBC ChE activity in the 0.1 mg/kg-day group. While significant reduction of plasma ChE activity was observed at 0.03 mg/kg-day, OEHHA agrees with U.S. EPA, as discussed in its chlorpyrifos re-evaluation document (U.S. EPA, 1999), that these effects were marginal and variable and were not statistically or

biologically significant at all time intervals. Therefore, any inhibition of plasma ChE activity seen at 0.03 mg/kg-day was not considered an effect. The calculation of the non-cancer chRD for chlorpyrifos is as follows:

$$\text{chRD} = \frac{\text{NOEL}}{\text{UF}} = \frac{0.03\,\text{mg/kg/day}}{300} = 0.0001\,\text{mg/kg/day}$$

Where, UF = Uncertainty factor of 300[10 for intra-species variation, 3 for extrapolation from dogs to humans, and 10 as an additional uncertainty factor for children, since young animals were not tested (OEHHA, 2008)] as discussed below.

Selected studies on the effects of chlorpyrifos on cholinesterase inhibition are listed in Table 2. Human studies are currently under review by U.S. EPA. Over 10 guideline studies were conducted in rats, dogs and mice. Among them, the dog is a sensitive indicator species for cholinesterase inhibition by chlorpyrifos (Zhao et al, 2006; U.S. EPA, 2000b; CDPR, 2000). U.S. EPA indicated in their chlorpyrifos reevaluation document that dogs appear to be the most sensitive species for cholinesterase inhibition (U.S. EPA, 2000b). The California Department of Pesticide Regulation stated in its risk characterization document for chlorpyrifos that the dog appeared to be more sensitive to chlorpyrifos than the rat (CDPR, 2000). The NOEL in 90-day and 2-year dog studies was one-third that in the human male as shown in Table 2. While the 20-day human NOEL is uncertain due to the small number of volunteer subjects and the short duration of this single human study, which prevented it from being used as the basis to derive the relevant chRD, the study itself still provided useful information. OEHHA believes that dogs appeared to be more sensitive than rats and possibly as sensitive as humans to the cholinesterase-inhibiting effect of chlorpyrifos; however, due to the limitation of the 20-day human study, uncertainty still exists. OEHHA recommends an interspecies uncertainty factor of 3 rather than the default value of 10, based on a comparison of the NOELs for blood cholinesterase inhibition in dogs, rats and humans.

As indicated above, there are age-related differences in the detoxification of chlorpyrifos; and the decreased levels of enzymes to detoxify chlorpyrifos in young vs. adult raises concerns regarding possible increased sensitivity in children compared to adults. Although available studies only demonstrated age-related differences in chlorpyrifos toxicity after acute exposure, there is uncertainty surrounding chronic low dose exposure to chlorpyrifos. The non-cholinergic mechanisms of chlorpyrifos observed in young animals also raised concern regarding the child-specific sensitivity. An additional safety factor for children is therefore necessary in terms of deriving a chRD (OEHHA, 2008). Due to above concerns, OEHHA applied a 10x safety factor for children [U.S. EPA's Office of Pesticide Program (OPP) applied a 10x safety factor for their Population Adjusted Dose in order to protect children and women who are at the childbearing age].

OEHHA also considered two rat studies as the basis for derivation of the chRD based on the overall data quality and dose selection. The first study is a 2-year rat study (Young and Grandjean, 1988). Fischer-344 rats (60 rats/sex/dose) were treated with chlorpyrifos by diets at 0, 0.05, 0.1, 1, or 10 mg/kg-day for 2 years starting at 6 weeks of age. Plasma and RBC cholinesterase activity was studied (10 rats/sex/dose) at 6, 12, 18, and 24 months, brain

cholinesterase was studied at 12 months (10 rats/sex/dose) and 24 months (20 rats/sex/dose). Chlorpyrifos treatment at 10 mg/kg-day for up to 2 years decreased cholinesterase activity in plasma, RBC, and brain, while 1 mg/kg-day of chlorpyrifos only decreased cholinesterase activity in plasma and RBC. A NOEL of 0.1 mg/kg-day was established based on the reduced plasma and RBC cholinesterase activity. These results are consistent with those of the McCollister et al (1974) rat study as discussed above.

OEHHA used the NOEL of 0.1 mg/kg-day for plasma and RBC cholinesterase inhibition (McCollister et al, 1974; Young and Grandjean, 1988) to develop a chRD. The calculation based on the rat studies is as follows:

$$\text{chRD} = \frac{\text{NOEL}}{\text{UF}} = \frac{0.1\,\text{mg/kg/day}}{1000} = 0.0001\,\text{mg/kg/day}$$

Where, UF = Uncertainty factor of 1000 (10 for intra-species variation, 10 for extrapolation from rats to humans, and an additional factor of 10 for children, since young animals were not tested).

Table 2. Chlorpyrifos cholinesterase inhibition studies

Study	Species	Route	Duration	Endpoint	NOEL (mg/kg-day)	LOEL (mg/kg-day)
Coulston et al., 1972	human	oral	20 days	plasma	0.03	0.1
				RBC	not determined*	not determined*
McCollister et al., 1974	dog	oral	2 years	plasma	0.01	0.03
				RBC	0.03	0.1
Barker, 1989	dog	oral	90 days	plasma	0.01	0.22
				RBC	0.01	0.22
Crown et al., 1985	rat	oral	90 days	plasma	not determined	0.025
Crown et al., 1990	rat	oral	2 years	plasma	0.014	0.35
				RBC	not determined	not determined
Hoberman et al., 1998a,b	rat	oral	developmental neurotoxicity	plasma	not determined	0.3
				RBC	not determined	0.3
Young and Grandjean, 1988	rat	oral	2 years	plasma	0.1	1
				RBC	0.1	1
McCollister et al., 1974	rat	oral	2 years	plasma	0.1	1
				RBC	0.1	1
Szabo et al., 1988	rat	oral	90 days	plasma	0.1	1
				RBC	0.1	1

* Unaffected at any dose examined

III. Conclusion

By this document, OEHHA establishes a child-specific reference dose of 0.0001 mg/kg-day for use in the assessment of risk at proposed or existing California school sites. This benchmark is based on cholinesterase inhibition in dogs and rats and supporting information on cognitive deficiencies in rats. Table 3 compares the proposed chRD for chlorpyrifos with existing health guidance values.

Table 3. Comparison of the proposed chRD with existing health criteria for chlorpyrifos

Organization	Endpoint	Study	Duration & Species	NOEL or LOAEL (mg/kg-day)	Uncertainty factor	Health Criterion (mg/kg-day)
U.S. EPA (IRIS) RfD	plasma ChE	Coulston et al., 1972	20 days human	0.03 (NOEL)	10	0.003
ATSDR MRL	plasma and RBC ChE	McCollister et al., 1974	2 years rat	0.1 (NOEL)	100	0.001
OEHHA chRD	plasma and RBC ChE	McCollister et al., 1974	2 years dog	0.03 (NOEL)	300	0.0001
	plasma and RBC ChE	Young and Grandjean, 1988; McCollister et al., 1974	2 years rat	0.1 (NOEL)	1000	0.0001
U.S. EPA (OPP[1]) RfD & PAD[2]	plasma and RBC ChE	McCollister et al., 1974; Barker, 1989; Crown, et al, 1985; Crown et al, 1990; Hoberman et al, 1998a, b	2 years dog; 90 days dog; 90 days rat; 2 years rat; developmental neurotoxicity rat	0.03 (NOEL)	100 (RfD)	0.0003 (RfD)
					1000 (PAD)	0.00003 (PAD)

1. OPP: Office of Pesticide Programs, U.S. EPA
2. PAD: Population Adjusted Dose (including additional FQPA safety factor = 10 for children and females 13-50 based on age-related difference in cholinesterase inhibition, qualitative difference between dams and adult offspring in the developmental neurotoxicity study, and uncertainties regarding the potential non-cholinergic adverse effects of chlorpyrifos, as described above).

As indicated in Table 3, the current chRD proposed by OEHHA is one-third of OPP's RfD but 3 times OPP's Population Adjusted Dose. OEHHA recommends a factor of 3 for the extrapolation from dogs to humans since dogs appear to be more sensitive than rats and possibly as sensitive as humans for cholinesterase inhibition. OEHHA also considered other rat studies (Young and

Grandjean, 1988; McCollister et al, 1974; Szabo et al, 1988) as the basis for deriving a chRD because of the consistent results observed in these studies.

As with all toxicity benchmarks, the chRD is subject to change if future studies indicate that changes are needed. Since an inhalation reference concentration or an inhalation reference exposure level has not been established, OEHHA recommends that the chRD be used for comparison with exposures from all routes.

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Appendix I: Toxicology Excellence for Risk Assessment (TERA) Comments on Draft

February 11, 2008

Regarding: Proposed child-specific reference dose (chRD) for school site risk assessment - Chlorpyrifos

Integrated Risk Assessment Branch
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Sacramento, California 95812-4010

Dear Colleagues:

We appreciate the opportunity to express our views on this important Agency action. Having conducted numerous noncancer dose response assessments for chemicals of environmental interest for our federal, state, industrial and environmental colleagues, we appreciate the difficulty of addressing controversial issues, such as appropriate literature to cite, judgment of critical effect, selection of critical study, and investigation of attendant uncertainties. As you know, we published a definitive analysis for a chlorpyrifos reference dose (RfD) in 2006 (Zhao et al., 2006). Unfortunately, you only cited our paper in an indirect way. We were most disappointed that our analysis was not given any discussion in yours, especially since we addressed similar issues and came to dramatically different conclusions.

The chlorpyrifos RfD that you propose is deficient in several ways. The first is that OEHHA misses significant literature, for example, on comparative cholinesterase inhibition in maternal, fetal and neonatal animals. Second, OEHHA does not discuss the concept of critical effect, choosing rather to display many toxicities as if they are somehow not related. Third, OEHHA selects an inappropriate critical study upon which to base its estimate of a safe dose. Finally, OEHHA judges uncertainties in its selections of NOAELs as if other data are somehow not available, when in fact the database for chlorpyrifos is replete.

We describe each of these deficiencies below. Also, we attach an unabridged set of comments to our federal EPA colleagues in 1999 on one of their early chlorpyrifos actions. This set of comments includes numerous data analyses that are relevant to your continuing effort. We would be more than happy to share

further analysis, raw data, excel spreadsheets, benchmark dose (BMD) modeling runs, or whatever else you need, and that we have, to conduct these revisions.

Our mission is to protect public health, as is yours. This mission, however, comes with a duty, as described perhaps best by Albert Einstein: The right to search for the truth implies also a duty; one must not conceal any part of what one has recognized to be true. We encourage OEHHA to hold fast to this principal and use the available and extensive information on chlorpyrifos in a scientific appropriate way. Such use will enable credible risk management decisions that ultimately best protects the public's health.

Sincerely,

Michael L. Dourson, Ph.D., DABT, ATS
Director

Bernard Gadagbui, M.S., Ph.D., DABT
Toxicologist

The opinions expressed in this commentary reflect those of *TERA* and do not represent the views of DowAgro Sciences, the sponsor of this work in part. A copy of this commentary was made available to DowAgro Sciences only after emailing to OEHHA.

Missing Literature

Literature Critical to Understanding Children's Health from Chlorpyrifos Exposure

Citing the following literature appears to us to be mandatory for any evaluation of chlorpyrifos toxicity to the young. The bioassay by Mattsson et al. (2000) (bold-printed below) is particularly important, since it compares cholinesterase inhibition in 5 different organs, at multiple times between dams, and their corresponding fetuses and neonates. Furthermore, the study measures levels of chlorpyrifos and a principal metabolite in blood, so that comparisons can be made on the basis of tissue dose (see Table 5 and Figures 1 and 2 of Zhao et al., 2005).

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Zhao, Q., Gadagbui, B., and Dourson, M. (2005). Lower birth weight as a critical effect of chlorpyrifos: a comparison of human and animal data. *Reg. Toxicol. Pharmacol.* 42(1):55-63.

Other Significant Literature For The Dose Response Assessment Of Chlorpyrifos

Reading and perhaps citing the following literature appears to us to be necessary for any dose response assessment of chlorpyrifos. Previous assessments include the work by Clegg and van Gemert (1999), van Gemert et al. (2001), Health Canada (2003), the UK ACP (2003), and WHO (2004),. The dose response human study of Kisciki et al. (1999) on cholinesterase inhibition should also be woven into the current assessment.

ATSDR (Agency for Toxic Substances and Disease Registry). (1997). Toxicological Profile for Chlorpyrifos. U.S. Department of Health and Human Services, Public Health Service.

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Nordstrandt, A.C., Padilla, S., and Moser, V.C. (1997). The relationship of oral chlorpyrifos effects on behavior, cholinesterase inhibition and muscarinic receptor density in rats. *Pharmacol. Biochem. Behav.* 58, 15-23.

Rauh VA, Garfinkel R, Perera FP, Andrews HF, Hoepner L, Barr DB, Whitehead R, Tang D, and Whyatt RW. (2006). Impact of prenatal chlorpyrifos exposure on neurodevelopment in the first 3 years of life among inner-city children. Pediatrics. 118(6):e1845-59.

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Discussion of the Critical Effect

Overview. Neurobehavioral effects are important for chlorpyrifos toxicity, but they are not the critical effect, that is, the first adverse effect, or its known and immediate precursor. Rather, cholinesterase inhibition, and specifically red blood cell (RBC) cholinesterase inhibition, is clearly the critical effect for chlorpyrifos toxicity. Moreover, whereas OEHHA suggests that the subcutaneous injection studies and the 2-year rat studies offered the best basis of the chRD, we demonstrate that human chlorpyrifos studies should be preferred (Zhao et al., 2006). These human studies provide an adequate and consistent picture of chlorpyrifos toxicity, and are consistent with many other world health organization decisions and guideline documents.[1] In addition, we demonstrate that the neurobehavioral effects will be observed only at higher chlorpyrifos oral doses than the NOAEL of 0.1 mg/kg/day (Zhao et al., 2006) reported for RBC cholinesterase inhibition in humans.

Choice of Critical Effect. Two recent publications, Zhao et al. (2005, 2006) determined a new chronic chlorpyrifos RfD, with particular close attention paid to developmental, including neurodevelopmental, toxicity from epidemiology and experimental animals studies, and the incorporation of information from human studies in the dose response assessment. Based on the weight of evidence analysis of the available chlorpyrifos toxicology and epidemiology data, together with issues associated with chlorpyrifos assessment, specifically in dose response assessment, Zhao et al. (2005) established that the most sensitive indicator of effect of chlorpyrifos is inhibition of cholinesterase in target tissues. In particular, these investigators show that red blood cell (RBC) cholinesterase inhibition, as opposed to cholinesterase inhibitions in the plasma or brain, is clearly the critical effect. Zhao et al. (2006) also established that the overall weight of evidence on fetal developmental, including neurodevelopmental, toxicity from animals and humans suggests that this effect does not precede RBC cholinesterase inhibition, the critical effect for chlorpyrifos. Based on their critical analyses of the available data, Zhao et al. (2006) developed chlorpyrifos RfD of 0.01 mg/kg/day, based on a dose of 0.1 mg/kg/day (the NOAEL) that observed no effect on RBC cholinesterase inhibition in human volunteers (Coulston et al., 1972), with supporting data from Kisciki et al. (1999) and Nolan et al. (1984) studies. A defensible composite uncertainty factor of 10 was applied to the NOAEL to arrive at the RfD.[2]

Table 1 in Zhao et al. (2006) lists the critical effects which international organizations or investigators used as the basis of safe doses for chlorpyrifos. All of these groups judge

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[1] It is worth commenting that the USEPA Office of Pesticide Programs was not able to consider the available human data during its deliberations of chlorpyrifos risk assessment due to its then pending decision on the applicability of human data. EPA has since confirmed its policy to use such data, after appropriate review.

[2] The OEHHA text does not acknowledge that Coulston et al. (1972) human study measured RBC cholinesterase inhibition but that the activity was not inhibited at any dose level. Instead, OEHHA incorrectly reports that no NOEL or LOEL was determined for RBC cholinesterase inhibition in the Coulston et al. (1972) study (see Table 2 of the draft chRD for chlorpyrifos report).

that cholinesterase inhibition is the critical effect for chlorpyrifos toxicity. And as OEHHA knows, as long as the RfD is based on the critical effect, neurobehavioral effects are not expected to occur.

In spite of the new analysis (Zhao et al., 2006) and the overwhelming agreement on cholinesterase inhibition as the critical effect, OEHHA does not discuss all the available data on chlorpyrifos toxicity and particularly the work published by these authors. Rather, OEHHA considers that the neurobehavioral effects observed in the subcutaneous study by Jett et al. (2001) are the critical effect upon which the chRD should be based, using the two-year rat studies conducted by Young and Grandjean (1988) and McCollister et al. (1974) as "supporting" studies. Not only is subcutaneous exposure an inappropriate route; it is not relevant for human risk assessment due to its associated potential uncertainties as discussed later in these comments.

***Recent epidemiological studies*.** Epidemiological studies continue to contradict each other with regard to whether or not chlorpyrifos exposure can result in neurobehavioral effects in humans. For example, a recent publication by Rauh et al. (2006) has evaluated the longer-term effects of prenatal chlorpyrifos exposure on preschool behavior and child neurodevelopment during the first three years of life. Using chlorpyrifos levels measured in umbilical cord blood as the dosimetric measure of prenatal exposure, Rauh et al. showed that highly exposed children with chlorpyrifos levels of 6.17 pg/g plasma scored in the range of mental and motor delays by three years of age, compared with children with lower exposures. However, the extent to which these measures of cord blood levels of chlorpyrifos reflect critical exposures throughout pregnancy is not clear. Additionally, Rauh et al. state that they did not have any information about early childhood chlorpyrifos and lead exposure, information that is critical for excluding lead exposure as causative agent. These authors did state that these exposures could affect estimates of dose-response relationship in their study. This again underscores the uncertainty associated with their observations.

Another recent epidemiology study by Eskenazi et al. (2007) also investigated the relationship between prenatal (maternal) chlorpyrifos urinary metabolite (TCP) levels with children's neurodevelopment, using the same tests as used by Rauh et al. (2006). In contrast to these latter investigators, however, Eskenazi et al. (2007) found no association between maternal TCP concentration (average of 3.54 μg/L) and mental development and pervasive developmental problems at 24 months of age. It may be recalled that in an earlier study, Zhao et al. (2005) evaluated *Whyatt et al.* (2004) epidemiological study that reported an association between prenatal chlorpyrifos exposure [using umbilical cord plasma chlorpyrifos levels as dosimetric measure of prenatal exposure] and birth weight and/or length]. This finding raised a concern on whether impaired fetal development could be the critical effect rather than the inhibition of acetyl cholinesterase as had been believed so far. When Zhao et al. (2005) conducted a side-by-side comparison of the Whyatt et al. study and two other epidemiology investigations [Eskenazi et al., 2004; Berkowitz et al., 2003] that also independently investigated the association between exposure to chlorpyrifos and fetal development by using different exposure biomarkers, the association between umbilical cord plasma chlorpyrifos levels and fetal birth weight

decreases observed by Whyatt et al. (2004) did not necessarily establish causality and that results from other available epidemiology studies and animal studies did not support their association. It is not surprising that Eskenazi et al. (2007) study contradicts the Rauh et al. (2006) study that draws its data from the same prospective cohort study used by Whyatt et al. (2004). Overall, there appears to be no evidence that oral exposure to chlorpyrifos can cause neurobehavioral effects at doses below those that cause RBC cholinesterase inhibition.

Some evidence from mice. Some information is available on whether or not prenatal or postnatal exposure to chlorpyrifos can elicit behavioral effects in mice. In a recent study, Venerosi et al. (2006) exposed mice, by gavage, to chlorpyrifos both prenatally (gestation days 15-18, doses 0, 3, or 6 mg/kg) and postnatally (postnatal days 11-14, doses 0, 1, or 3 mg/kg) and used a balanced design to evaluate in female offspring the behavioral effects of prenatal or postnatal chlorpyrifos exposure. These authors also assessed the possibility of synergic/additive or contrasting effects of combined prenatal + postnatal exposure. In this study, when females were four months old, the authors measured social recognition test in which ultrasound vocalizations (USVs) and social investigation behavior emitted by a resident female in the presence of a female partner. Results showed that females prenatally treated with 6 mg/kg chlorpyrifos (the LOAEL) exhibited a marked increase in USVs in the social recognition test. The NOAEL was 3 mg/kg. The authors concluded that developmental exposure to chlorpyrifos induces long-lasting alterations in the social behavior repertoire of the mouse. This study provides additional evidence that neurobehavioral effects are likely to occur at chlorpyrifos concentrations following oral exposure that are higher than those needed to evoke the critical effect, that is RBC cholinesterase inhibition.

