Lack of Differential Sensitivity to Cholinesterase Inhibition in Fetuses and Neonates Compared to Dams Treated Perinatally with Chlorpyrifos

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Received November 2, 1998; accepted August 20, 1999

Pregnant Sprague-Dawley rats were exposed to chlorpyrifos (CPF; O,O-diethyl-O-[3,5,6-trichloro-2-pyridinyl] phosphorothioate) by gavage (in corn oil) from gestation day (GD) 6 to postnatal day (PND) 10. Dosages to the dams were 0 (control), 0.3 (low), 1.0 (middle) or 5.0 mg/kg/day (high). On GD 20 (4 h post gavage), the blood CPF concentration in fetuses was about one half the level found in their dams (high-dose fetuses 46 ng/g; high-dose dams 109 ng/g). CPF-oxon was detected only once; high-dose fetuses had a blood level of about 1 ng/g. Although no blood CPF could be detected (limit of quantitation 0.7 ng/g) in dams given 0.3 mg/kg/day, these dams had significant inhibition of plasma and red blood cell (RBC) ChE. In contrast, fetuses of dams given 1 mg/kg/day had a blood CPF level of about 1.1 ng/g, but had no inhibition of ChE of any tissue. Thus, based on blood CPF levels, fetuses had less cholinesterase (ChE) inhibition than dams. Inhibition of ChE occurred at all dosage levels in dams, but only at the high-dose level in pups. At the high dosage, ChE inhibition was greater in dams than in pups, and the relative degree of inhibition was RBC > plasma > heart > brain (least inhibited). Milk CPF concentrations were up to 200 times those in blood, and pup exposure via milk from dams given 5 mg/kg/day was estimated to be 0.12 mg/kg/day. Therefore, the dosage to nursing pups was much reduced compared to the dams exposure. In spite of exposure via milk, the ChE levels of all tissues of high-dose pups rapidly returned to near control levels by PND 5.

Key Words: Dursban; chlorpyrifos; chlorpyrifos-oxon; trichlopyridinol; blood; milk; cholinesterase; rats; dams; fetuses; pups.

Chlorpyrifos (CPF) is a widely used organophosphorous insecticide, which has engendered much debate about whether to apply an additional safety factor to protect fetuses and neonates (Campbell et al., 1997; Gibson et al., 1999; Moser and Padilla, 1998; Schardein and Scialli, 1999). Effects in young animals appear to vary with route of exposure and with dosage levels, and single versus repetitive doses. Direct exposure of nursing rats to single doses of CPF has resulted in lethality, toxicity, and significant inhibition of ChE at appreciably lower doses than in adult rats (Moser and Padilla, 1998; Moser et al., 1998; Pope and Chakraborti, 1992; Pope et al., 1991; Pope and Liu, 1997; Whitney et al., 1995). The differences in sensitivity, however, appeared to be dose-related. Pope et al. (1991) found the maximum-tolerated dose (MTD) of subcutaneously (sc) administered CPF in 7-day-old pups to be about 6 times less than in adults (pup MTD = 45 mg/kg; adult MTD = 279 mg/kg), while Pope and Chakraborti (1992) reported about a two-fold difference in sensitivity at the dose necessary to inhibit 50% of brain ChE (pup EDso = 19.9 mg/kg; adult EDso = 44 mg/kg).

Recent information on repeated dosing, available only by abstract, indicates that oral CPF exposure of 7-day-old rat pups and adults for 14 days resulted in ChE inhibition EDso values of 1 to 2 mg/kg/day for pups (similar for all tissues), and 0.5 to 6.6 mg/kg/day for adults (RBC < plasma < brain < dia phragm). Brain ChE EDso values were comparable for pups and adults (1.9 to 2.4 mg/kg/day) (Zheng et al., 1999). In a similar repeated-exposure paradigm, heart muscle ChE was also comparably inhibited in pups and adults (EDso ≈ 1 mg/kg/day) (Stiles and Pope, 1999).

When pregnant rats were treated daily with CPF, their fetuses, exposed via maternal circulation, typically had less ChE inhibition than their dams (Chanda and Pope, 1996; Chanda et al., 1995; Lassiter et al., 1998). When rat dams were given a single dose of CPF, and fetal and maternal tissues were sampled at the time of peak inhibition (5 h post-exposure), brain ChE inhibition was only slightly less for fetuses than for dams (Lassiter et al., 1998).