Inappropriate Critical Study

***Overview*.** OEHHA's choice of critical study, the Jett et al. (2001) subcutaneous study, is not defensible. Although extrapolation of results from subcutaneous to oral exposure for chlorpyrifos can be scientifically defended if the routes/pathways of metabolism of chlorpyrifos in humans and/or animals and the spectrum of metabolites formed following oral exposures are the same as in subcutaneous exposure, OEHHA does not convincingly demonstrate this. These conditions, among others, must be met before one can reliably conclude that the neurobehavioral effects, observed following subcutaneous exposure to chlorpyrifos, are to be considered to be more sensitive than cholinesterase inhibition found after numerous oral studies.

***Pharmacokinetic Data*.** As OEHHA knows, several regulatory agencies have evaluated the utility of parenteral routes of exposure (including subcutaneous injection). Specifically, the U.S. EPA's OPP reevaluated chlorpyrifos toxicity and has rejected subcutaneous injection as a relevant pathway of human exposure, stating that such dosing regimen cannot be reliably compared to the oral route in the absence of pharmacokinetic data. However, OPP suggested that such studies still provide important qualitative information. OEHHA disagrees with OPP on this conclusion, but does not provide the convincing pharmacokinetic data.

For example, OEHHA argues that the Jett et al. (2001) subcutaneous injection study still provides pivotal information on the developmental neurotoxicity of chlorpyrifos *because* studies in both humans and animals have indicated that chlorpyrifos is well absorbed following oral exposure. In humans, between 70% and 93% of the orally administered dose are absorbed (Nolan et al., 1984; Griffin et al., 1999), while in rats, an average of 90% of the orally applied dose is absorbed (Bakke et al., 1976). OEHHA presumes that chlorpyrifos is similarly well absorbed from the subcutaneous dose in oil. However, the Jett et al. (2001) study does not measure chlorpyrifos or its metabolites in tissues. Thus, comparable time course absorption is not known between the oral and subcutaneous routes.

OEHHA also expects that the first-pass metabolism of chlorpyrifos will transform a large amount of chlorpyrifos into its metabolites. Assuming that the first-pass metabolism of chlorpyrifos is only mediated through its active metabolite chlorpyrifos oxon, OEHHA believes that this metabolism should make chlorpyrifos appear more potent after oral exposure since it makes the oxon available earlier and in higher concentration when compared with subcutaneous injection. However, ATSDR (1997) states that metabolism of chlorpyrifos is rapid and extensive, with the parent compound and the oxon not detected or found only in trace concentrations in blood or urine, except following very high exposures. This clearly shows that chlorpyrifos or chlorpyrifos oxon are not likely to be detected in significant concentrations following oral exposure. This statement is supported by the Nolan et al. [1984] that detected $\leq$30 ng/g of chlorpyrifos in the blood of human volunteers orally exposed to 0.5 mg/kg of chlorpyrifos. From this latter study, it is clear that the subcutaneous administration may likely result in effective chlorpyrifos

levels in blood that are several folds higher than dose levels observed in critical studies for chlorpyrifos that identified cholinesterase inhibition as the critical effect. Because blood levels of chlorpyrifos and its metabolites were not measured in the Jett et al. (2001) study, however, it is difficult to determine with certainty whether such levels were higher or lower than those observed in blood of fetuses or pups that result in cholinesterase inhibition (Mattsson et al., 2000). Additionally, the Jett et al. (2001) subcutaneous injection study did not measure RBC cholinesterase inhibition, the critical effect. This is another shortcoming in the use of this latter study as the basis for a chlorpyrifos RfD.

It is worth pointing out that a recent study in which dams were orally dosed at extremely high concentrations of chlorpyrifos (7 mg/kg/day) on gestation days 14-18 did not detect chlorpyrifos nor its oxon metabolite in the fetal nor maternal brain (Hunter et al. 1999), indicating that the oxon is not likely to be found in significant concentrations in fetal brain following oral exposure.

OEHHA also states that "The amniotic effect of chlorpyrifos may lead to the loss of neural cells, deterioration of brain function, and eventually, behavioral changes." As rightly stated (page 23, the chlorpyrifos chRD Report), "the first-pass metabolism of chlorpyrifos after oral administration may lead to a relatively lower toxicity and higher LOAEL for neurobehavioral effects compared to the subcutaneous injection." This is a huge area of uncertainty that precludes the use of a subcutaneous study to determine the critical effect until appropriate studies are conducted to establish the oral dose levels that are likely to produce neurobehavioral effects. The selection of such a study as the basis of the RfD, then, flies in the face of all previous dose response assessments and demands a higher level of evidence than currently offered.

It is also worth noting that several reviews and studies have shown that the metabolite, 3,5,6-trichloro-2-pyridinol (TCP), is the principal form of chlorpyrifos found in the circulation (ATSDR 1997; FAO/WHO 1999), and not chlorpyrifos oxon. TCP may affect both neurons and glia *in vitro* (page 20, the chlorpyrifos chRD Report). TCP, but not chlorpyrifos or its oxon, was found to accumulate at a concentration 3-fold higher in fetal brain than in adult brain after dams were orally exposed to extremely high concentrations of chlorpyrifos (7 mg/kg/day) on gestation days 14-18 (Hunter et al. 1999).

We presume that subcutaneous injection is considered to achieve 100% bioavailability. Although the bioavailability of chlorpyrifos has not been well estimated following oral exposure in humans or animals, it would be inappropriate to assume that bioavailability following oral exposure is the same (or higher) than that following subcutaneous injection, given that the parent compound and the oxon are not detected or are found only in trace concentrations in blood or urine after oral dosing (ATSDR 1997; Nolan et al., 1984). Again, measurements of tissue concentration in the Jett et al. (2001) study would have been helpful in this regard.

Judging Uncertainties Appropriately

***Overview*.** We believe that OEHHA inappropriately judged uncertainties in its selections of NOAEL, almost as if other data are not available. Zhao et al. (2006) have extensively evaluated the choice of uncertainty factors in their paper and this publication can be consulted for a defensible application of many of these uncertainty factors for developing a safe dose for chlorpyrifos, including the use of the FQPA safety factor. The following text is excerpted from Zhao et al. (2006) on two of these factors.

***Database Uncertainty Factor (UFD)*.** Based on US EPA's non-cancer risk assessment methodology the database for deriving a high confidence RfD should include a minimum two chronic bioassays testing systemic toxicity by the appropriate route of exposure in different species, one 2-generation reproductive toxicity study, and two developmental toxicity studies in different species. The minimal database required for deriving an RfD is a single subchronic bioassay, which includes a full histopathology examination. The database factor is used when a potentially more sensitive health effect may not be identified if the database is missing a particular type of study. This factor may also be used if the existing data indicate the potential for a heath effect, for example, neurotoxicity or immunotoxicity, but this effect is not fully characterized in the available standard bioassays. If the database is complete for deriving a high confidence RfD, a value of 1 is considered appropriate. Otherwise, a default factor of as high as 10 is used.

The database for chlorpyrifos includes a large number of experimental animal studies, including multiple chronic studies in several species (Figs. 1 and 2 of Zhao et al., 2006), numerous shorter-term bioassays, developmental toxicity studies in various species (e.g., Breslin et al., 1996; Deacon et al., 1980; Rubin et al., 1987a,b), and 1- or 2-generation reproduction studies (e.g., Breslin et al., 1991, 1996; James et al., 1988; Mattsson et al., 2000). The database also includes human clinical, epidemiology, and occupational studies. The weight of evidence from all of these studies suggests that inhibition of ChE is the most sensitive effect in all animal species evaluated and in humans, regardless of route or duration of exposure, and humans are no more sensitive to chlorpyrifos than the most sensitive non-human species tested, the dog. Moreover, a recent evaluation of either birth weight decrease or cholinesterase inhibition as a critical effect reaffirmed the latter as being critical (Zhao et al., 2005). Even though chlorpyrifos can cause neurotoxic effect at high dose, preventing the cholinesterase inhibition would protect humans and animals from further neurotoxic effects. Therefore, the overall chlorpyrifos database appears to be complete and any new studies that are done to fine tune our knowledge of the chlorpyrifos mode of action will not likely identify lower points-of-departure than can be estimated from the existing database. An appropriate value for this factor is likely to be 1.

***Use of FQPA safety factor*.** For the purposes of developing an RfD, a concern exists for the toxicity of chlorpyrifos in neonatal and young animals because of their potentially greater sensitivity than adults. This concern has to be focused on the critical effect, cholinesterase inhibition, and not effects of different severities that occur at higher chlorpyrifos doses. This is because one of the basic assumptions of the RfD is that if the

critical effect is prevented, then other more severe effects are prevented as well (Barnes and Dourson, 1988; US EPA, 2002a). Fortunately, a wealth of data and analyses are available on this critical effect in adults, neonatal and young animals. The definitive study for this comparison appears to have been done by Mattsson et al. (2000), who specifically tested cholinesterase activity in five different organs in dams and their fetuses or pups at five different time points, and at three different doses and control. A unique aspect of the Mattsson et al. (2000) work is that they also measured levels of chlorpyrifos and one of its principal metabolites, TCP, in the blood of both the dam and corresponding fetus or pup. Thus, direct comparisons of sensitivity (i.e., toxicodynamics) to the critical effect between these divergently aged groups are possible on a tissue-dose, rather than an administered-dose-specific basis. No other study comparing adult and neonatal chlorpyrifos toxicity has this unique feature.

As analyzed by Zhao et al. (2005) and briefly summarized above, the results of the repeated-dose study of Mattsson et al. (2000) unequivocally show that neonatal and young animals are equally or perhaps less sensitive than adults to the cholinesterase inhibition on a tissue dose and tissue response specific basis. Similarly, BMD analysis of the Zheng et al. (2000) study (Table 3 of Zhao et al., 2006) would suggest that neonatal experimental animals are no more sensitive to repeated exposure to chlorpyrifos than are adults. In reviewing all of this information, our overall judgment is that an FQPA safety factor is not needed (or at least its toxicity component). This is because:

• The critical effect is considered to be RBC cholinesterase inhibition and not brain or plasma inhibition. Our BMD analysis of the acute exposures in the Zheng et al. (2000) study did not show a difference between the neonatal and adult experimental animals for RBC cholinesterase inhibition.

• Our BMD analysis of the repeated exposures in the Zheng et al. (2000) study did not indicate that neonatal experimental animals were more sensitive than adult experimental animals for any cholinesterase inhibition.

• Our toxicodynamic analysis of the Mattsson et al. (2000) study unequivocally shows that neonates are not more sensitive than their mothers to cholinesterase inhibition in five tissues and for multiple time measurements. See footnote 1 for reference to an analysis of the complete dataset.

• Our review of the overall database for chlorpyrifos indicates that a database uncertainty factor is not needed. US EPA (2002b) suggests that an FQPA factor is also not needed when the database factor has been considered.

In summary, we believe that there is compelling evidence that there is no increased sensitivity of infants relative to adults and that the database for chlorpyrifos is complete, thereby warranting the removal of the database and FQPA safety (uncertainty) factors for chlorpyrifos safe dose.

Comments To EPA In 1999 On One Of Their Early Chlorpyrifos Actions

Attached, we reproduce unabridged, and publicly available comments we sent to our colleagues at the US EPA in 1999 on one of their early chlorpyrifos actions with the belief that this may offer insight into the derivation of OEHHA chRD. OEHHA scientists are welcome to any of this information, including the raw data analyses and excel spreadsheets. In addition, OEHHA is welcome to any and all analyses from the Zhao et al. (2005 and 2006) publications.

Appendix II: Dow Agrosciences Comments on Draft

Dow AgroSciences LLC
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11 February 2008

via electronic transmission

Mr. Leon Surgeon
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DOW AGROSCIENCES COMMENTS ON
PROPOSED CHILD-SPECIFIC REFERENCE DOSE (chRD)
FOR SCHOOL SITE RISK ASSESSMENT- Chlorpyrifos

Dear Mr. Surgeon;

Dow AgroSciences LLC is the primary manufacturer and registrant for chlorpyrifos-containing products and appreciates the opportunity to comment on the "Proposed Child-Specific Reference Dose (chRD) for School Site Risk Assessment – Chlorpyrifos – External Draft Report November 2007" (Draft Report). Chlorpyrifos is an important pest management tool for the control of insect pests in California's $30 billion agricultural industry. As we discuss in this cover letter and detailed in the Comments, we have identified several areas of concern with OEHHA's draft document and its conclusions. We request that OEHHA consider these issues and revise the proposed rationale for developing a chRD for chlorpyrifos and, accordingly, revise the proposed chRD. We look forward to our continued interactions with OEHHA on this process.

Dow AgroSciences respectfully contends there is no compelling scientific basis or public health need for the development of a chRD based on the rationale and studies presented by OEHHA and the weight of evidence from studies which adhere to sound toxicological principles and appropriate study design for use in risk assessment. Enclosed please find the attached comments to the draft "Proposed Child-Specific Reference Dose for School Site Risk Assessment - Chlorpyrifos":

- "Dow AgroSciences Comments in Response to: Proposed Child-Specific Reference Dose (chRD) for School Site Risk Assessment – Chlorpyrifos – External Draft Report November 2007" (OEHHA, California Environmental Protection Agency). D.R. Juberg and J.L. Mattson. 11 February 2008. 41 pp.

We would also like to make two additional points on the Draft Report regarding the section "What characteristics make chlorpyrifos of concern pursuant to Health & Safety Code Section 901(g)?" (p. 10-11). As cited in the Draft Report, the June 2000 Memorandum of Agreement with U.S. EPA discontinued domestic uses of chlorpyrifos. Subsequently, the potential exposure scenarios at school sites have been significantly reduced. The table below shows the dramatic decrease in domestic uses since some of the data in this section of the Draft Report were collected.

Pounds chlorpyrifos applied in CA from urban site applications (DPR Pesticide Use Reporting)								
YEAR	1999	2000	2001	2002	2003	2004	2005	2006
LANDSCAPE MAINTENANCE	158,187	13,600	9,087	5,591	4,877	4,728	2,960	2,458
STRUCTURAL PEST CONTROL	526,298	428,917	251,069	103,805	103,099	120,487	69,370	5,325

Secondly, Dow AgroSciences disagrees with the characterization in this section that "Additional problems have now surfaced with chlorpyrifos, as it has been found at National Priorities List (NPL) sites." Such a statement implies that the presence of chlorpyrifos at such sites is either indicative of a public risk or at worst implies it was a causative agent in the site being listed. The National Priorities List is a list of superfund sites prioritized for remediation. To characterize chlorpyrifos as being present at NPL sites, and therefore a "problem," and to use this as part of rationale for establishment of a ChRD is, in our opinion, inappropriate. Furthermore, it misstates the facts regarding chlorpyrifos. Chlorpyrifos is not listed in the current U.S. EPA Superfund Chemical Data Matrix (http://www.epa.gov/superfund/sites/npl/hrsres/tools/scdm.htm), however it is listed (as no longer being listed) in the EPA's Substances No Longer Listed in SCDM (http://www.epa.gov/superfund/sites/npl/hrsres/tools/remscdm.pdf)

Thank you once again for the opportunity to provide comment. Please contact me at blbret@dow.com or Dr. Juberg at drjuberg@dow.com if you have further questions or wish additional information.

Sincerely,

Brian L. Bret

Brian L. Bret, Ph.D.
State Regulatory Manager

Enclosure

Dow AgroSciences LLC

Comments in Response to:

Development of Health Criteria for School Site Risk Assessment Pursuant to Health and Safety Code Section 901(g):

Proposed Child-Specific Reference Dose (chRD) for School Site Risk Assessment – Chlorpyrifos – External Draft Report November 2007 (OEHHA, California Environmental Protection Agency)

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Date:
February 11, 2008

Total Pages:
41

Executive Summary

Evaluation of child sensitivity to chemical exposure for regulatory purposes, in this case, the possible development of a child-specific reference dose, should use the most relevant and robust study available. In the case of chlorpyrifos, the Hoberman developmental neurotoxicity study (DNT) is the only study conducted under global standards and regulatory guidelines (Hoberman (1998a) and supplements 1 (1998b), 2 (1999) and 3 (2000); Maurissen et al., 2000) which would meet the standard required in this case.

Equally important, OEHHA should not rely on toxicology studies that are inappropriate for risk assessment. The studies selected and used to support the proposed chRD by OEHHA have both unusual dosing regimes and/or routes of exposure, characteristics which render these studies inappropriate for use in establishing Reference Doses that are intended to be relevant to actual exposures to humans. Fortunately, there is a wealth of literature on how to judge the merit of studies for consideration in risk assessment. Identification of toxicological principles and guidance on their application have been published by the Society of Toxicology, World Health Organization, International Life Sciences Institute, US Environmental Protection Agency, Organization for Economic Cooperation and Development, and others. A common theme in the guidance publications is the importance of studies that use dose levels and routes of exposure that are relevant to expected human exposures. In addition, dose-related transitions in mechanisms of toxicity are an 'obligate' consideration of risk assessment. The usefulness of pharmacokinetics is recognized.

In June 8, 2000, the USEPA (2000a) risk assessment of chlorpyrifos concluded that a 10X FQPA factor was warranted based upon their particular interpretation of the chlorpyrifos DNT study and supported by publications that created uncertainty in their minds about non-cholinergic mechanisms of toxicity. It is notable that WHO (1999) did not feel additional protection was needed for children, even though a senior USEPA toxicologist (Dr. Penny Fenner-Crisp) participated in the WHO evaluation. Nor did the Australian toxicology review of chlorpyrifos recognize a need for additional protection of children. Both the WHO and Australian toxicologists were more rigorous than the USEPA (2000a) in application of principles of toxicology concerning dose, route, maternal toxicity, and relevancy for risk assessment.

The USEPA position on chlorpyrifos and differential sensitivity for children has shifted since 2000, and the uncertainty factor (i.e., for differential sensitivity) currently embraced by the USEPA is now 1X (from 10X formerly; Organophosphate Cumulative Risk Assessment, USEPA 2002a and 2006). In making this change, the USEPA (2002a) reviewed many studies including several from the laboratory of Slotkin and colleagues, and concluded that there was little evidence of toxicological effects at doses that did not inhibit cholinesterase. USEPA (2002a) also analyzed data from oral gavage of chlorpyrifos to pups and adults and concluded there was no meaningful difference in sensitivity of brain cholinesterase to inhibition from repeated doses of chlorpyrifos. USEPA (2006) reaffirmed these earlier analyses (USEPA, 2002a), and included an analysis of differential sensitivity from differences in PON1 activity, and concluded that PON1 mechanisms of detoxification were dose related and not a significant factor at

environmental levels of exposure. Consequently, USEPA Cumulative Risk Assessment retained the FQPA factor at 1X for chlorpyrifos.

Since 2000, there have been several additional publications on principles of toxicology for risk assessment. There have been additional publications on chlorpyrifos pharmacokinetics and characterization of an important dose-related transition in toxicological mechanism that is central to the question of extrapolation of high-dose data to expected environmental exposures. The UK Advisory Committee on Pesticides, after review of many open literature publications, including several from the Slotkin laboratory, determined in 2003 that no modification of current regulatory values was indicated (UK/ACP, 2003). The NOAEL for chlorpyrifos was 1 mg/kg/day. Chlorpyrifos was re-registered in the EU in 2005.

Scientifically-based regulatory conclusions cannot be achieved without rigorous application of the principles of toxicology as they relate to risk assessment. Sound public policy should not be based on the use of inappropriate data or science.

Dow AgroSciences LLC submits the following document to address issues that are central to the interpretation of the Hoberman (1998a, 1998b, 1999, 2000) chlorpyrifos DNT study and to the question of differential sensitivity of the young.

- Section A. Rigorous application of widely-recognized principles of toxicology is essential to valid conclusions in risk assessment. While we appreciate the expertise within OEHHA on the principles of toxicology and the development of hazard identification documents and risk assessments, as a foundation to our comments we feel it is important for the first section to provide an update of universally accepted principles of toxicology for risk assessment and provide a detailed example of the need for sound application of the principles of toxicology.
- Section B. Age- Related Differences in Detoxification and Cholinesterase Inhibition. The second section is an update on relevant information on age-related and dose-related susceptibility to chlorpyrifos.
- Section C. Non-Cholinesterase Mechanisms of Chlorpyrifos Neurotoxicity. The third section addresses the regulatory significance of non-cholinergic mechanisms and how USEPA (2002a, 2006) and UK ACP (2003) considered whether protection of cholinesterase would protect against non-cholinergic mechanisms of toxicity.
- Section D. Late Arising Deficits in Young Animals During Development. The last section builds upon the previous three sections. A key issue for the USEPA in 2000a was their conclusion that, in the guideline DNT study, mid-dose female pups at 2 months of age had a treatment-related decrease in thickness of the parietal cortex in the absence of any history of inhibition of plasma, RBC or brain cholinesterase. The study director and study pathologist were very explicit that the difference should not be attributed to treatment. No other regulatory agency shared the USEPA 2000a interpretation. Since June 2000, there are key additional data that have been developed including DNT morphometric historical control data from the same laboratory and same scientists which have to be considered when examining the potential significance of this finding.

Dow AgroSciences is confident that if OEHHA evaluates the lack of biological plausibility of a treatment-related effect on the parietal cortex, in the context of the principles of toxicology, the now available morphometric historical control data, the authors' arguments on the lack of biological coherence of the data, and if OEHHA utilizes the additional data on dose-related biology on cholinergic and non-cholinergic mechanisms, that OEHHA will be satisfied that the no-observed adverse effect level in the chlorpyrifos DNT study is 1 mg/kg/day for both dams and pups.

The chlorpyrifos DNT study, properly interpreted, in combination with the current USEPA and previous and current world-wide regulatory reviews of chlorpyrifos, will provide OEHHA with a very strong weight-of-evidence that there is no differential risk of children from the appropriate and labeled use of chlorpyrifos. The NOAEL to derive an RfD for adults and children should be 1 mg/kg/day based upon the chlorpyrifos DNT study, and brain cholinesterase inhibition data from subchronic and chronic toxicity studies of chlorpyrifos. Data from human kinetic studies provide assurance that the potency to inhibit RBC cholinesterase is similar between humans and the animal species studied. Uncertainty factors of 10X for within-species differences in sensitivity, and 10X for between species uncertainty should apply. A resulting RfD of 0.01 mg/kg/day for humans, including children, would be consistent with WHO and the EU.

Introduction

Dow AgroSciences LLC appreciates the opportunity to comment on the proposed child-specific Reference Dose (chRD) for chlorpyrifos and offers the following points as to why it contends there is no scientific basis or public health need for the development of a chRD based on the rationale and studies presented by OEHHA: (1) OEHHA's selection and reliance on *in vitro* studies as the basis for why a chRD should be considered ignore global efforts and consensus on the principles of toxicology and how they should be applied in risk assessment efforts; (2) historical review of all chRDs developed to date show that this is the first time OEHAA has departed from the traditional and standard practice of selecting a study and NOAEL/LOAEL using a relevant route of exposure in deference to one that used a subcutaneous route of administration for which there is no available historical, mechanistic or pharmacokinetic data for a rational interpretation; (3) an in-depth scientific analysis of potential age-related sensitivity (to children) and presence of non-cholinergic effects reveals that there is insufficient basis for concern; (4) there is a clear omission of several whole animal studies that evaluated developmental effects, whose inclusion is mandatory to a weight of the evidence evaluation of chlorpyrifos and developmental concerns; and (5) existing regulatory reviews on the non-cholinergic effects and toxicity of organophosphate insecticides have not been recognized or considered by OEHHA.

Section A. Global efforts to clarify principles of toxicology as they apply to risk assessment

A1. The Principles of Toxicology for Risk Assessment

Chlorpyrifos has been regulated world-wide for several decades. All countries have regulated chlorpyrifos on the basis of inhibition of plasma, RBC or brain cholinesterase, in humans and/or animals. The World Health Organization, European Union, and Australia utilize cholinesterase inhibition data from human chlorpyrifos kinetic/biomarker studies to aid in setting exposure standards. Other regulatory bodies have sometimes ignored the human cholinesterase kinetic/biomarker data on chlorpyrifos. However, ignoring relevant human data creates an elevated perception of interspecies uncertainty that does not exist.

Regulatory conclusions about the safety of chlorpyrifos are dependent on the degree to which an agency applies the basic principles of toxicology for use in risk assessment. The less rigorous the agency is in applying widely recognized standards, the greater the claim of uncertainty and the application of even more uncertainty factors. The World Health Organization, European Union, United Kingdom Advisory Committee on Pesticides, and Australian regulatory agencies have been the most rigorous and transparent in adhering to, and applying toxicology principles in hazard and risk assessment of pesticides.

The problems generated by a lack of rigor in the application of basic toxicologic principles were noted in a publication (Neal and Doull, 1995) by two past-presidents of the Society of Toxicology (SOT), one of the authors (J. Doull) whom is an icon in toxicology. The article criticized toxicologists in general, and federal, state and local agencies specifically, for a "… lack of rigor in application of standard principles for interpretation of toxicology data." They also noted "… toxicology has attracted a number of special interest groups whose concern is not primarily scientific." Subsequent to this article, the SOT convened an "SOT Task Force to Improve the Scientific Basis of Risk Assessment."

Society of Toxicology

In 1998, the SOT sent a Special Issue of the Society of Toxicology Communiqué to all members that summarized the deliberations of the SOT 'Risk Assessment' Task Force. The dissemination of the Communiqué was followed by publication in Toxicological Sciences of the task force deliberations (Conolly RB, Beck BD, and Goodman JI. Stimulating research to improve the scientific basis of risk assessment. Toxicological Sciences 49: 1-4, 1999). Notably, Dr. Goodman is also a past-president of the Society of Toxicology. The main points of the Communiqué and the open literature publication were:

- It is important to use realistic doses and routes of administration of chemicals to avoid generating data that 'raise serious questions of relevance.'
- Toxicologists too often use dose levels and routes of exposure because of their convenience rather than because of their relevance to risk assessment.
- Oral gavage (in corn oil) was used as an example of a convenient but unrealistic route that can cause unrealistic kinetics in the test animal.

- The use of data generated at unrealistic doses or unrealistic routes of exposure can predict risks that "…have little or no relationship to risk in the real world."
- It is important to acknowledge that dose affects mechanism, and it can be expected that mechanisms will change with dose.
- It is the responsibility of the toxicologist to design studies to be relevant to risk assessment.
- "Given that a major, and possible *the* major, application of toxicology data today is protection of the public health via its application to risk assessment, use of routes of exposure and high-dose levels, set primarily for purposes of experimental convenience, should be avoided (Conolly et al., 1999)."

The principles, as enunciated by the SOT, have subsequently been restated several times by scientific and governmental organizations. The main additions to the SOT principles have been a call for the application of scientific expertise in data interpretation and the use of statistical analyses only as tools and not as determinants of treatment-related effects. That is, the toxicologist is responsible for integrating the biological information from multiple sources, looking for patterns and coherence of data across doses and across endpoints that are logically related. The SOT recognition that dose affects mechanism has been developed to the point that evaluation of dose-related transitions in mechanism are considered an essential part of interpreting data for risk assessment.

World Health Organization

In 1999, the World Health Organization published "Principles for the Assessment of Risks to Human Health from Exposure to Chemicals" (WHO/IPSC, 1999). They stated clearly that the studies most relevant to 'hazard identification for risk assessment' are those that use a route of exposure that is similar to that of humans. They also spoke to the need for several dose levels to identify dose-response information 'relevant to hazard identification.'

In 2001, the World Health Organization published "Neurotoxicity Risk Assessment for Human Health: Principles and Approaches" (WHO/IPSC, 2001). Their recommendations were similar to those stated above, speaking to the importance of using doses, routes, and durations that reasonably approximate human exposure. They also spoke to the importance of using available pharmacokinetic and dynamic data.

U.S. Environmental Protection Agency

In 2002, the USEPA published "A Review of the Reference Dose and Reference Concentration Processes" (USEPA, 2002b). They speak to the need to characterize databases for possible human effects in the context of "dose, route, duration, and timing of exposure." They stated that the most appropriate route of exposure is the route for which an evaluation is to be made. They emphasized the 'weight-of-evidence' approach that "requires a critical evaluation of the entire body of available data for consistency and biological plausibility." Importantly, the study should be evaluated for possible alterations in metabolism at higher exposure levels.

The Importance of Dose in Affecting Mechanism of Toxicity

Slikker et al. (2004) published the consensus conclusions from two workshops sponsored by the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) on the impact of dose-dependent transitions on the risk-assessment process. Co-sponsors were the ATSDR, American Chemistry Council, NIEHS, SOT, and USEPA.

- A 'transition' was described as a "change with increasing dose in key underlying kinetic and/or dynamic factors that influence the mechanism responsible for the observed toxicity, resulting in a change in the relationship of the response rate as a function of dose."
- "A transition usually occurs over a range of doses, and reflects a continuum of change, rather than a single point of departure."
- "The demonstration and characterization of a dose-dependent transition should influence the evaluation of data both above and below the transition." p.204.
- "… consideration of dose-dependent transitions in the mechanism of toxicity is an obligate example of integrating the "best science" into the decision making process." p.221.

OECD

In 2006, the Organization for Economic Cooperation and Development (OECD) published a draft for an updated Guideline 426, Developmental Neurotoxicity Study (OECD, 2006).

- The route of administration of the test substance should be that most relevant to potential human exposure.
- Data interpretation: Importance of expert judgment. Weight of evidence. Patterns of behavior. Relationships between behavior and morphology. Dose response.
- Statistics: Tools that guide interpretation. Not a sole determinant for presence or for absence of a treatment-related effect.

Considerations When Interpreting/Using Neurodevelopmental Endpoints/Outcomes

Tyl et al. (2007) published a report from the ILSI research foundation/risk science institute expert panel on neurodevelopmental endpoints. Neurotoxicol. Teratol. (e-publication).

- "The goal of this manuscript is to provide guidance to researchers and reviewers of DNT studies on the interpretation of the data generated in these studies." p. 2.
- Biological relevance must consider all available data: historical controls, positive controls, offspring toxicity, effects in offspring in relation to maternal toxicity, …
- Dose, route, vehicle: Consider pharmacokinetics, mode of action, other toxicity studies, data regarding known or expected human exposure.
- The concept of olfactory sensations was raised in context of husbandry. This concern should realistically be expanded to include the olfactory effects of test

materials. For example, those agents that contain sulfur molecules would be expected to have high likelihood of olfactory influences on behavior, particularly on maternal/neonatal interactions. If odor affected behavior, it will affect neurochemistry.

- Interpretation of treatment-related effects to include evaluation of the shape of the dose-response curves and concordance (or lack thereof) between genders, across time points, and across parameters that are logically related.
- Historical control data "can be used to determine whether the results for treated animals are well within the range of historical control values [31,46,49,58], which may indicate that the differences from concurrent control values may be unrelated to treatment." p. 5.
- Maternal vs offspring toxicity: "Since the offspring are dependent on the mother throughout gestation and lactation until weaning, maternal toxicity can be very detrimental to the pups' growth and survival and can potentially affect nearly every endpoint." p. 27.
- "The kind and timing of maternal toxicity and the degree of the effect are important in trying to interpret the role, if any, of the maternal effects on the offspring." p. 28.
- Section 4. Conclusions and recommendations [there are 12]. p. 28.

  … #6. "Though statistical significance is a powerful tool for evaluating toxicological data, it is just a tool that should be used in conjunction with an evaluation of biological relevance and scientific judgment. In this context, a modest difference from control that is not statistically significant may still suggest a relationship to treatment (e.g., if it occurs in a dose-related manner, in both sexes, and in conjunction with other DNT effects or with evidence of other types of toxicity). Contrarily, a modest difference from control that is statistically significant but inconsistent with a pattern of effect (i.e., does not occur in a dose-related manner and is not accompanied by any other DNT or toxic effects) may be considered an incidental finding that is unrelated to treatment." p. 28-29. …

  … #8. "Evaluate the biological relevance of neurodevelopmental findings in the context of other available data, including historical and positive controls, offspring and maternal systemic toxicity, and other toxicity data." p. 29.

OECD

In 2007, OECD published a draft Guidance Reproductive Toxicity Testing. OECD Environment, Health and Safety Publications #43 (OECD, 2007).

- The need for scientific judgment.
- Pharmacokinetic/dynamic profiles important in design, dose selection, data interpretation and data extrapolation.
- Section 64. "Statistical significance does not need to be present to validate the biological significance of treatment-related effects." … "In the same way,

statistical significance does not necessarily signify biological significance, and scientific judgment and relevant historical control data should be used to distinguish between fortuitous and real findings."

- Section 65. Points out that the value of historical control data depends on the similarity of experimental circumstances to the study in question (date, laboratory, personnel, species, strain, source, age, vehicle, route, etc.).
- Sections 181 and 186 state that a treatment-related effect is indicated by both a dose-response trend and a statistically significant effect.

The Challenge in Relying on Studies Not Designed for Use in Risk Assessment

In 2004, Slotkin described his views on differences between academic toxicology and regulatory toxicology (Slotkin, 2004). In academia, Slotkin stated a primary emphasis was 'novelty' of findings, publication in top journals, obtaining current funding and opening pathways to funding in the future. In contrast, Slotkin stated:

"Practical issues that are critical to standardized testing are de-emphasized, such as …"

- [De-emphasize] "pharmacokinetics/toxicokinetics".
- [De-emphasize] "matching of routes of exposure to those of humans".
- [De-emphasize] "development of biologically-based dose response models of established hazards".
- "In that sense, the academic approach is entirely deficient in those attributes that are necessary components of the application of research findings to regulatory science." (p. 633)."

In summarizing, there clearly exists, on a global scale, scientific unanimity on the need to recognize and apply toxicological principles in risk assessment settings, particularly the need to consider dose and route in experimental studies when extrapolating to humans.

OEHHA, in its derivation of a proposed chRD for chlorpyrifos, has not adhered to this global perspective and guidance and has relied almost exclusively on *in vitro* studies as the basis for the developmental concern, a fact which not only is at odds with global guidance on the inappropriateness of such, but also ignores the wealth of existing animal studies, many of which were not cited or discussed by OEHHA.

A2. An example from the chlorpyrifos literature that shows the importance of principles of toxicology (Jameson RR, Seidler FJ, and Slotkin TA. Nonenzymatic functions of acetylcholinesterase splice variants in the developmental neurotoxicity of organophosphates: Chlorpyrifos, chlorpyrifos oxon, and diazinon. Environ. Health Perspect. 115:65–70, 2007).

Chlorpyrifos has been extensively investigated by a wide array of techniques using a variety of standardized and novel approaches. A recent paper from the laboratory of Slotkin and colleagues (Jameson et al., 2007) is a good example of a study where the

principles of toxicology for risk assessment were either not considered or were weakly applied. Universally accepted principles of toxicology as described above include:

- The toxicologist is responsible for proper design of a study for risk assessment.
- Dose and route of exposure for the convenience of the investigator should be avoided.
- The study should match as closely as possible the expected routes of exposure.
- If the route of exposure is not realistic to expected exposure, then pharmacokinetics can be helpful to bridge from route to route.
- Multiple dose levels should be used to define dose response and no-observed-adverse effect levels.
- The possible role of the vehicle on kinetics and the study results should be understood.
- The relationship of dose to dose-related transitions in mechanisms is important.
- The conclusions should flow from the data.

Design of the *in vitro* portion of the study - utility for risk assessment?: Drs. Jameson, Seidler and Slotkin (2007) evaluated the expression of mRNA for AChE-R and AChE-S isoforms of AChE as markers of developmental neurotoxicity in PC12 cells and *in vivo*. The *in vitro* methods (p. 66) indicate that the culture RMPI-1640 medium was initially supplemented with horse and fetal bovine serum, and then the medium was replaced with new medium with Nerve Growth Factor and chlorpyrifos added, but no mention is made of adding horse or fetal bovine serum to the fresh medium when chlorpyrifos was added. The concentrations of chlorpyrifos in the culture medium were very high (30 uM), and Slotkin's laboratory (Qiao et al., 2001) has previously shown the importance of physiological levels of protein for mitigating *in vitro* toxicity of chlorpyrifos. These *in vitro* protective effects of physiological levels of protein have been further explored and confirmed by Geter et al., 2008. The very high concentrations of chlorpyrifos *in vitro* by Jameson et al., especially if protein was removed from the medium, compromise toxicological interpretation of these data.

Issues of study design for risk assessment - Considerations of route of exposure and dosing vehicle. Chlorpyrifos (CPF) and diazinon (DZN) were the test organophosphates. Our discussion will focus on CPF results and interpretation. The Jameson et al. study used the customary experimental design employed by Slotkin and colleagues. CPF at 1 mg/kg/day was injected subcutaneously in DMSO at 1 mL/kg into pups on post-natal days 1-4 (four injections over four days). The *in vivo* studies from Slotkin's laboratory, like the Jameson et al. study, used subcutaneous injection of chlorpyrifos in DMSO with an assumption of rapid and complete absorption, but no data are provided to test this assumption.

The assumption of rapid and complete absorption is not supported by recently generated data. Marty et al. (2007) evaluated the kinetics of several oral-gavage dosing strategies in 5-day old pups, and also evaluated pups injected subcutaneously with chlorpyrifos at 1 mg/kg/day (in DMSO 1 mL/kg). Because the kinetic profile from subcutaneous injection

in DMSO did not show evidence of a high level of absorption, a subsequent study examined the absorption and distribution of radiolabeled chlorpyrifos using the same route, dose and vehicle in an attempt to clarify the issue of the missing chlorpyrifos. Radiolabeled chlorpyrifos that was administered by subcutaneous injection in DMSO showed that more than half the radiolabel remained at the injection site after two hours, and about 40% was in the carcass (i.e., not in blood, heart, brain, liver, or at injection site). Four percent of radiolabel was measured in the liver, and less than 0.5% in the brain. The point here is that effects, previously attributed to chlorpyrifos following exposure via subcutaneous injection, may have little to do with chlorpyrifos itself, but rather may be influenced by dosing vehicle, local response (i.e., site of injection), and peripheral injury, among other factors that have to be considered.

Current PBPK models do not fit the subcutaneous/DMSO data. The biological fate of the chlorpyrifos 'depot' was not determined by Marty et al. (2007), as measurements did not extend beyond two hours (based upon an expectation of rapid absorption). The chlorpyrifos PBPK/PD model was used to attempt a simulation of the chlorpyrifos and TCP kinetics following subcutaneous/DMSO injection, but the PBPK model did not reasonably simulate the data "… without substantial optimization of model parameters (data not shown)."

Evaluation of possible non-chlorpyrifos CNS consequences of subcutaneous injection of chlorpyrifos in DMSO has not yet been conducted. The likelihood of a subcutaneous depot of chlorpyrifos from four daily injections, in context of chlorpyrifos as a recognized mild irritant when applied to the surface of the skin, raises a number of questions that need to be evaluated when assessing associative relationships between chlorpyrifos and reported effects. There is a growing literature on biochemical changes in the brain that occur from stimuli and cytokines from peripheral irritation and injury. In addition, there is a well established and growing literature on the biological and toxic effects of DMSO itself, at dose levels comparable to 1 mL/kg as used by Slotkin and associates. Then there is the known ability of DMSO to form interactions with pharmaceuticals. Interactions between test agent and DMSO cannot be controlled by having only a DMSO control group. Thus, at this point in time the mechanism for changes in neurochemistry and behavior that result from subcutaneous injection of chlorpyrifos (in 1 mL/kg DMSO) are unknown, but the recent data point to the wisdom of applying the principles of toxicology for risk assessment. There is no scientifically rational way that data from subcutaneous injection of chlorpyrifos in high doses of DMSO can be considered relevant to risk assessment until this host of questions is adequately addressed.

Apparent inaccuracy in citing cholinesterase inhibiting potential of the subcutaneous/DMSO method. (From Jameson et al., Methods): "This CPF treatment and the higher dose of DZN produce neurotoxicity in developing rat brain while eliciting <20% AChE inhibition …". In contrast to this statement, another publication from Slotkin's laboratory by Dam et al. (2000) demonstrated that a single subcutaneous injection in 1-day old pups of chlorpyrifos at 1 mg/kg in 1 mL/kg of DMSO, at 2 hr post injection, caused up to 60% inhibition of brain cholinesterase with less inhibition in female pups (data in Dam et al., Fig. 4). At 24 hr after the last of four injections, brain ChE was reported from Slotkin's laboratory (Song et al., 1997) to be nearly 25%

inhibited. It appears that a subcutaneous dose of chlorpyrifos at 1 mg/kg/day (in DMSO 1 mL/kg) can cause appreciably more than 20% inhibition of brain cholinesterase in post-natal day 1-4 pups.

Incomplete description of the data: Jameson et al. state in their results: "Daily treatment of neonatal rats with 1 mg/kg CPF evoked slight increments in AChE-R expression in brainstem and fore-brain of male rats (Figure 4A), an effect that did not achieve statistical significance (no significant main treatment effect or interaction of treatment x region)." … "Similarly, for AChE-S (Figure 4B), DZN evoked significant increases in expression at either 0.5 or 2 mg/kg ($p < 0.05$ and $p < 0.003$ for main treatment effects), but the effects for CPF were insufficient to achieve statistical significance…". These statements focus on male rats and leave the impression that CPF produced the expected results, an increase in mRNA for AChE-R and AChE-S, but not at a statistically significant level.

If one examines Figure 4A (*in vivo* AChE-R results) a different potential interpretation emerges. For chlorpyrifos, the percent change from control values in AChE-R showed small increases in males that were matched in magnitude by decreases in females (brainstem and forebrain AChE-R). Visual inspection indicates the average AChE-R across both sexes and both areas of brain would be near zero difference from control. There were no significant ($p = 0.05$) main effects of treatment, no sex-by-treatment effects, nor a treatment-by-brain region effect.

Fig. 4B depicts the AChE-S data. For chlorpyrifos, the male brainstem AChE-S was about 5% above control, the male forebrain about 8% above, female brainstem about 1% below control, and female forebrain about 2.5% above control. These differences from control were not statistically significant for any comparison (treatment, treatment-by-sex, treatment-by-brain region). Overall, it is scientifically inappropriate to conclude that mRNA for AChE-R or AChE-S was affected by a challenging, subcutaneous, four-day treatment regime of CPF in neonatal rats.

Inaccurate characterization of positive findings relative to chlorpyrifos: (From Jameson et al., Discussion): "The present results in developing neuronotypic cells *in vitro* and in neonatal rat brain regions *in vivo* indicate that, during development, exposures to OPs instead elicit a pattern associated with progressive neurotoxicity, namely co-induction of both AChE-R and AChE-S (Cohen et al. 2002; Perrier et al. 2005; Shohami et al. 2000; Sternfeld et al. 2000); more specifically, our in vivo findings indicate that this pattern emerges in the developing brain even with lower, nonsymptomatic exposures. Equally important, our results support the idea that the increases in AChE expression—especially that of AChE-S, the critical factor that determines the balance between repair and neurotoxicity — are unrelated to the ability of the OPs to inhibit catalytic activity." p. 68.

This statement, for CPF, is simply wrong for the mRNA *in vivo* results, and misleading relative to the degree of CPF-induced inhibition that this same laboratory has reported for this specific treatment regime.

Inappropriate linkage of diazinon findings to previous chlorpyrifos studies: (Jameson et al., Discussion): "Finally, if the expression pattern of AChE variants plays a role in the developmental neurotoxicity of OPs, then, based on our findings, it would be expected that males would be affected to a greater extent by the DZN in vivo treatment regimens examined here. Indeed, this prediction is consistent with earlier results for effects of CPF

on neuronal structural proteins (Garcia et al. 2003), for long-term changes in central and peripheral nervous system synaptic function (Aldridge et al. 2004; Meyer et al. 2004), for structural abnormalities such as cortical thinning (Byers et al. 2005), for tests of cognitive performance (Aldridge et al. 2005; Levin et al. 2002), and for locomotor activity (Dam et al. 2000)."

Thus, this publication by Jameson et al. (2007) ends with an inappropriate juxtaposition of CPF results and DZN results. A critical examination of the *in vivo* CPF mRNA expression data indicates that no meaningful changes occurred. The magnitude of CPF differences from control for AChE-R and AChE-S were small and inconsistent, sometimes in opposite directions, and not statistically significant in any measure. In spite of the lack of positive findings for chlorpyrifos, Jameson et al. stated that the pattern of mRNA AChE-R/AChE-S findings from DZN were consistent with their laboratories earlier results with CPF. In reality, the chlorpyrifos mRNA AChE-R/AChE-S findings in this study did not support their laboratories earlier results with CPF.

Broader implications of no positive findings for CPF. If Jameson et al. (2007) had applied the principles of toxicology to their evaluation of the data, their conclusions would almost certainly have been different. From the lack of a measurable induction of AChE-R and AChE-S from chlorpyrifos at 1 mg/kg/day injected subcutaneously in 1 mL/kg DMSO from post-natal days 1-4, it would be reasonable to conclude there was no measurable injury-based response in the brains of these pups. If this method of screening for neurotoxicity is to have merit, the lack of AChE-R and AChE-S effects in pups after subcutaneous exposure to chlorpyrifos, at doses high enough to cause 25 to 60% inhibition of brain ChE (i.e., reported in other studies), should provide clear impetus for the need to rectify these dichotomous findings and exert caution when evaluating and interpreting neurotoxicity studies involving chlorpyrifos.

Section B. Age-related Differences in Detoxification and Cholinesterase Inhibition

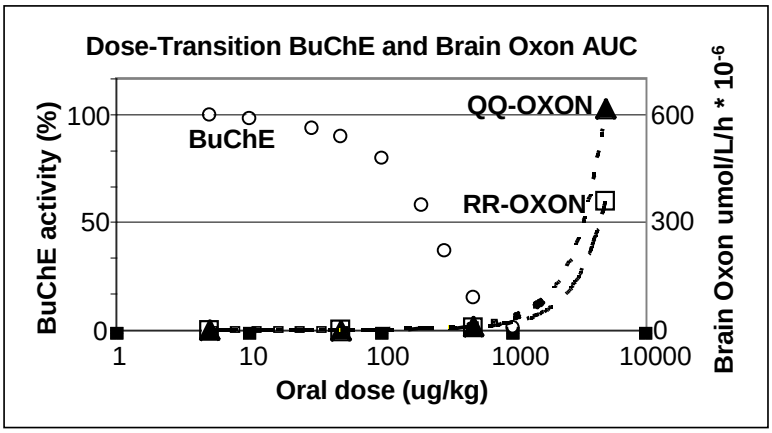
Because the very essence of the proposed chRD for chlorpyrifos is based on putative "child-specific physiological sensitivities" (OEHHA, 2007), it is relevant to evaluate studies and data that exist which enable a 'weight of the evidence' review and conclusion on this point.

PON1 (Chlorpyrifos-oxonase), a high-dose, dose-related mechanism of detoxification.

Recent data indicate that genetic differences in human PON1 activity and detoxification of chlorpyrifos have no practical significance in the real world. PON1 has a modest role in detoxification of chlorpyrifos at very high doses, and no apparent role at environmentally relevant doses. Chlorpyrifos detoxification is layered, and mechanisms independent of PON1 are operational at environmentally relevant doses (Timchalk et al., 2002; Cole et al., 2005). The importance of factoring dose-related transitions in mechanisms of toxicity into risk assessment has recently been emphasized by two ILSI-sponsored workshops (Slikker et al., 2004):

> "… consideration of dose-dependent transitions in the mechanism of toxicity is an obligate example of integrating the "best science" into the decision making process." p.221.

An article by Timchalk et al. (2002) demonstrated, with PBPK modeling, the role plasma butyrylcholinesterase (BuChE) has as a dose-related mechanism for chlorpyrifos toxicity. Substantial amounts of plasma BuChE must be inhibited before significant increases in brain oxon exposure occurs. The single oral dose necessary to inhibit substantial BuChE is above 500 ug/kg. The article also demonstrated that PON1, and genetic Q versus R differences in PON1, have little influence at doses below 500 ug/kg on exposure of the brain to oxon. The text figure combines data on BuChE inhibition (visual BuChE estimate) from Figure 2 and brain oxon AUC data from table 2 of Timchalk et al. (2002). The curves linking the four data points for QQ-oxon AUC or the four data points for the RR-oxon AUC were accomplished by a polynomial fit solely to assist the reader track the AUC data across doses. The polynomial is not intended to describe the dose-response between 500 and 5000 ug/kg doses.



The USEPA 2006 Organophosphate Cumulative Risk Assessment applied two principles of toxicology for risk assessment when considering the role of age-related differences in PON1 activity. The USEPA 2006 first considered the fact that the role of PON1 as an oxonase was dose-related (consistent with both Conolly et al. 1999 and Slikker et al., 2004), and recognized the purpose of the risk assessment was the protection of children from environmental levels of OP exposure:

USEPA, 2006. b. Intra-species extrapolation (Section I.B - Page 55 of 522).

"Interpreting the variability in enzyme levels in the context of increased sensitivity to OPs needs to be done cautiously. Timchalk et al. (2002) used a physiologically-based pharmacokinetic model (PBPK) model for chlorpyrifos to evaluate the impact of variability associated with chlorpyrifos-oxonase polymorphisms on the theoretical concentrations of chlorpyrifos -oxon in the human brain over a range of chlorpyrifos doses. The authors reported that over a range of dose-levels, the response was relatively insensitive to changes in oxonase activity at low doses. However, chlorpyrifos-oxonase status may be an important determinant of sensitivity with increasing dose. The authors further suggest that other esterase detoxification pathways may adequately compensate for lower chlorpyrifos-oxonase activity; hence an increased sensitivity to low chlorpyrifos-oxonase is not observable until other detoxification pathways or esterases have been appreciably depleted or overwhelmed." … "For risk assessment purposes, human responses at low, environmental levels are the most relevant." … *"In conclusion, the standard 10-factor for intra-species extrapolation has been applied to the OP CRA."*

The dose-related role of PON1 in chlorpyrifos detoxification was confirmed by Cole et al. (2005) in genetically-modified mice. The data in Cole et al. also demonstrate that, even at very high dermal doses of chlorpyrifos in mice (which have very thin skin compared to human skin), that tolerance to chlorpyrifos exposure was high even in the absence of PON1.

Cole et al. modified mice to express normal levels of human PON1 of either $hPON1_{\mathbf{R192}}$ or $hPON1_{\mathbf{Q192}}$. The activity of $hPON1_{\mathbf{R192}}$ is slightly greater against chlorpyrifos-oxon than $hPON1_{\mathbf{Q192}}$. Other mice were PON1 knockout, and expressed no PON1 activity. The <u>mice with no PON1 activity</u> (knock-out $PON1^{-/-}$ mice) that were exposed dermally to high doses of chlorpyrifos had practically no difference in brain ChE inhibition compared to genetically-modified mice that had active expression of normal amounts of human PON1 (Cole et al., 2005, Fig. 4). There was minimal inhibition of brain ChE in $PON1^{-/-}$ mice, or mice expressing the more active human $hPON1_{\mathbf{R192}}$ or the less active human $hPON1_{\mathbf{Q192}}$, at a 50 mg/kg dermal dose. In Cole et al. the dermal NOAEL approximated 50 mg/kg regardless of PON1 presence or absence. For comparison, the USEPA short-term dermal NOAEL is 5 mg/kg/day, based on rat dermal exposure data.

The chlorpyrifos dermal dose necessary to inhibit 50% of brain ChE in Cole et al. was about 100 mg/kg. At the ED50, there was less than a 20% difference between those mice with no PON1 activity and those mice with normal amounts of human PON1 activity.

The data of Cole et al. (2005) are consistent with computer modeling of PON1 position 192 Q/R differences and dose-response (Timchalk et al., 2002). Physiologically-based pharmacokinetic/pharmacodynamic (PBPK/PD) modeled differences in PON1-192 Q/R activity (QQ, QR or RR genetics) had practically no effect on estimates of brain oxon exposure when oral exposures were below 500 ug/kg/day (text and text figure above).

CDC estimates of children's exposure to chlorpyrifos are very low

For an exposure context, CDC scientists (Barr et al., 2005) estimate the chlorpyrifos 95 percentile exposure of children is 0.06 ug/kg/day. Thus, environmental exposures are several thousands of times less than the dose necessary to begin to discern small differences in PON1 effects on chlorpyrifos detoxification. When PON1 differences in brain oxon did occur at oral 5000 ug/kg, the brain oxon differences were less than 3X (Timchalk et al., 2002). Cole et al. (2005) and Timchalk et al. (2002) stated the PBPK/PD model predicts that lower-level exposures have other esterase detoxification pathways that would compensate for the inter-individual differences in chlorpyrifos-oxonase activity due to the PON1-Q192R polymorphism.

In summation, PON1 has a modest role in detoxification of chlorpyrifos at very high doses, and no apparent role at environmentally relevant doses. Chlorpyrifos detoxification is layered, and mechanisms independent of PON1 are operational at environmentally relevant doses.

Lack of differential sensitivity of the fetus during maternal exposure

Consistent with the work of Timchalk et al. (2002) and Cole et al. (2005), a USEPA study by Lassiter et al. (1998) demonstrated that the rat fetus had slightly less inhibition of brain cholinesterase than their dams when their dams were administered chlorpyrifos on gravid-day 18 by single-dose oral gavage at both 7 and 10 mg/kg body weight. At these same dose levels, maternal exposure on gravid-days 14 to 18 caused much greater maternal brain cholinesterase inhibition than fetal brain cholinesterase inhibition. The greater tolerance of fetal than maternal brain cholinesterase to inhibition from maternal gavage exposure was again demonstrated by Mattsson et al., 2000.

Lack of differential sensitivity of cholinesterase of the neonate exposed via nursing of milk from treated dams

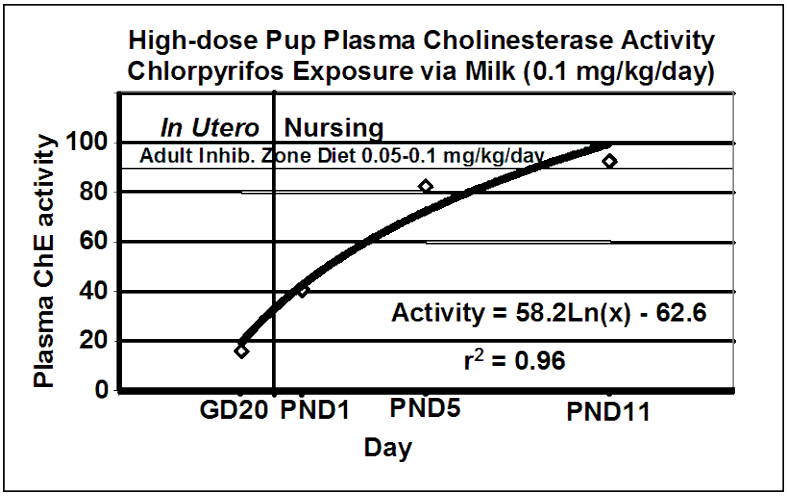
The only data on sensitivity of neonatal rat pups to chlorpyrifos from a natural route of exposure was evaluated in Mattsson et al., 2000. This evaluation of maternal, fetal and neonatal chlorpyrifos kinetics was the 'companion' study to the chlorpyrifos developmental neurotoxicity study (Hoberman, 1998a and supplements 1998b, 1999, 2000; Maurissen et al., 2000). Dams were treated from gravid-day 6 to lactation-day 10 by gavage (in oil) at 0, 0.3, 1 and 5 mg/kg/day. Maternal, fetal or pups cholinesterase activity was evaluated on gravid-day 20, and post-natal days 1, 5, 11, and 21 (birth = PND 0). Fetal and pup cholinesterase inhibition occurred only at the high maternal dose, and the amount of inhibition was less than in dams.

Chlorpyrifos concentrations in milk were measured, and by integration of blood pharmacokinetic information and published algorithms on milk consumption, an estimate of pup dose from nursing was determined (Mattsson et al. 2000). On post-natal days 1-11, pups of high-dose dams were exposed to approximately 0.1 mg/kg/day of chlorpyrifos via milk. During post-natal days 1-11, brain and plasma cholinesterase activity returned to or very close to control values. RBC cholinesterase recovered more slowly, presumably due to the different mechanism for recovery of RBC cholinesterase activity by replacement of RBC in circulation. At this 0.1 mg/kg/day dose level, differential sensitivity is best addressed by examination of plasma cholinesterase activity

as this is the most sensitive to inhibition by chlorpyrifos.

The following information is presented in the next text figure. The kinetic principle involved in the following analysis is that different doses above the threshold for inhibition of cholinesterase will still cause measurable inhibition, but at a different percentage according to dose. The most meaningful adult comparison to pup exposure from milk would be adult exposure to chlorpyrifos by diet. Subchronic and chronic dietary doses of chlorpyrifos to adult rats causes roughly 10% inhibition of plasma cholinesterase at 0.1 mg/kg/day, and no inhibition at 0.05 mg/kg/day (Yano et al., 2000). In Mattsson et al. (2000), gravid-day 20 fetal plasma cholinesterase activity was about 15% of control values in the 5 mg/kg/day maternal dosing group. The dose in mg/kg/day to the fetus is unknown. When born, the estimated dose to the high-dose neonate via nursing was 0.1 mg/kg/day.

Plasma cholinesterase activity rapidly rose from 15% activity in the fetus to just above 90% on post-natal 11 (Text figure). The last day of gavage treatment of dams was post-natal 10. At the lowest dose tested, at maternal gavage 0.3 mg/kg/day, these dam's plasma cholinesterase activity on post-natal day 11 was 84% of control (Mattsson et al, 2000, Figure 2). Thus, post-natal day 11 pup plasma cholinesterase activity increased to 90+ % of control values during exposure to 0.1 mg/kg/day chlorpyrifos via milk, a value higher than dams administered 0.3 mg/kg/day by gavage and a value comparable to adult dietary exposure to 0.05 to 0.1 mg/kg/day. The rapid recovery of high-dose pup plasma cholinesterase activity to near control levels during lactation exposure is not consistent with a biologically meaningful increased sensitivity to chlorpyrifos.



<u>No meaningful differences in sensitivity of nursing-age pups (post natal days 7 to 21) from repeated oral gavage (in oil) of chlorpyrifos</u>

USEPA 2006 Organophosphate Cumulative Risk Assessment, Section I.B - Page 61 of 522: "Regarding chlorpyrifos, the Agency has not performed a BMD analysis but has generated a plot of the data from Zheng et al (2000). Dr. Carey Pope of Oklahoma State University provided the data in Figure I.B-3 to the Agency. The estimated dose to result in 10% brain ChE inhibition is noted as the dotted line in the graph. At this dose, there is

no difference in response between pups and adult rats. Thus, the FQPA factor for chlorpyrifos in the OP CRA for repeated exposures is 1X."

Of the 33 organophosphates considered in the 2006 USEPA risk assessment, chlorpyrifos was one of only 5 that merited a 1X FQPA factor based upon comparable brain ChE inhibition in pups vs adult rats to repeated doses. Eleven had FQPA between 1 and 10, and the others had FQPA=10.

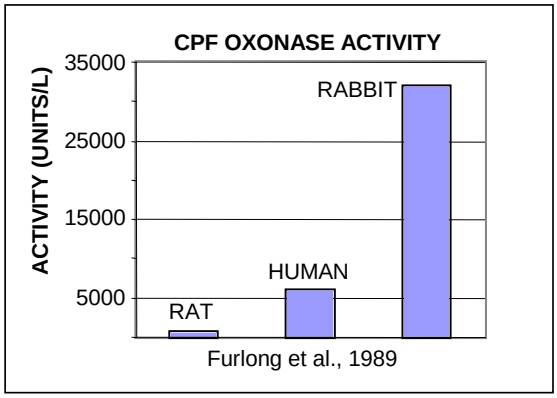
The repeated-dose pup and adult gavage data of Zheng et al. (2000) was evaluated by the bench-mark dose (BMD) method for a 20% inhibition of brain cholinesterase by Zhao et al. (2006). The repeated-dose BMD20 for pups was 1.2 mg/kg/day, and for adults was 1.5 mg/kg/day, indicating a very similar sensitivity at the BMD20. Zhao et al. recommended using inhibition of RBC cholinesterase as the point of departure for risk assessment. There was little RBC difference in either acute or repeated dose BMD20 between pups and adults.

The need for high quality scientific interpretation of data from gavage studies

As will be promptly recognized by OEHHA toxicologists, except for the nursing exposure data reported in Mattsson et al. (2000), which is in reality an indirect gavage study, the other data about pup's sensitivity to chlorpyrifos were from oral gavage (in oil) of chlorpyrifos, either to dams or directly to pups. As the Society of Toxicology has made clear (1998 Communiqué, and Conolly et al., 1999), gavage is a route of exposure that is a convenient but unrealistic route of exposure that can cause unrealistic kinetics. The magnitude of the 'gavage distortion' in kinetics has been evaluated.

Marty et al. (2007) reported an approximate 13X increase in blood chlorpyrifos Cmax in lactating dams administered 5 mg/kg/day chlorpyrifos by oral gavage (in oil), versus the same daily dose via the diet. One would expect a similar distortion in systemic Cmax from oral gavage in pups versus exposure from milk, diet, or contact with the environment. The use of the oral gavage route of exposure in these studies places a special burden on the toxicologist to judge the impact of both dose and route on risk assessment.

The rat as a model of low PON1 activity



It is also relevant to risk assessment that the test species, the rat, has appreciably lower PON1 chlorpyrifos oxonase activity than humans (Furlong et al., 1989). Thus, the use of rats in the risk assessment process uses an animal model that is deficient in chlorpyrifos oxonase as compared to humans.

Conclusion.

The toxicology literature on risk assessment states the weight-of-evidence approach should be used in risk assessment. Of those toxicology studies most relevant to risk assessment, the fundamental science and the weight-of-evidence demonstrate the young are not at additional risk of harm from chlorpyrifos under any realistic exposure scenario.

Section C. Non-Cholinesterase Mechanisms of Chlorpyrifos Neurotoxicity

Because there is significant attention presently directed at putative non-cholinergic effects from chlorpyrifos, particularly in many of the *in vitro* (not whole animal) studies, it is important not only to evaluate this potential concern, but to illustrate that this concern is not new or unique, but rather, has been evaluated and addressed by global regulatory authorities in recent years.

The following three publications provide useful information for evaluation of organophosphate insecticides for non-cholinergic mechanisms of toxicity. The recommendations are to use all available data and compare dose-response characteristics to see if appreciable toxicity occurs in the absence of inhibition of cholinesterase, and major reviews in 1998 and 2004 concluded that acetylcholinesterase [inhibition] is the primary mechanism of toxicity.

Mileson et al. (1998) published the opinions of an expert working group, convened by ILSI Risk Science Institute, to address whether the anticholinesterase organophosphate pesticides act by a common mechanism of toxicity. In addition, the working group addressed the problem of how to evaluate organophosphate pesticides for a significant level of non-cholinergic toxicity.

- "Organophosphorus insecticides share a common action of inhibiting acetylcholinesterase; the resulting excess acetylcholine accumulation underlies the principal mechanism of toxicity, …".
- The working group discussed an approach to evaluating the importance of non-cholinergic mechanisms by looking for appreciable toxicity in the absence of significant inhibition of AChE.

In 2000, the USEPA (2000b) issued a science policy document on the use of cholinesterase inhibition data in risk assessment. The most relevant cholinesterase for risk assessment is brain cholinesterase, followed by RBC and then plasma cholinesterase. This document makes a clear statement that non-cholinergic events must be carefully considered in risk assessment.

- "When applying the weight-of-the-evidence approach for selecting critical effect(s) for derivation of a reference dose (RfD) or concentration (RfC),

however, the entire toxicological data base on a pesticide must be evaluated (i.e., there also must be consideration of endpoints not related to the cholinergic consequences of anticholinesterase activity, for instance, liver or developmental toxicity or carcinogenicity).

- "It is possible that, for one or more of the exposure scenarios being evaluated, the non-cholinergic effects will be identified as critical or co-critical, and they may become a more appropriate basis for deriving RfDs or RfCs." pp. 2-3.

In 2004, Casida et al. published an extensive review of cholinergic versus non-cholinergic mechanisms of toxicity. This review included 13 *in vitro* and *in vivo* publications from the Slotkin laboratory. Casida et al. concluded:

- "High-dose laboratory experiments with animal models (e.g., mice, rats, and chickens) are difficult to relate to low-dose, long-term environmental exposure and particularly to actual risks for people.
- The findings reviewed reconfirm the importance of AChE as the primary target and NTE-LysoPLA as the secondary target of greatest interest (Figure 1)(p. 993)."
- Chlorpyifos IC_{50} was about 9x lower for AChE than for NTE-LysoPLA (Table 2), indicating inhibition of NTE-LysoPLA cannot occur without very high levels of inhibition of AChE.

Chlorpyrifos is regulated world-wide based upon the inhibition of cholinesterase

Different agencies regulate based upon inhibition of plasma, RBC or brain cholinesterase, depending on the regulatory goals of the agency. A key question in the safety regulation of organophosphate pesticides is 'will protection of cholinesterase provide protection against possible non-cholinergic mechanisms of toxicity'? USEPA policy requires that non-cholinergic mechanisms of toxicity be considered in the risk assessment of cholinesterase inhibiting pesticides (USEPA, 2000b).

While it is generally accepted that the principle mechanism or key event for the toxicity of organophosphate pesticides is the inhibition of acetylcholinesterase (AChE) in muscle and the nervous system (Mileson et al., 1999; USEPA, 2000b; Casida et al., 2004), numerous publications have indicated that some mechanisms of toxicity of chlorpyrifos may be mediated by non-cholinergic mechanisms.

An expert-working group convened by ILSI Risk Science Institute (Mileson et al., 1998) discussed the issue of OP pesticides having mechanistic subgroups based upon toxicological actions other than, or in addition to, inhibition of AChE (Mileson et al., alternate hypothesis 2, p. 15-16). "This hypothesis could be tested by looking for indicators that inhibition of AChE does not correlate with toxicity as might be expected (p. 15)." Mileson et al. discussed using relationships between clinical, pharmacokinetic and *in vitro* data (rate constants against AChE, IC50 versus whole animal ED50, LD50, and the like). There was not enough data available to the committee to reach final conclusions about non-cholinergic subgroups, but they did present the potentially useful

concept of evaluating correlations between AChE inhibition and other clinical or biological effects.

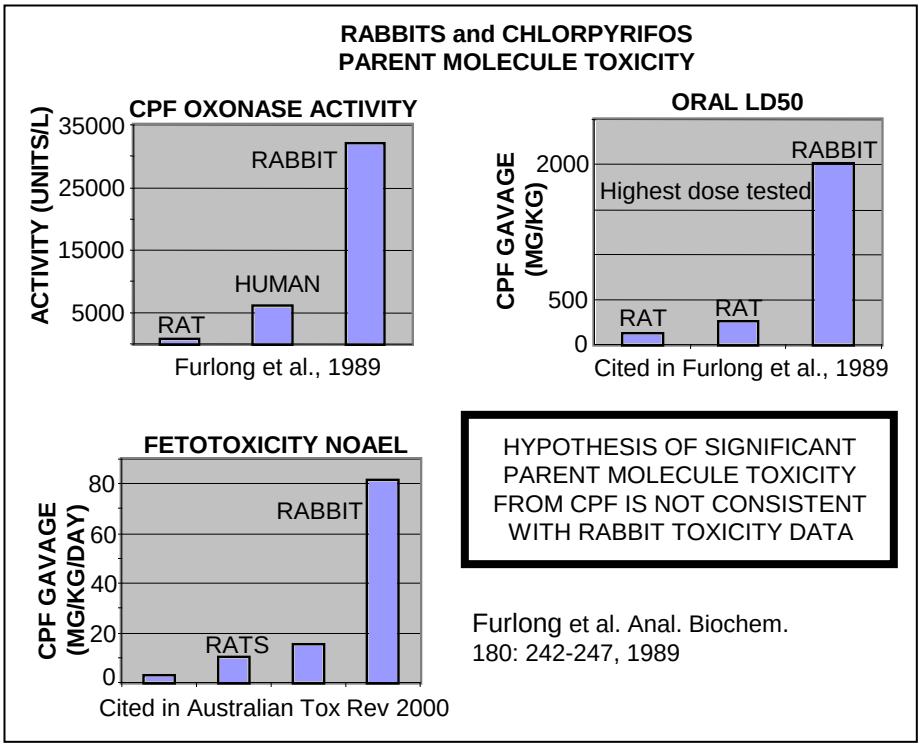
Data that Refute Chlorpyrifos Parent Molecule as a Significant Toxicant

Several publications from Slotkin's laboratory have stated that the parent chlorpyrifos molecule, in contrast to the oxon, causes clinically important developmental toxicity in experimental animals (e.g. review, Slotkin, 1999). Other data on chlorpyrifos are available to test the strength of the conclusion that the parent chlorpyrifos molecule contributes significantly to chlorpyrifos toxicity.

Rabbits have high levels of chlorpyrifos-oxonase compared to rats, and rabbits are much more tolerant of chlorpyrifos than are rats (Furlong et al., 1989). Information of susceptibility of rats and rabbits to chlorpyrifos toxicity and developmental toxicity are available.

> Evaluated in USEPA Toxicity Chapter for Chlorpyrifos (9/28/1999), the WHO (9/1999) chlorpyrifos toxicity assessment, and the Australian NRA Toxicology Assessment of chlorpyrifos (9/2000), and in Furlong et al. Anal Biochem. 180(2):242-247, 1989.

The following text figure shows the relative tolerance of rats and rabbits to fetotoxic effects and to adult lethal effects of chlorpyrifos by oral-gavage:



The rabbit developmental toxicity data demonstrate a dramatically higher NOAEL than occurs in the rat. These rat and rabbit LD50 and developmental data are consistent with oxon toxicity, which is the mechanism for inhibition of cholinesterase. A chlorpyrifos oral gavage dose of 140 mg/kg/day to rabbits during gestation days 7-19 caused fetotoxicity but not teratogenicity. Given the large amount of chlorpyrifos given to rabbits before fetotoxicity occurs, any inherent toxicity of the parent molecule to the developing fetus of rabbits must have been small.

USEPA (2002a) and UK ACP (2003) Determine Chlorpyrifos has no Non-cholinergic Effects That Would Affect Regulation Based Upon Cholinesterase Inhibition

The USEPA, in the 2002a revised OP Cumulative Risk Assessment, appeared to follow the guidance of the USEPA 2000b policy document on cholinesterase inhibitors and evaluated chlorpyrifos for non-cholinergic developmental effects. Whether intended or not, the USEPA (2002a) evaluation also followed the proposal of Mileson et al. (1999) and looked for evidence of developmental effects at doses below those expected to inhibit cholinesterase. Papers cited in USEPA (2002a) for a variety of possible treatment-related effects: Johnson *et al.*, 1998; Crumpton *et al.*, 2000; Dam *et al.*, 1999, Dam *et al.*, 2000; Slotkin *et al.*, 2001a,b; Levin *et al.*, 2001; Slotkin *et al.*, 2002).

- "In the few prenatal studies where ChE activity was assessed, however, few of these effects occur at dose levels that do not inhibit ChE activity in the fetal brain, and probably none of these effects occur in the absence of ChE inhibition in maternal tissues. In both the studies assessing prenatal effects of chlorpyrifos, effects on brain development were noted at dosages (1 mg/kg/day) that did not inhibit fetal brain ChE (Lassiter *et al.*, 2002; Qiao *et al.*, 2002), but would be predicted to show inhibition of maternal blood and brain ChE activity (Maurissen *et al.*, 2000)."
- "In postnatal studies, there are no reports of effects in the absence of ChE inhibition. In some cases, this assertion is made by the authors, but the authors fail to ascertain that the ChE measurements were taken at the time of peak effect. Often the measurements are taken 24 hours after the last dose, rather than assessing ChE activity during the entire dosing period."

Thus, in 2002a, the USEPA considered the publications from Slotkin's laboratory and noted the apparent lack of developmental effects at doses below those that inhibit cholinesterase. In addition, the USEPA (2002) also evaluated the chlorpyrifos gavage repeated-dose, adult versus neonate, brain inhibition data of Zheng et al. (2000). USEPA (2002a) concluded there was no meaningful NOAEL difference in sensitivity of pup brain cholinesterase. This is the first time that dose-related mechanisms were a factor in USEPA chlorpyrifos regulation. The chlorpyrifos repeated-dose FQPA factor was reduced to 1X for this assessment.

In 2003, the United Kingdom (UK) Pesticide Safety Directorate (PSD) and the UK Advisory Committee on Pesticides (UK/ACP 2003) reviewed some 25 additional

publications, including many from Slotkin's laboratory, for impact on chlorpyrifos reference doses.

2. Introduction. … "For this update, key considerations have therefore been:"

• "Do the studies report effects at dose levels that could impact on the currently proposed regulatory reference dose levels which are based on NOAELs of 1 mg/kg bw/day in humans and dogs (with LOAELs of 2 and 3 mg/kg bw/day, respectively)?"

• "Do the studies provide evidence of greater sensitivity of fetuses and/or pups than adults to the effects of chlorpyrifos (particularly effects other than cholinesterase inhibition)?"

Section 3.2 "In vivo studies (subcutaneous dosing). All of these papers, except for papers by Liu and Pope (1996) and Jett and Navoa (2000), are from the same research group at Duke University, USA."

"The dosing route and the vehicle used (subcutaneous injection in DMSO – designed to maximise exposure) mean that the dose levels used cannot be directly compared with chlorpyrifos reference values (which were derived from oral studies). The pharmacokinetics of chlorpyrifos would also be expected to be completely different following oral ingestion, with first pass metabolism by the liver. Additionally, dermal exposure of operators would not involve such rapid and complete absorption of chlorpyrifos (1% has been proposed based on human data) as occurs following direct subcutaneous injection in DMSO, resulting in a different rate of absorption of chlorpyrifos into the systemic circulation and possible resulting differences in the extent of metabolism. These factors limit the value of the following studies using subcutaneous dosing."

The PSD review of the additional 25 publication was evaluated by the ACP. Minutes of the 299th Meeting of the Advisory Committee on Pesticides (ACP) on 10 April 2003. Representatives from the following Departments and other organizations were present: The Pesticides Safety Directorate (PSD), Department of Health (DH), Health & Safety Executive (HSE), Food Standards Agency (FSA), Scottish Agricultural Science Agency (SASA). … Section 4. Chlorpyrifos Human Health Review. Evaluation of further papers requested by the ACP [ACP 6 (299/2003)].

- 4.1 "As part of this review, members were asked to consider additional papers on developmental neurotoxicity and prenatal exposure in rats."
- 4.2 "The Committee concluded that the papers did not affect their advice on reference doses reached at the meeting in <u>November</u>. They also decided to discuss outside the meeting whether some clarification was required from the company on one point."

In summary, both the USEPA (2002a) and the UK ACP (2003) specifically evaluated publications from Slotkin's laboratory for non-cholinergic effects relative to cholinesterase inhibition. Neither agency recognized non-cholinergic effects of a magnitude that caused them to reconsider using cholinesterase inhibition NOAELs for regulation of chlorpyrifos.

Furthermore, the USEPA (2002a) determined an FQPA factor of 1X for repeated exposure to chlorpyrifos, bringing the USEPA in agreement with the WHO and EU on the issue of differential sensitivity of the young.

Inappropriateness of selecting a study (Jett et al., 2001) using a subcutaneous route of administration

In a review of the historical practice of OEHHA (2007) when proposing chRDs for various chemicals, it is particularly noteworthy that in all previous cases, the study or studies selected for use in establishing a chRD has/have always encompassed the oral route (Text Table 1). Never has a subcutaneous route of administration study been used and it is inappropriate in the case for chlorpyrifos because (1) it ignores global guidance on selecting a relevant route of exposure when human risk assessment is being considered and (2) qualified and sufficient whole animal toxicity studies exist (for chlorpyrifos) which were not discussed or considered by OEHHA.

Text Table 1. Study Summary of OEHHA Derived chRDs.

Report Date	Chemical	Study(ies) Used & Reported effect(s)	Route*	Point of Departure	UF
12/05 – Final Report	Cadmium	Renal tubular dysfunction in humans	Oral	LOAEL of 0.001 mg/kg/d	90
12/05 – Final Report	Chlordane	Sex-steroid mediated behavioral changes in rats	Oral	LOAEL of 0.1 mg/kg/d	3000
12/05 – Final Report	Heptachlor	Decreased cognitive function in rats; suppression of immune function in rats	Oral	LOAEL of 0.03 mg/kg/d (both studies)	1000
12/05 – Final Report	Heptachlor epoxide	Altered liver to body weight ratio in dogs	Diet	LOAEL of 0.0125 mg/kg/d	1000
12/05 – Final Report	Methoxychlor	Increased urine marking in mice; increase in prostate size in mice		LOAEL of 0.02 mg/kg/d (both studies)	1000
12/05 – Final Report	Nickel	Pup (rat) mortality	Diet	NOAEL of 1.1 mg/kg/d	100
6/06 – Final Report	Manganese	Ambiguous GI absorption data in humans – not well-defined toxicity	Not defined	NOAEL of 0.086 mg/kg/d	3
6/06 – Final Report	Pentachloro-phenol	Decrements in thyroxin and effects on thyroid in mink/lamb	Diet	LOAEL of 1 mg/kg/d	1000
10/07 – Final Report	Atrazine	Attenuation of LH surge in rats	Diet	NOAEL of 1.8 mg/kg/d	300
10/07 – Final Report	Deltamethrin	Nerve degeneration from 2-year rat study	Diet	NOAEL of 0.1 mg/kg/day	1000
11/07	Malathion	Insufficient data for chRD development; acknowledged concern with low, non-cholinergic doses	NA	No chRD established – will maintain RfD based on RBC/plasma AchE	Not applicable
11/07	Dieldrin	Cite DNT effects but cannot determine NOAEL for these effects	NA	No chRD – NOAEL cannot be established	Not applicable
11/07 – External Draft Report	Chlorpyrifos	NB Endpoint: Behavioral/cognitive alterations in rats; ChEI Endpoint: plasma/RBC cholinesterase inhibition	SC	NB: LOAEL of 0.3 ChEI: NOEL of 0.1	NB: 3000 ChEI: 1000

*NA – Not available; SC – Subcutaneous
NB - Neurobehavioral

There is no scientifically rational basis to use Jett et al. (2001) for regulatory decisions. As previously noted, the route of administration was subcutaneous injection of chlorpyrifos in vegetable oil. The authors present no data showing that chlorpyrifos was even absorbed, but do show data that too little was absorbed to affect brain ChE and muscarinic binding. There are no data to demonstrate that the kinetics were appropriate to environmental exposures. There are many publications of the SOT and other organizations that point out the use of unrealistic routes of administration can distort regulatory decisions.

In addition to an inappropriate route of exposure, there are interpretive problems within the Jett et al. (2001) study. Jett et al. misinterpret the cognitive data reported from early exposures (PND 7, 11, 15). Examination of the data in Figure 2 (Jett et al) will show that the rate of learning for pups treated subcutaneously at 0, 0.3 and 7 mg/kg/day were all nearly the same (the slope of the learning curves are similar). The starting location of the curves were different, indicating a problem other than learning the Morris swim maze. If the data are adjusted for day 1 differences in starting location, then the similarity of the learning curves becomes readily apparent.

In addition, it is important to note in Figure 2 that the controls had a decrement in learning on day 3 that was comparable in magnitude to the difference between control vs 0.3 mg/kg groups on day 1. There is an odd flattening of the learning curve for the 7 mg/kg dose group on days 4 and 5 of testing. A treatment-related effect on learning should have produced a flatter curve than controls starting from day 1. Given the size of the decrement in controls on day 3, and the size of the shift in the high-dose curve on days 4 and 5, it is again important to consider factors inherent in the testing paradigm for the differences between groups. In Figure 2, it is also important to consider the degree of difference among groups, given a 23X increase in dose. If chlorpyrifos is the mechanism for the differences between groups, then a 23X increase in dose should have had dramatic effects on performance throughout the test. This did not occur.

The only data supportive of a treatment-related effect from pre-weaning treatment is in Table 1, in the high-dose ‘time in training quadrant’. But, this ‘time in training quadrant’ finding also needs critical examination since it was measured after test day 5. The high-dose pups had a rate of learning which exceeded that of controls on days 1 to 3, due to a decrement in learning in controls on day 3. The rate of learning of high-dose pups then became flat on days 3 to 5 (Figure 2), followed by a low ‘time in training quadrant’. If the training paradigm is weak enough to allow a transient reversal in learning in controls, the training paradigm is weak enough to account for the shift in behavior in the high-dose pups.

Jett et al. again misinterpret the data from Figure 3. This figure demonstrates a break-down in the testing paradigm. You cannot evaluate learning if the test does not work. Once again, there is a 23X difference in dose, but no evidence of this difference in dose was apparent in behavior or in swim speed.

In summary, the Jett et al. study is inappropriate as it employed a route of exposure not relevant to humans, did not reveal any cholinesterase inhibition (i.e., which prevents a direct evaluation of non-cholinergic effects relative to degree of cholinesterase inhibition), and the data presented have fundamental interpretive problems which

preclude OEHHA's conclusion that this study is useful for evaluating potential non-cholinergic effects from chlorpyifos exposure. Moreover, regulatory agencies have considered non-cholinergic effects as a potentially more sensitive marker of potential toxicity resulting from chlorpyrifos exposure, but have consistently concluded that protection against cholinesterase inhibition is protective against all other potential effects, including non-chlolinergic effects.

Section D. Late Arising Deficits in Young Animals During Development

This section addresses key aspects of the history of the chlorpyrifos developmental neurotoxicity study, the study report and its three supplements. This section addresses the study director's and study pathologist's conclusions that high-dose effects in pups was a consequence of undernutrition because of significant maternal toxicity at birth, and that the slightly thinner parietal cortex in mid-dose and high-dose female pups at 2 months of age were not at any time considered to be treatment related.

Hoberman and Garman (2000), Supplement 3, historical control data for morphometrics, was accumulated just after the USEPA June 2000 risk assessment, precluding the use of this information by the USEPA at that time. Supplement 3 showed the parietal cortex measurements were comfortably within the historical control range. In addition, the authors provided a detailed biological rationale for the implausibility of the small differences in parietal cortex being due to chlorpyrifos treatment. All of the authors' statistical and biological arguments were consistent with the world-wide publications on principles of toxicology for risk assessment. The conclusions of major regulatory agencies world-wide are consistent with the authors' conclusions.

The only chlorpyrifos developmental neurotoxicity (DNT) study available today that best meets the study-design requirements of regulatory agencies world-wide is the guideline-compliant, Good-Laboratory-Practices compliant, chlorpyrifos DNT study conducted by Drs. Alan Hoberman (study director) and Robert Garman (pathologist) at Argus Laboratories in 1998. Not only does this study meet global standards and requirements for study design to evaluate neurotoxicity, sensitivity, and non-chlolinergic effects in young animals, it also supercedes all *in vitro* and other laboratory animal studies that use inappropriate doses and routes of administration, key factors when considering relevance to humans.

Background

Because of the newness of guideline-based developmental neurotoxicity (DNT) studies in 1997, the chlorpyrifos DNT study was conducted under a protocol developed by Dr. Jacques Maurissen and other toxicologists at The Dow Chemical Company (Dow) in consultation with USEPA toxicologists. Although the study was conducted according to the 1991 DNT guidelines, the 1998 DNT guidelines were under preparation and the purpose of the consultation with USEPA was to design a study that would meet all current expectations for a state-of-the-art DNT study. Although the draft protocol recommended dietary exposure to chlorpyrifos, the USEPA strongly recommended oral gavage. The use of oral gavage would turn out to be an unfortunate decision because of

confounding of pup data from maternal toxicity. Dietary dosing would have generated significant inhibition of maternal brain cholinesterase without causing clinical toxicity.

The maternal doses were 0, 0.3, 1 or 5 mg/kg/day. The route of exposure was oral gavage in vegetable oil of dams from gestation day 6 to lactation day 10 (birth = lactation day 0). The decision to administer chlorpyrifos by oral gavage in vegetable oil would be key to high-dose maternal toxicity at the time of birth, and interpretation of growth and developmental effects in high-dose pups. A recent publication by Marty et al. (2007) demonstrates that oral gavage of chlorpyrifos in vegetable oil to pregnant rats causes a blood chlorpyrifos Cmax approximately 13X higher than chlorpyrifos administered in diet.

The USEPA analyzed samples from the chlorpyrifos DNT study for maternal plasma, RBC and brain ChE activity. Because of his experience with chlorpyrifos and cognitive testing, Dr. Mark Stanton of the USEPA was consulted on the design of the cognitive test that was conducted just after weaning and again when the pups were about 2 months old (a T-maze spatial-delayed alternation task to evaluate learning and memory).

Dow contracted the DNT study to Argus Laboratories with Dr. Alan Hoberman as study director and Dr. Robert Garman as study pathologist. Drs. Hoberman and Garman were highly experienced in reproduction and development studies, but DNT studies were new. The final report was released as Hoberman, 8/19/1998. The pathology report for the pups at 2 months of age was inadvertently not included in the final report, and was submitted as report Supplement 1, Hoberman, 9/23/1998. The USEPA requested a statistical reanalysis of the morphometric data. The reanalysis was done in consultation with the USEPA and submitted as Supplement 2, Hoberman and Garman, 3/19/1999. The chlorpyrifos DNT study was published in the open literature (Maurissen et al., 2000).

No historical DNT morphometric control data were available at the time the chlorpyrifos DNT study was conducted, but Drs. Hoberman and Garman conducted 5 DNT studies soon after the chlorpyrifos study, at the same laboratory and using the same methods, and issued a Supplement 3, Historical control morphometric data (Hoberman and Garman, 10/9/2000). The morphometric historical control data was submitted five months after the USEPA June 8, 2000 risk assessment was released.

Companion Pharmacokinetic Study

Dow conducted a companion pharmacokinetic study at nearly the same time as the DNT study (Report Mattsson et al., 1998; Publication Mattsson et al., 2000). Dams were dosed as in the DNT study; 0, 0.3, 1 or 5 mg/kg/day oral gavage in corn oil from gestation day 6 to lactation day 10. Dams and fetuses were evaluated on day 21 of pregnancy, and dams and pups on lactation days 1, 5, 11 and 22. Endpoints were cholinesterase inhibition (plasma, RBC, heart, two areas of brain), blood chlorpyrifos levels, blood TCP levels, and milk chlorpyrifos levels. Dams had a high level of inhibition of brain cholinesterase at the high dose and minor inhibition at the middle dose. Fetuses had inhibition of brain cholinesterase only at the high-dose, and the per-cent inhibition was less than occurred in their dams. High-dose newborn pups had a very rapid post-natal recovery of cholinesterase activity, and plasma and brain cholinesterase activity was comparable to

controls in 5 days. RBC cholinesterase recovery was slightly less, perhaps because RBC cholinesterase recovery is due to replacement of old RBCs with new, rather than a resynthesis of inhibited cholinesterase. The no-observed-adverse effect level for inhibition of brain cholinesterase was 0.3 mg/kg for dams and 1.0 mg/kg maternal-dose for fetuses and pups.

Nursing pups of high-dose dams ingested chlorpyrifos from milk at approximately 0.1 mg/kg/day. Plasma cholinesterase is the most sensitive to inhibition by chlorpyrifos. The recovery of pup plasma cholinesterase to approximate those of controls in 5 days while ingesting 0.1 mg/kg/day of chlorpyrifos indicated this dose level was near or below the threshold for inhibition of adult plasma cholinesterase. If the plasma cholinesterase threshold had been exceeded, than the pup plasma cholinesterase would have attained a new level of inhibition during lactation exposure, and would not have recovered to control values during exposure.

Chlorpyrifos DNT Summary of Results

High-dose dams had clinically-evident toxic signs just before and for 4 days subsequent to giving birth (muscle fasciculations, hyperpnea, hyperactivity, diminished weight and weight gain). Several pups of high-dose dams died at this time, some in entire litters and some without milk in their stomachs. When maternal clinical signs abated, no more pup deaths occurred. Pups from high-dose dams gained weight more slowly than controls, and several of the developmental measures showed effects consistent with slightly delayed maturation. Although there were many signs of delayed maturation, pups of high-dose dams performed as well as controls in post-weaning tests of learning and memory (T-maze spatial delayed-alternation task). There was no evidence of maternal toxicity at 1 mg/kg/day, and pups of these dams had no differences from control that were attributed to treatment. Small but statistically significantly differences in the thickness of the parietal cortex of high- and mid-dose female pups at 2 months of age were considered to be random effects and not treatment related for several reasons (discussed below). The DNT study concluded the maternal and developmental NOAEL was 1 mg/kg/day.

All adverse effects in offspring of high-dose dams in this study were interpreted by Drs. Hoberman and Garman as secondary to pup undernutrition due to excessive maternal toxicity in high-dose dams.

Interpretation of DNT Results by Regulatory Agencies

The interpretations of the biological and statistical results of this DNT study by regulatory agencies have been variable, but most agencies interpretations were consistent with the DNT study's conclusions. An examination of the published records of the agencies indicate their respective conclusions were highly dependent on the rigor of application of toxicological principles for data evaluation of the chlorpyrifos DNT study and of published chlorpyrifos studies that were not designed for use in risk assessment.

The World Health Organization (1999), UK Advisory Committee on Pesticides (UK/ACP 2003), Australia (2000a,b), CalEPA/DPR (2001), and EU (2005 European Re-registration) were in general concurrence with the study authors that pups had no

treatment-related effects at 1 mg/kg/day (maternal gavage dose), and the adverse effects in pups of high-dose dams were consistent with maternal toxicity and diminished maternal care. In contrast, the USEPA (2000a) concluded pups did show signs of differential sensitivity (more severe effects in pups than in dams), and that the 5% thinner parietal cortex in 2-month old high- and mid-dose female pups was treatment related.

Dow AgroSciences toxicologists strongly disagree with the USEPA (2000a) conclusions. As a caveat, Dow AgroSciences toxicologists also recognize that the USEPA, in June 2000, did not have an important morphometric historical-control supplement to the DNT study that clearly showed the female parietal cortex measurements in question were comfortably with historical control limits. A comprehensive 'lack of biological-plausibility' argument was also presented in the morphometric historical control supplement.

It is important to note that the USEPA did not consider the chlorpyrifos DNT study an issue during the revised organophosphate cumulative risk assessment (USEPA, 2002a) or in their final cumulative risk assessment (USEPA, 2006). Both of these subsequent USEPA reviews considered published literature on chlorpyrifos developmental toxicity and the USEPA showed evidence of application of sound toxicological principles in the review of this literature. For repeated-exposures to chlorpyrifos, the FQPA factor was considered 1X.

Dow AgroSciences toxicologists are confident that OEHHA toxicologists will concur that application of sound toxicological principles is critical to risk assessment. And further, we are confident that application of sound toxicological principles to interpretation of the chlorpyrifos DNT study and the many chlorpyrifos studies not designed for risk assessment will convince OEHHA of the validity of the conclusions of the DNT authors and the WHO (1999), UK/ACP (2003), Australia (2000a,b), CalEPA/DPR (2001), and EU re-registration (2005).

Summary of Toxicological Principles

The following is a summary of the recommendations for study design, conduct, and interpretation of toxicity studies as published by the Society of Toxicology, the World Health Organization, USEPA, OECD and ILSI (references below):

- The toxicologist is responsible for designing relevance for risk assessment into the toxicology study.
- Use realistic dose levels compared to expected human exposure.
- Use realistic routes of exposure (oral, dermal, inhalation), matched as closely as possible to expected human exposure.
- Gavage is not a realistic route of exposure (Conolly et al., 1999). Therefore, one should evaluate impact of gavage exposure on the study.
- Need PK/PD data to extrapolate from unrealistic to realistic doses and routes of exposure.
- There needs to be an emphasis on scientific evaluation and judgment.

- Statistics are tools, and not sole determinates of presence or absence of treatment-related effects. Plausible treatment-related effects can occur without statistical significance, and statistical-significance can occur for differences that are not caused by treatment.
- Use 'weight-of-evidence' approach. Other studies, other endpoints, systemic toxicity.
- Evaluate data for consistency and biological plausibility.
- Evaluate data for dose-response patterns, LOAEL, NOAEL, BMD, etc.
- Evaluate patterns among logically related endpoints (between genders, doses, clinical signs, body and organ weights, patterns of behavior, relationship of motor function and operant behavior, across time points, etc).
- Evaluate for dose-related transitions in mechanisms (recognize that dose affects mechanisms, and mechanisms that occur at high doses may not be relevant to low doses).
- Evaluate relationship of maternal toxicity to effects in offspring. Maternal toxicity can have profound effects on offspring and can affect nearly every endpoint.
- Evaluate historical control data, which may indicate that differences from concurrent controls may be unrelated to treatment.
- Be alert to odor (and taste) as a potential confounder in developmental studies (many test agents, vehicles, and metabolites have taste and/or odor; what is the effect on grooming, maternal-pup interactions, pup-pup interactions, etc.).

The Issue of a Thinner Parietal Cortex in Female Offspring at 2-Months of Age

Dow AgroSciences toxicologists will present considerable information on this topic because of the importance of these data to regulation of chlorpyrifos.

The following statement about parietal cortex measurements in the June 8, 2000, USEPA Human Health Risk Assessment of Chlorpyrifos (USEPA, 2000a) is contrary to the conclusion of Drs. Hoberman and Garman, the study director and pathologist for the chlorpyrifos DNT study. This USEPA (2000a) conclusion is also contrary to conclusions of WHO (1999), Australia (2000a,b), CalEPA/DPR (2001). Furthermore, the chlorpyrifos DNT study was not an issue in the USEPA organophosphate cumulative risk assessment, where the FQPA factor was reduced to 1X for repetitive exposures (USEPA 2002a, 2006).

USEPA June 8, 2000:

> "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." p. 16.

Hoberman, A. Supplement 1. 23 Sep 1998:

> "Considering the fact that only six rats/sex were evaluated from each treatment group and that a 5% to 6% intragroup variation is seen for many of the morphometric measurements (including within the control group), these differences between the female control and high dose groups were considered to represent random variation." … "Further, no neuropathologic

alterations were found in either male or female rats from the maternal high dose group. There is, therefore, no evidence that exposure to the test substance, both *in utero* and during the postnatal lactational period and under the conditions of this study, produce any morphologic neurotoxic effects." pp. 11-12.

Hoberman, A. and Garman, R. Supplement 2. 19 Mar 1999.

"For adult (day-66) female rats, all ANOVAs were non-significant except for the parietal cortex. Dunnett's test for the female parietal cortex indicated significantly smaller measures for both mid-dose and high-dose rats versus control females (Table 6). Although statistically significant, the parietal cortices were only 5.1% less than controls in high-dose females, and 4.2% less in mid-dose females (Table 2). Thus, parietal cortical measurements were minimally different from controls and were virtually the same in both mid- and high-dose females, indicating a lack of dose-response relationship. In addition, there was no apparent relationship of adult female parietal cortical measurements and the same measurements of pups." p. 9. …

There was a section in Supplement 2 on "Inherent Variability in Morphometric Measurements" p. 10. The authors addressed:

- Even the use of "miter box/brain matrix" cutting molds does not eliminate the problems with positioning brains of varying sizes, and movement of brains while cutting. Knife slots are at fixed intervals.
- Variation in young-animal brain size is a particular problem.
- There are difficulties in obtaining the same plane of section among different brains. Dimensions will change as plane of section changes.
- Tissue dehydration can differ if all brains are not processed in the same run of the processor.
- Different degrees of 'facing in' of microtome sections of tissue in paraffin blocks results in sections taken at different levels.
- The histology technician has to periodically soak the paraffin block in cold water to re-hydrate the cut surface layer. This causes varying degrees of paraffin block swelling, producing sections of slightly different thickness.

"Based on the fact that a moderate degree of inter-animal variation may exist in the dimensions of certain brain structures and that there are inherent difficulties in obtaining highly standardized coronal sections on the same brains that are used for histopathologic evaluation, **a groups size of six animals/sex/dosage level is considered to be inadequate for definitive morphometric analyses."** p. 11.

"To use the initial morphometry data set to drive a regulatory decision about the potential neurotoxicity of a chemical is inadvisable unless the inter-group morphometric differences are substantial or are also associated with histopathologic alterations." p. 11. [bold emphasis added]

Dr. Garman's discussion of issues in Supplement 2 about variability in brain linear morphometrics in developmental neurotoxicity studies were incorporated into an ILSI/USEPA/NIEHS sponsored workshop publication (Garman et al., 2001), and the Garman et al. recommendations for larger sample sizes and for 'same batch' processing of tissues are incorporated into the 2006 version of the OECD 426 DNT guideline. Dr.

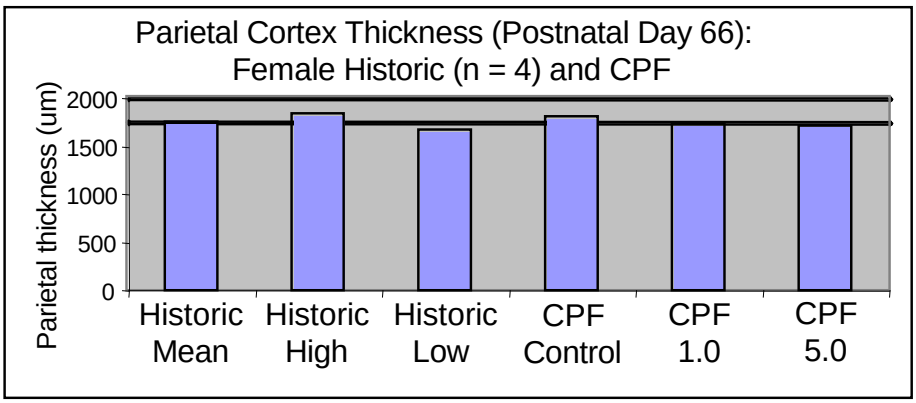
Garman is a recognized authority on developmental neuropathology, and his conclusions upon examination of the actual tissues in the chlorpyrifos DNT study should have been taken more seriously by USEPA (2000a).

Supplement 3 (Hoberman and Garman) to the chlorpyrifos DNT study, 9 Oct. 2000.

Supplement 3 was submitted four months after the USEPA issued their June 8, 2000 chlorpyrifos risk assessment. Supplement 3 contained a thorough biological plausibility evaluation of the parietal cortex findings, and reported the results of five subsequent DNT studies for historical reference values. Four of the historical control studies contained data relevant to the parietal cortex.

In summary, examination of the female post-natal day 66 parietal cortex data (Figure and Table below) shows how small the differences are between chlorpyrifos controls and low- and high-dose pups. Given a 5X difference in doses, it is difficult to argue that a 5.1% difference from concurrent control (high-dose) is truly different from 4.2% for mid-dose females. Of four historic control studies, the low historic value was 7.6% smaller than the chlorpyrifos control mean. It is readily apparent that the female day-66 parietal cortex measurements from four historic control studies and from the chlorpyrifos DNT study are all within the range of normal variation.

Data from Hoberman and Garman, Supplement 3, 10/9/2000.



Female Day 66	Parietal Thickness (um)	% historic mean	% control mean
Hist mean	1738	100.0	97.0
Hist high	1824	104.9	101.8
Hist low	1656	95.3	92.4
CPF Cont	1792	103.1	100.0
1 mg/kg/d	1716	98.7	95.8
5 mg/kg/d	1700	97.8	94.9

The following are extensive quotations from Hoberman, A. and Garman, R. Supplement 3 to the Final Report: Historical Control Morphometric Data, 9 Oct 2000. It will be seen that Drs. Hoberman and Garman rigorously used the principles of evaluation of toxicology studies for risk assessment in their evaluation of the post-natal day 66 female parietal cortex data. In particular, the authors summarize their rationale for considering as biologically implausible any relationship of differences in adult female parietal cortex thickness to be related to treatment.

From pages 7-8:

"… In separate papers, the issues of dose, time of exposure, route of exposure and sensitivity of the adult and pup rats to Chlorpyrifos and cholinesterase inhibition have been extensively investigated. All of these investigations have added weight to the original authors conclusions. Specifically, the original report attributes all effects of chlorpyrifos, including those that resulted in morphometric differences in brain areas of high dosage group pups, to slower growth caused by undernutrition of the offspring and not to any type of defective growth or effect on brain development. These effects on brain weight and morphometric measurements were clearly limited to the maternal high dosage group. A statistically significant reduction in the parietal cortex of the F1 generation female adult rats in the middle (1 mg/kg) dosage group was never considered to be related to the test article."

"The rationale for concluding that the slightly thinner parietal cortex in the adult females was not related to treatment was as follows:

A. There was a greater than 50% likelihood of a statistical false-positive conclusion (16 ANOVAs on the adult morphometric data).

B. The difference was small (approximately 5%), which was well within the differences that could occur from embedding the brains at different times (one day difference in time between the control and high dose, and one month difference in time between the control and middle dosage group adult females). This would be a "batch" effect.

C. There was a persuasive lack of biological plausibility in the other data. If the slightly thinner parietal cortex were due to a pathological process, then one would expect to find supporting biological data. None were found among the following data sets:

1. When cortical thickness was corrected for body weight, the relative cortical thickness of high-dose females was slightly greater than for middle dosage females. The relative thickness of the parietal cortex in high dose females was not statistically significant as compared to controls.
2. There was no difference in the parietal cortex in high-dose adult males. While differences in dose response often occur between males and females, these differences in sensitivity are seldom large. A 5X difference in dose would be highly likely to affect males if the effect were true.
3. There was no effect on the parietal cortex in the middle dosage group males or females on PD 12. Since the greatest exposure occurred *in utero*, and neocortical neuronogenesis and migration occur *in utero*, an effect on the PD 12 pups would be expected.
4. There was no effect on the frontal cortex, even at a dose that was 5X higher. The frontal cortex is adjacent to the parietal cortex, is seen on the same plane of section, and undergoes development by the same process as the parietal cortex (Bayer et al., Neurotoxicol 14(1): 83-144, 1993). It would be very unusual for pathological processes to alter development of the parietal cortex and not the frontal cortex, given that *in utero* exposure encompasses the development phases of both cortical areas.
5. No histopathological changes were seen in any brain tissue, including the parietal cortex. Aberrant cortical neuronogenesis and migration would be expected to result in altered cytoarchitecture, especially over a 5X-dose range. The adult female middle and high dosage group parietal cortex had a normal cytoarchitecture when examined by light microscope.
6. There were no changes in complex behaviors. The learning and memory of the delayed spatial alternation task is a set of complex behaviors that were not affected by treatment, in males or in females, even at the high dose. There is a substantial literature concerning the complex role of the parietal cortex on spatial memory. These functions of the parietal cortex increase the likelihood that if the pathological changes had occurred in the parietal cortex in the middle dosage group, then the high dosage group should have demonstrated performance effects on the delayed spatial alternation task. This did not occur."

"While one could argue that a particular biological effect might not be detected concurrently with a pathological change in the parietal cortex, it is very difficult to argue that a

pathological change in the parietal cortex would no affect any of the above parameters." pp. 7-8.

Page 9, topic 2. "The subject of this supplement to the developmental neurotoxicity report is the accumulation of relevant historical control data collected after the original study. The authors have since conducted 5 more studies, and have now demonstrated that these parietal-cortical values were well within historical control ranges. The historical average (range) for this value was 1738 micrometers (um) (1656 to 1824 um). The average thickness of the adult female parietal cortex in the 1 mg/kg group was 1716 ± 36.4 um, and the concurrent control value was 1792 ± 36.1 um." p. 9.

Last two paragraphs: "The parietal cortex thickness of middle dosage group females was 1716 um, which was 1.3% smaller than the historical average control and 3.6% thicker than the smallest historical control. Thus, although slightly (about 5%) thinner than concurrent controls (statistically significant), the parietal cortex of the middle dosage group adult females was comfortably within the range of historical control values."

"In conclusion, the historical control data have provided an important perspective to the normal variability of morphometric data under the circumstances used in these experiments. In addition, the data have provided additional support to the original conclusions of the authors that the statistically significant differences in adult female parietal cortex were within normal variation and were not treatment related." Signed A. Hobeman and Robert H. Garman, 9 Oct 00.

Comments from Regulatory Agencies

WHO 1999 toxicology assessment of chlorpyrifos: "The NOAEL for toxic effects in the pups was 1 mg/kg bw per day on the basis of the decreased viability index, relative brain weight, and delayed sexual maturity, possibly associated with maternal toxicity and subsequent diminished maternal care at the high dose. Cognitive function (learning, memory, and habituation) in the pups were not affected by treatment (Hoberman, 1998)."

From 2001 California EPA, DPR, Summary of Toxicology Data, page 22. Concluding comments about Supplement 3: "In the context of the demonstrated high maternal and neonatal toxicity of this dose, the supplemental data reinforce the lack of demonstrated special toxicity of the test article toward the developing nervous system. Supplemental to a previously acceptable study with no adverse effects." Aldous, 9/26/01.

The UK Advisory Committee on Pesticides (ACP 6(299/03): "By contrast, the OECD Guideline-compliant developmental neurotoxicity study performed with chlorpyrifos covered similar endpoints and established a clear NOAEL (1 mg/kg bw/day) for effects on pups following oral exposure (see Appendix 2, Hoberman, 1998 at section 5.1.7.1 (q), and the evaluation of a supplement to this study at Appendix 3)." p. 3.

APPENDIX 2 - Taken from ACP 264 (277/00) considered by ACP 6 July 2000: "The NOAEL for effects on pups was 1 mg/kg bw/day, based on decreased viability, lower pup bodyweights and brain weights and delayed sexual maturity at 5 mg/kg bw/day. These effects were consistent with being secondary to maternal toxicity. Cognitive functions in the pups (learning, memory and habituation) were not affected

by treatment at any dosage. There were no neuropathology findings in pups at 12 or 66 days of age." pp. 92-

APPENDIX 3 - Taken from ACP 23 (281/01) considered by ACP 18 January 2001. Supplement 3: a) A supplement to the developmental neurotoxicity study in rats (ACP 264 (277/00) Section 5.1.7.1 (q)) Hoberman AM (1998) which provides an analysis of the morphometric data (with reference to historical control data)-requested by the US EPA.

"PSD comment: The analysis of the morphometric data provided by the company gives a detailed argument as to the 'lack of biological plausibility' of the apparent treatment related effects of chlorpyrifos on pup brain sizes. The paper provides some limited historical control data (given the limited length of time such studies have been conducted). Overall there appears to be little consistency to the effects, and since they are no marked differences from control values the overall significance of the findings is unclear."

Australia 2000a chlorpyrifos toxicology assessment (Supplement 3 not included): "The morphometric measurements reveal minor variations (ca. 5%) which might be expected for such a small sample (6 animals). The neuropathological microscopical examinations (generally 48 sites/tissues reported) were restricted to the control and high dose animals and no effects of treatment were evident. While data comprising the morphometric measurements were provided for mid-dose DPP 66 females (1 mg/kg/d), no neuropathological examinations were reported for this group. These results suggest that the animals had generally recovered from the delayed development that was evident at DPP 12."

Australia 2000b NRA chlorpyrifos summary (based upon analyses in Australia 2000 chlorpyrofos toxicology review): "There was no evidence that significant developmental or neurological effects were caused by chlorpyrifos in young animals at doses below those that inhibited plasma cholinesterase activity." … "The data on effects of chlorpyrifos in young or developing animals have been reviewed and infants and children are not considered to be at an increased risk from chlorpyrifos products that are used according to label instructions."

Concluding Remarks

In addition to the historical control and the biological implausibility analysis of a treatment-related effect on mid-dose female parietal cortex by Drs. Hoberman and Garman (above, supplement 3), fetal and pup cholinesterase inhibition data from the companion study (Mattsson et al., 2000) show this mid-dose parietal cortex effect, if true, would have had to be non-cholinergic in nature.

Section C on non-cholinergic effects presents rabbit data on the implausibility of a major non-cholinergic effect at any dose, and regulatory agency analyses of several other studies show that, regardless of the route of exposure, no treatment-related effects have been reliably demonstrated below those exposures that cause inhibition of cholinesterase.

Thus, the lack of a plausible non-cholinergic mechanism to affect only the parietal cortex at doses that do not affect cholinesterase activity is but another dimension of biological implausibility. Application of the array of principles of toxicology (Section A) to the issue of a treatment-related mechanism for the mid-dose female parietal cortex effect leads to a considerable weight-of-evidence against such a treatment-related conclusion. In summary, the only biologically rational conclusion is that the slightly thinner parietal cortex of mid-dose female rats on 2 months of age was not treatment related.

While the application of the principles of toxicology summarized in Section A are very demanding of toxicologists, a rigorous application of the principles would greatly enhance the scientific stature of this chlorpyrifos toxicology evaluation by OEHHA.

The rigorously evaluated, cumulative weight-of-evidence across Sections B, C and D, in the context of all of the chlorpyrifos safety studies on reproduction and development, acute, subchronic and lifetime studies, and kinetic studies, conducted on several thousand rats, mice rabbits, dogs, monkeys and humans and submitted to regulatory agencies world-wide, should be compelling that the past and current regulation of chlorpyrifos to prevent inhibition of cholinesterase is protective of the health of children as well as adults.

The chlorpyrifos DNT study, properly interpreted, in combination with the current USEPA and previous and current world-wide regulatory reviews of chlorpyrifos, will provide OEHHA with a very strong weight-of-evidence that there is no differential risk of children from the appropriate and labeled use of chlorpyrifos. The NOAEL to derive an RfD for adults and children should be 1 mg/kg/day based upon the chlorpyrifos DNT study and brain cholinesterase inhibition data from subchronic and chronic toxicity studies of chlorpyrifos. Data from human kinetic studies provide assurance that the potency to inhibit RBC cholinesterase is similar between humans and the animal species studied. Uncertainty factors of 10X for within-species differences in sensitivity, and 10X for between species uncertainty should apply. A resulting RfD of 0.01 mg/kg/day for humans, including children, would be consistent with WHO and the EU.

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Appendix III: OEHHA Response to Public Comments on Draft

Response to Comments from Toxicology Excellence for Risk Assessment (TERA)

TERA Comment 1:

The first is that OEHHA misses significant literature, for example, on comparative cholinesterase inhibition in maternal, fetal and neonatal animals.

Citing the following literature appears to us to be mandatory for any evaluation of chlorpyrifos toxicity to the young. The bioassay by Mattsson et al. (2000) (bold-printed below) is particularly important, since it compares cholinesterase inhibition in 5 different organs, at multiple times between dams, and their corresponding fetuses and neonates. Furthermore, the study measures levels of chlorpyrifos and a principal metabolite in blood, so that comparisons can be made on the basis of tissue dose (see Table 5 and Figures 1 and 2 of Zhao et al., 2005).

OEHHA Response 1:

The current OEHHA document is a focused review on studies that explore critical windows of development, child-specific physiological sensitivities, and child-specific exposure parameters for use in the risk assessment. In the process, OEHHA reviewed all available literature and discussed studies that best fit OEHHA's criteria.

Mattsson et al. (2000) exposed female rats to chlorpyrifos by gavage at 0, 0.3, 1, or 5 mg/kg-day from gestational day (GD) 6 to postnatal day (PN) 10. Chlorpyrifos-induced cholinesterase inhibition and the level of chlorpyrifos and its major metabolites in dams and their offspring were studied on GD20, PN1, PN5, and PN11. In the same study and also in a separate article by Zhao et al. (2005), a comparison of cholinesterase inhibition has been made in maternal rats and their offspring on the basis of the blood level of chlorpyrifos. This study and the subsequent analysis by Zhao et al. (2005) were not considered by OEHHA as primary studies for the school site chRD based on the following concerns:

1. The Mattsson et al. (2000) study is based on perinatal chlorpyrifos exposure in rats. The route of exposure in this study was the indirect dosage to the fetus and neonate via the placenta and mother's milk, which creates uncertainties regarding the real form and dose of chlorpyrifos received by the offspring from the maternal rats.

2. Although blood concentrations of chlorpyrifos have been measured so a direct comparison for the pharmacodynamic differences can be achieved at the same blood level of chlorpyrifos, the pharmacokinetic differences still can not be

identified from this type of comparison. In order to draw a conclusion on the age-related difference in chlorpyrifos-induced cholinesterase inhibition, one has to assume that the same absorption and distribution of chlorpyrifos occur in dams and offspring, which is factually incorrect.

3. Samples were collected from dams and fetuses 4 hours postdosing, but from pups only 2 hours after dosing of the dams. It is indicated that the time to peak concentration of chlorpyrifos in the blood following oral exposure is 3 hours; it may also take a few additional hours for the pups to digest the ingested milk (Mattsson et al, 2000; Mendrala and Brzak, 1998; Byczkowski et al, 1994). While peak blood chlorpyrifos level and maximum cholinesterase inhibition may be achieved in dams when analyzed 4 hours postdosing, peak blood levels may not be achieved in pups 2 hours after dosing of the dams. Therefore, cholinesterase inhibition and blood chlorpyrifos levels in dams and their pups, when analyzed at current postdosing times, are not comparable.

TERA Comment 2:

Second, OEHHA does not discuss the concept of critical effect, choosing rather to display many toxicities as if they are somehow not related.

OEHHA Response 2:

OEHHA discussed the concept of critical effect, including cholinesterase inhibition and non-cholinesterase mechanisms of chlorpyrifos, and as OEHHA indicated in the draft, they may not be related.

TERA Comment 3:

Third, OEHHA selects an inappropriate critical study upon which to base its estimate of a safe dose.

OEHHA's choice of critical study, the Jett et al. (2001) subcutaneous study, is not defensible.

In spite of the new analysis (Zhao et al., 2006) and the overwhelming agreement on cholinesterase inhibition as the critical effect, OEHHA does not discuss all the available data on chlorpyrifos toxicity and particularly the work published by these authors. Rather, OEHHA considers that the neurobehavioral effects observed in the subcutaneous study by Jett et al. (2001) are the critical effect upon which the chRD should be based, using the two-year rat studies conducted by Young and Grandjean (1988) and McCollister et al. (1974) as "supporting" studies.

OEHHA Response 3:

OEHHA never used the word *"supporting"* in the document. Young and Grandjean (1988) and McCollister et al. (1974) are not "supporting" studies. OEHHA discussed the use of Jett et al. study and its limitations, including uncertainty regarding the effective dose. The Jett et al. (2001) study is only one of four studies upon which the proposed chRD was based. However, in view of the uncertainty regarding the effective dose, in the current revised draft, OEHHA has reduced the status of the Jett et al. (2001) study to a supporting role.

TERA Comment 4:

As you know, we published a definitive analysis for a chlorpyrifos reference dose (RfD) in 2006 (Zhao et al., 2006). Unfortunately, you only cited our paper in an indirect way. We were most disappointed that our analysis was not given any discussion in yours, especially since we addressed similar issues and came to dramatically different conclusions.

Moreover, whereas OEHHA suggests that the subcutaneous injection studies and the 2-year rat studies offered the best basis of the chRD, we demonstrate that human chlorpyrifos studies should be preferred (Zhao et al., 2006). These human studies provide an adequate and consistent picture of chlorpyrifos toxicity, and are consistent with many other world health organization decisions and guideline documents.

Based on their critical analyses of the available data, Zhao et al. (2006) developed chlorpyrifos RfD of 0.01 mg/kg/day, based on a dose of 0.1 mg/kg/day (the NOAEL) that observed no effect on RBC cholinesterase inhibition in human volunteers (Coulston et al., 1972), with supporting data from Kisciki et al. (1999) and Nolan et al. (1984) studies. A defensible composite uncertainty factor of 10 was applied to the NOAEL to arrive at the RfD.

OEHHA Response 4:

The human studies cited by TERA are limited by small sample size, short exposure duration, the investigation of only one sex, and lack of knowledge on study protocol, which make them inappropriate for use in risk assessment or endpoint selection. Specifically:

- The Coulston et al. (1972) study was conducted on volunteers from a correctional facility in Dannemora, New York. The 25 days recovery period is exceptionally long for plasma ChE. The short exposure period (20 days in the critical exposure groups) adds some uncertainty concerning the NOAEL for long term human exposure. While Zhao et al. (2006) have assumed that the 20-day human exposure in Coulston et al. (1972) study is comparable to the potential longer-term human exposure, they also admit that "this supposition has some uncertainty".

- The Nolan et al. (1984) pharmacokinetic study investigated only one dose level (a single oral dose of 0.5 mg/kg). Moreover, there are concerns regarding the

reliability of the study since the day 3 and day 4 plasma and RBC cholinesterase activity data are considered analytical artifacts by the registrant.

- The Kisicki et al. (1999) study is limited by the lack of plasma cholinesterase inhibition data and the low and variable absorption. The absorbed doses ranged from 19 to 94 percent with an average of 34 percent, which is only half of the absorption rate observed from other human and rodent studies.

TERA Comment 5:

Finally, OEHHA judges uncertainties in its selections of NOAELs as if other data are somehow not available, when in fact the database for chlorpyrifos is replete.

In summary, we believe that there is compelling evidence that there is no increased sensitivity of infants relative to adults and that the database for chlorpyrifos is complete, thereby warranting the removal of the database and FQPA safety (uncertainty) factors for chlorpyrifos safe dose.

OEHHA Response 5:

The question of uncertainty has been extensively discussed in the draft document. Numerous studies provided sufficient evidence to raise concern about the age-related difference in the susceptibility to chlorpyrifos exposure. OEHHA believes that the uncertainty factor of 10 for children is justified under the current evaluation.

TERA Comment 6:

Overall, there appears to be no evidence that oral exposure to chlorpyrifos can cause neurobehavioral effects at doses below those that cause RBC cholinesterase inhibition.

…… *neurobehavioral effects are likely to occur at chlorpyrifos concentrations following oral exposure that are higher than those needed to evoke the critical effect, that is RBC cholinesterase inhibition.*

OEHHA Response 6:

A wealth of information demonstrated the existence of non-cholinergic mechanisms of chlorpyrifos toxicity, which were extensively discussed in the draft document. OEHHA agrees that there is no direct evidence that oral exposure to chlorpyrifos can cause neurobehavioral effects at doses below those that cause RBC cholinesterase inhibition. However, as indicated in the draft document, due to the limited dose selection, many of these studies (the Jett et al. study in particular) failed to identify a NOAEL, which creates uncertainty regarding whether protection against cholinesterase inhibition will also protect against other effects of chlorpyrifos.

TERA Comment 7:

The OEHHA text does not acknowledge that Coulston et al. (1972) human study measured RBC cholinesterase inhibition but that the activity was not inhibited at any dose level. Instead, OEHHA incorrectly reports that no NOEL or LOEL was determined for RBC cholinesterase inhibition in the Coulston et al. (1972) study (see Table 2 of the draft chRD for chlorpyrifos report).

OEHHA Response 7:

In a detailed discussion of the Coulston et al. (1972) study on page 11 of the draft document, OEHHA states, "The RBC cholinesterase activity was unaffected at any dose examined." OEHHA will add this information to Table 2 to ensure that it is more visible.

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Response to comments from Dow AgroSciences (DAS)

***DAS Comment 1:* Global efforts to clarify principles of toxicology as they apply to risk assessment**

OEHHA, in its derivation of a proposed chRD for chlorpyrifos, has not adhered to this global perspective and guidance and has relied almost exclusively on in vitro studies as the basis for the developmental concern, a fact which not only is at odds with global

guidance on the inappropriateness of such, but also ignores the wealth of existing animal studies, many of which were not cited or discussed by OEHHA.

While we appreciate the expertise within OEHHA on the principles of toxicology and the development of hazard identification documents and risk assessments, as a foundation to our comments we feel it is important for the first section to provide an update of universally accepted principles of toxicology for risk assessment and provide a detailed example of the need for sound application of the principles of toxicology.

OEHHA Response 1:

As indicated in the introduction, the current OEHHA document is a focused review of studies that explore critical windows of development, child-specific physiological sensitivities, and child-specific exposure parameters for use in the risk assessment. In the process, OEHHA reviewed all available literatures and discusses both in vitro and in vivo studies that best fit OEHHA's criteria. The critical studies are in vivo studies, with in vitro studies serving a supporting role.

***DAS Comment 2:* Age-related Differences in Detoxification and Cholinesterase Inhibition**

Of those toxicology studies most relevant to risk assessment, the fundamental science and the weight-of-evidence demonstrate the young are not at additional risk of harm from chlorpyrifos under any realistic exposure scenario.

Recent data indicate that genetic differences in human PON1 activity and detoxification of chlorpyrifos have no practical significance in the real world. PON1 has a modest role in detoxification of chlorpyrifos at very high doses, and no apparent role at environmentally relevant doses.

OEHHA Response 2:

Chlorpyrifos detoxification is a complex process involving different enzymes and serum proteins. As discussed in the draft document, a wealth of information demonstrated age-related differences in the detoxification of chlorpyrifos, which include but are not limited to PON1 (paraoxonase-1). The FIFRA Scientific Advisory Panel, during its meeting on September 16-18, 2008, reviewed U.S. EPA's evaluation of the toxicity profile of chlorpyrifos. After review of available data, the Panel agreed with U.S. EPA that PON1 status should not be ruled out as a determinant of chlorpyrifos toxicity, especially for the fetus and young child. In terms of chlorpyrifos-induced cholinesterase inhibition, despite the fact that age-related differences were not evident in some individual studies, numerous studies provided sufficient evidence to raise concern about the differential susceptibility to chlorpyrifos exposure in young vs. adults. OEHHA believes the evidence should not be excluded from the risk assessment process.

***DAS Comment 3:* Non-Cholinesterase Mechanisms of Chlorpyrifos Neurotoxicity**

In summary, both the USEPA (2002a) and the UK ACP (2003) specifically evaluated publications from Slotkin's laboratory for non-cholinergic effects relative to cholinesterase inhibition. Neither agency recognized non-cholinergic effects of a magnitude that caused them to reconsider using cholinesterase inhibition NOAELs for regulation of chlorpyrifos.

Moreover, regulatory agencies have considered non-cholinergic effects as a potentially more sensitive marker of potential toxicity resulting from chlorpyrifos exposure, but have consistently concluded that protection against cholinesterase inhibition is protective against all other potential effects, including non-chlolinergic effects.

OEHHA Response 3:

A wealth of information demonstrated the existence of non-cholinergic mechanisms of chlorpyrifos toxicity, which were extensively discussed in the draft document. OEHHA does not dispute the contention that protection against cholinesterase inhibition may be protective against other potential effects, including non-cholinergic effects. However, as indicated in the draft document, due to the limited dose selection, many of the studies on the non-cholinergic effects of chlorpyrifos failed to identify a NOAEL, which creates uncertainty regarding whether protection against cholinesterase inhibition will also protect against other effects of chlorpyrifos.

***DAS Comment 4:* The Jett et al. study and routes of exposure**

Equally important, OEHHA should not rely on toxicology studies that are inappropriate for risk assessment. The studies selected and used to support the proposed chRD by OEHHA have both unusual dosing regimes and/or routes of exposure, characteristics which render these studies inappropriate for use in establishing Reference Doses that are intended to be relevant to actual exposures to humans.

In summary, the Jett et al. study is inappropriate as it employed a route of exposure not relevant to humans, did not reveal any cholinesterase inhibition (i.e., which prevents a direct evaluation of non-cholinergic effects relative to degree of cholinesterase inhibition), and the data presented have fundamental interpretive problems which preclude OEHHA's conclusion that this study is useful for evaluating potential noncholinergic effects from chlorpyifos exposure.

OEHHA Response 4:

OEHHA discussed the use of Jett et al. study and its limitations, including uncertainty regarding the effective dose. The Jett et al. (2001) study is only one of four studies upon which the proposed chRD was based. However, in view of the uncertainty regarding the effective dose, OEHHA reduced the status of the Jett et al. (2001) study to a supporting role in the present draft.

***DAS Comment 5:* The Hoberman developmental neurotoxicity study (DNT)**

The chlorpyrifos DNT study, properly interpreted, in combination with the current USEPA and previous and current world-wide regulatory reviews of chlorpyrifos, will provide OEHHA with a very strong weight-of-evidence that there is no differential risk of children from the appropriate and labeled use of chlorpyrifos. The NOAEL to derive an RfD for adults and children should be 1 mg/kg/day based upon the chlorpyrifos DNT study and brain cholinesterase inhibition data from subchronic and chronic toxicity studies of chlorpyrifos. Data from human kinetic studies provide assurance that the potency to inhibit RBC cholinesterase is similar between humans and the animal species studied. Uncertainty factors of 10X for within-species differences in sensitivity, and 10X for between species uncertainty should apply. A resulting RfD of 0.01 mg/kg/day for humans, including children, would be consistent with WHO and the EU.

OEHHA Response 5:

While the resulting RfD of 0.01 mg/kg-day from the Hoberman study would be consistent with WHO and the EU, it would not be consistent with ATSDR and USEPA, two leading agencies in the United States; lower RfDs were derived by both agencies which are more consistent with the OEHHA-proposed chRD. The Hoberman developmental neurotoxicity study (DNT) is based on perinatal chlorpyrifos exposure (from gestational day 6 to postnatal day 11) in rats. OEHHA has identified additional uncertainty not considered by DAS, the route of exposure in this study was the indirect dosage to the fetus and neonate via the placenta and mother's milk, which creates uncertainties regarding the real form and dose of chlorpyrifos received by fetus and neonate from the maternal rats and makes it difficult for the study to be used for the calculation of the school site chRD.

***DAS Comment 6:* Chlorpyrifos as a chemical of concern at school sites**

We would also like to make two additional points on the Draft Report regarding the section "What characteristics make chlorpyrifos of concern pursuant to Health & Safety Code Section 901(g)?" (p. 10-11). As cited in the Draft Report, the June 2000 Memorandum of Agreement with U.S. EPA discontinued domestic uses of chlorpyrifos. Subsequently, the potential exposure scenarios at school sites have been significantly reduced. The table below shows the dramatic decrease in domestic uses since some of the data in this section of the Draft Report were collected.

Secondly, Dow AgroSciences disagrees with the characterization in this section that "Additional problems have now surfaced with chlorpyrifos, as it has been found at National Priorities List (NPL) sites." Such a statement implies that the presence of chlorpyrifos at such sites is either indicative of a public risk or at worst implies it was a causative agent in the site being listed. The National Priorities List is a list of superfund sites prioritized for remediation. To characterize chlorpyrifos as being present at NPL sites, and therefore a "problem," and to use this as part of rationale for establishment of a ChRD is, in our opinion, inappropriate. Furthermore, it misstates the facts regarding chlorpyrifos. Chlorpyrifos is not listed in the current U.S. EPA Superfund Chemical Data

Matrix (http://www.epa.gov/superfund/sites/npl/hrsres/tools/scdm.htm), however it is listed (as no longer being listed) in the EPA's Substances No Longer Listed in SCDM (http://www.epa.gov/superfund/sites/npl/hrsres/tools/remscdm.pdf).

OEHHA Response 6:

OEHHA appreciates the update. The relevant sentences have been revised according to the updated information. Despite the decrease in domestic uses of chlorpyrifos, chlorpyrifos is still a concern in California. Many schools in Central Valley agricultural communities are located adjacent to groves with high pesticide use. At OEHHA's DARTIC (Developmental and Reproductive Toxicant Identification Committee) meeting on December 10, 2007, residents from Central Valley agricultural communities expressed their concerns about chlorpyrifos use: "We can taste it." "We can smell it." "There are schools across the street." "We live across the street." While these statements do not prove a threat from chlorpyrifos exists at these locations, the former and continued use of chlorpyrifos does indicate it may need to be considered when assessing potential new and current school sites.

DAS Comment 7:

The USEPA position on chlorpyrifos and differential sensitivity for children has shifted since 2000, and the uncertainty factor (i.e., for differential sensitivity) currently embraced by the USEPA is now 1X (from 10X formerly; Organophosphate Cumulative Risk Assessment, USEPA 2002a and 2006). In making this change, the USEPA (2002a) reviewed many studies including several from the laboratory of Slotkin and colleagues, and concluded that there was little evidence of toxicological effects at doses that did not inhibit cholinesterase. USEPA (2002a) also analyzed data from oral gavage of chlorpyrifos to pups and adults and concluded there was no meaningful difference in sensitivity of brain cholinesterase to inhibition from repeated doses of chlorpyrifos. USEPA (2006) reaffirmed these earlier analyses (USEPA, 2002a), and included an analysis of differential sensitivity from differences in PON1 activity, and concluded that PON1 mechanisms of detoxification were dose related and not a significant factor at environmental levels of exposure. Consequently, USEPA Cumulative Risk Assessment retained the FQPA factor at 1X for chlorpyrifos.

OEHHA Response 7:

The DAS comment is not accurate. The USEPA organophosphate cumulative risk assessment is an analysis of all organophosphates instead of individual chemicals. In term of chlorpyrifos, the document is limited in scope. It focused only on a subset of data, which is mainly cholinesterase inhibition in the brain following repeated exposure (USEPA, 2006; Zheng et al, 2000). The USEPA position on chlorpyrifos is not changed, and the FQPA factor of 10 is still retained by USEPA for any individual risk assessment of chlorpyrifos (personal communication with USEPA on April 7, 2008).

References:

Berkowitz, G. S., J. G. Wetmur, E. Birman-Deych, J. Obel, R. H. Lapinski, J. H. Godbold, I. R. Holzman, and M. S. Wolff. 2004. In utero pesticide exposure, maternal paraoxonase activity, and head circumference. *Environ Health Perspect* 112 (3):388-91.

U. S. EPA (2000) Chlorpyrifos - reevaluation based on phase 3 (public comments) of the TRAC process-Report of the Health Identification Assessment Review Committee, 6 April 2000. HED DOC. No. 014088.

U. S. EPA (2006) Organophosphorus cumulative risk assessment – 2006 update. August 2006.

Zheng, Q., K. Olivier, Y. K. Won, and C. N. Pope. 2000. Comparative cholinergic neurotoxicity of oral chlorpyrifos exposures in preweanling and adult rats. *Toxicol Sci* 55 (1):124-32.

Appendix IV: Dow Agrosciences Comments on Final Draft

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Dow AgroSciences

March 1, 2010

Mr. Leon Surgeon
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DOW AGROSCIENCES COMMENTS ON PROPOSED CHILD-SPECIFIC REFERENCE DOSE (chRD) FOR SCHOOL SITE RISK ASSESSMENT – CHLORPYRIFOS – EXTERNAL DRAFT REPORT, FEBRUARY 2010

Dear Mr. Surgeon;

Dow AgroSciences LLC appreciates the opportunity to respond to the OEHHA's External Draft Report (February 2010): Development of Health Criteria for School Site Risk Assessment Pursuant to Health and Safety Code Section 901(g): Proposed Child-Specific Reference Dose (chRD for School Site Risk Assessment – Chlorpyrifos.

Dow AgroSciences provided comments in February 2008 in response to the External Draft Report dated November 2007 and appreciates the subsequent responses provided by OEHHA in the 2010 External Draft Report. In our comments provided in February 2008, we stated that "Evaluation of child sensitivity to chemical exposure for regulatory purposes, in this case, the possible development of a child-specific reference dose, should use the most relevant and robust study available." We remain steadfast in this opinion and it is our hope that the comments included below will provide the impetus for increased dialogue and a better understanding of the types and breadth of studies which comprise what OEHHA defines as "best fit OEHHA's criteria" (From External Draft, February 2010: "In the process, OEHHA reviewed all available literature and discussed studies that best fit OEHHA's criteria").

Chlorpyrifos possesses a robust and complete toxicological database, as defined by FIFRA requirements and reflecting upon the depth and scope of studies that have been conducted. Dow AgroSciences concurs with OEHHA that when considering development of a chRD that the attention remain on "a focused review on studies that explore critical windows of development, child-specific physiological sensitivities, and child-specific exposure parameters for use in risk assessment." In this regard, while we

believe that a robust set of data and studies exist that inform on the question of young sensitivity from exposure to chlorpyrifos, it is not clear, from a review of the OEHHA documentation related to development of chRDs, what criteria exist or how those criteria are defined which ultimately result in identification of studies considered acceptable for chRD development. To the extent that OEHHA can provide further clarification and delineation of those criteria used for the evaluation of studies, this will enable a greater understanding by the regulated community and may even offer the opportunity for more targeted and relevant testing that in turn can be used by regulatory agencies.

In order to provide an example of the need for greater transparency on what OEHHA defines, and by what criteria, to be a relevant study for use in chRD establishment, we would point to the first comment (posed by TERA) and first response (provided by OEHHA) included in Appendix I of the February 2010 External Draft Report. The essence of this exchange was that respondents from Toxicology Excellence for Risk Assessment (TERA) commented that OEHHA "misses significant literature" specifically the study by Mattsson et al (2000) which included extensive evaluations aimed at identifying differential sensitivity between the young and adults. Specifically, this study compared cholinesterase inhibition (the most sensitive toxicological endpoint of relevance for risk assessment) in 5 different organs, at multiples times, between dams and their corresponding fetuses and neonates.

In response, OEHHA stated that their review focused on studies that 'best fit OEHHA's criteria' and furthermore stated that the Mattsson et al (2000) study was not considered by OEHHA as a primary study for the school site chRD based on the following concerns:

1. The Mattsson et al (2000) study is based on perinatal chlorpyrifos exposure in rats. The route of exposure in this study was the indirect dosage to the fetus and neonate via the placenta and mother's milk, which creates uncertainties regarding the real form and dose of chlorpyrifos received by the offspring from the maternal rats.

2. Although blood concentrations of chlorpyrifos have been measured so a direct comparison for the pharmacodynamic differences can be achieved at the same blood level of chlorpyrifos, the pharmacokinetic differences still cannot be identified from this type of comparison. In order to draw a conclusion on the age-related difference in chlorpyrifos-induced cholinesterase inhibition, one has to assume that the same absorption and distribution of chlorpyrifos occur in dams and offspring, which is factually incorrect.

3. Samples were collected from dams and fetuses 4 hours postdosing, but from pups only 2 hours after dosing of the dams. It is indicated that the time to peak concentration of chlorpyrifos in the blood following oral exposure is 3 hours; it may also take a few additional hours for the pups to digest the ingested milk

(Mattsson et al, 2000; Mendrala and Brzak, 1998; Byczkowski et al 1994). While peak blood chlorpyrifos level and maximum cholinesterase inhibition may be achieved in dams when analyzed 4 hours postdosing, peak blood levels may not be achieved in pups 2 hours after dosing of the dams. Therefore, cholinesterase inhibition and blood chlorpyrifos levels in dams and their pups, when analyzed at current postdosing times, are not comparable.

While there exist a number of studies on chlorpyrifos that can inform on the question of young sensitivity, for which OEHHA has assigned a 10X factor (noting that young were not tested in the studies selected for chRD derivation), until study selection criteria are clearly delineated, questions will remain over what constitutes the best study (studies) for use in risk assessment. This notwithstanding, Dow AgroSciences appreciates the opportunity to share with OEHHA an ongoing study that specifically addresses the three points noted above, in addition to providing sentinel insight on potential differential sensitivity to cholinesterase inhibition. As ongoing support for the federal reregistration review of chlorpyrifos, Dow AgroSciences is conducting a comparative cholinesterase assay, required by the USEPA. In the "Chlorpyrifos Final Work Plan, Registration Review, September 2009", the USEPA notes that an acute and repeated comparative cholinesterase assay (CCA) with the acute and repeated exposures administered to post-natal day 11 (PND11) rats and to young rats for both chlorpyrifos and chlorpyrifos-oxon, will be required. This study will provide information on the cholinesterase-inhibiting activity of chlorpyrifos and chlorpyrifos oxon as well as whether juvenile animals are more susceptible to the cholinesterase inhibition than adult animals. Furthermore, this study will inform on the need for and possible magnitude of a child-specific uncertainty factor which may be helpful in refinement of the child-specific RD for chlorpyrifos. Finally, this study will provide critical insight on the 3 points noted by OEHHA as to why the Mattsson study was not considered useful in derivation of a chRD. Specifically:

1. Direct dosing of chlorpyrifos will occur to both young (required by EPA to be of age PND11) and adult rats, thus, there should be no uncertainty around the real form and dose of chlorpyrifos received by the young animals.

2. This study will be collecting pharmacokinetic data at the time of peak cholinesterase inhibition across different chlorpyrifos dose levels in both young and adult rats; thus insight on pharmacokinetic differences and/or similarities should be apparent.

3. This study has evaluated the time to peak inhibition and Dow AgroSciences has provided these data to EPA which has concurred that this time point selected is valid. Thus, those concerns raised by OEHHA regarding uncertainty around whether peak cholinesterase inhibition had been identified and measured in the Mattsson et al (2000) study will be addressed.

We appreciate the opportunity to contribute to the ongoing dialogue and assessment of chlorpyrifos during OEHHA's chRD development and hope that a more transparent process and delineation of study selection criteria can be made available.

Sincerely,

Daland R. Juberg, Ph.D.
Research Leader/Senior Toxicologist
317-337-3787

cc: Brian L. Bret, Ph.D. State Regulatory Manager

References:

Byczkowski et al. 1994. Computer simulation of the lactational transfer of tetrachloroethylene in rats using a physiologically-based model. Toxicol. Appl. Pharmacol. 125:228-236.

Mattsson et al. 2000. Lack of differential sensitivity to cholinesterase inhibition in fetuses and neonates compared to dams treated perinatally with Chlorpyrifos. Toxicol. Sci. 53:438-446.

Mendrala and Brzak. 1998. Chlorpyrifos: Part A – Concentration-time course of Chlorpyrifos and Chlorpyrifos-oxon in blood. Project study ID 971187A. MRID 44648102.

Appendix V: OEHHA Response to Dow Agrosciences Comments on Final Draft

Dow Comment 1:

……it is not clear, from a review of the OEHHA documentation related to development of chRDs, what criteria exist or how those criteria are defined which ultimately result in identification of studies considered acceptable for chRD development.

In order to provide an example of the need for greater transparency on what OEHHA defines, and by what criteria, to be a relevant study for use in chRD establishment, we would point to the first comment (posed by TERA) and first response (provided by OEHHA) included in Appendix I of the February 2010 External Draft Report. The essence of this exchange was that respondents from Toxicology Excellence for Risk Assessment (TERA) commented that OEHHA "misses significant literature" specifically the study by Mattsson et al (2000)……

OEHHA Response 1:

OEHHA detailed our criteria and methodology for the development of the chRDs in the introduction part of the document. However, OEHHA's concern with the Mattsson et al (2000) study is with the overall limitation and uncertainties associated with the study, as indicated in OEHHA's response to TERA's comments. U.S. EPA also has the same concern regarding the experimental design and uncertainties of the study (U.S. EPA, 2000).

Dow Comment 2:

As ongoing support for the federal Reregistration review of chlorpyrifos, Dow AgroSciences is conducting a comparative cholinesterase assay, required by the USEPA.

This study will provide information on the cholinesterase-inhibiting activity of chlorpyrifos and chlorpyrifos oxon as well as whether juvenile animals are more susceptible to the cholinesterase inhibition than adult animals. Furthermore, this study will inform on the need for and possible magnitude of a child-specific uncertainty factor which may be helpful in refinement of the child-specific RD for chlorpyrifos.

OEHHA Response 2:

OEHHA is aware of the comparative cholinesterase assay (CCA). The comparative cholinesterase assay will be helpful. When the study is completed and the results are available for review, OEHHA will evaluate the findings. Should the evaluation find that the new information has a significant impact on our child-specific RD, OEHHA will, as resources allow, reevaluate our child-specific RD.

We do want to point out that this new study will not cover the non-cholinesterase mechanism of chlorpyrifos neurotoxicity, which is also a big concern for the development of the child-specific reference dose of chlorpyrifos.

References

U. S. EPA (2000) Chlorpyrifos - reevaluation based on phase 3 (public comments) of the TRAC process-Report of the Health Identification Assessment Review Committee, 6 April, 2000. HED DOC. No. 014088.