Schardein and Scialli (1999) reviewed a number of CPF reproduction and developmental studies, which included teratology studies in 3 species, two 2-generation studies, a 3-generation study with a teratology component, and a developmental neurotoxicity study. In guideline U.S. EPA reproduction and teratology studies, the dams are treated and fetuses are exposed via maternal circulation and nursing pups via milk. The authors concluded that CPF “...did not adversely affect reproduction and was not developmentally neurotoxic or teratogenic, and no selective toxicity or sensitivity of the fetus or young animals was apparent in any guideline studies that were scientifically acceptable.”

There appears to be a significant difference in sensitivity of
the young when exposed directly versus exposure via maternal circulation and via nursing. The differences in routes of exposure raise the question of the level exposure to fetuses and neonates when they are exposed via the mother. To address this question, we have utilized recent advances in the ability to quantify low concentrations (>0.7 ng/g) of CPF and CPF-oxon in blood and milk (Brzak et al., 1998). CPF is metabolized by P-450 enzymes to either CPF-oxon (which inhibits cholinesterase, ChE) or to 3,5,6-trichloro-2-pyridinol (TCP). CPF-oxon is hydrolyzed to TCP by A-esterases. Consequently, blood and urine contain TCP from both the parent CPF and from CPF-oxon (Sultatos et al., 1984). The following experiment, therefore, addresses fetal and nursing pup exposure to CPF and CPF-oxon, and consequent ChE inhibition, as results from daily gavage of dams with CPF during gestation and lactation. The doses and time points were selected to match those of a recently conducted U.S. EPA guideline developmental neurotoxicity study of CPF (Hoberman, 1998).

MATERIALS AND METHODS

Chlorpyrifos (O,O-diethyl, O-(3,5,6-trichloro-2-pyridyl)phosphorothioate), purity 99.8% ± 0.1%, was dissolved in corn oil and administered by gavage at a volume of 1 ml/kg to Sprague-Dawley rats (CD; Charles River Breeding Laboratories, Portage, MI). Rats were gavaged daily from gestation day (GD) 6 to postnatal day (PND) 10. Dosage levels of CPF were 0 (control), 0.3 (low), 1 (middle) or 5 mg/kg/day (high). Dosages were adjusted daily for body weight changes and given at approximately the same time each morning. In dams and fetuses/pups, laboratory evaluations were made on GD 20, PNDs 1, 5, and 11 as follows: blood CPF, CPF-oxon, and 3,5,6-trichloro-2-pyridinol (TCP); and ChE analyses of plasma, RBC, heart and brain. CPF and CPF-oxon analyses were conducted on milk from PNDs 1, 5, and 11. Only ChE analyses were conducted on dams and pups on PND 22, and on pups only, as adults, on PND 65. Separate groups of time-mated dams were used for each time period. GD 0 was the day on which a vaginal plug was detected. PND 0 was the day of birth.

Dams were randomly assigned to treatment groups using a computer program and were uniquely identified via subcutaneously implanted transponders (BioMedic Data Systems, Searford, DE). Pups randomly selected for the study were identified on PND 4 by a tattoo. After weaning, the pups were uniquely identified via subcutaneously implanted transponders. Allocation of the animals to the different subgroups of activities or measurements (i.e., CPF vs. ChE determination for dams and pups, age at testing) was done randomly, using a computer program. Rats were provided Purina Certified Lab Diet #5002 (PMI Feeds Inc., St. Louis, MO) in meal form and municipal drinking water ad libitum. This study followed the requirements of the Guide for the Care and Use of Laboratory Animals (NRC, 1996), and complied with Good Laboratory Practice regulations (USEPA, 1989). In response to the U.S. Animal Welfare Act that was promulgated by the U.S. Department of Agriculture (CFR, 1985), the Animal Care and Use Activities that were required for the conduct of this study were reviewed and approved by the Institutional Animal Care and Use Committee.

Daily Observations

The exposure regimen in this study was the same as the one used in a developmental neurotoxicity study (Hoberman, 1998) done according to United States Environmental Protection Agency guidelines (USEPA, 1991). A major difference between studies was that clinical examinations were conducted 3 to 4 h post-exposure in the developmental neurotoxicity study, and just before daily gavage in this study. Observations 3 to 4 h post-exposure were optimized to record the presence of daily transient cholinergic effects, while observations before daily gavage in this study were limited to those necessary to care for the animals and were not designed to be dependent variables. All animals were evaluated for morbidity, moribundity, mortality, and the availability of feed and water. Alterations in fecal and urine output, skin, fur, mucous membranes, respiration, central nervous system function, and animal behavior were recorded. Visible physical abnormalities or behavioral changes in the neonates were recorded daily during the lactation period.

Litter Information

Litters born overnight were considered to have been delivered on the morning they were found. On PND 4, the goal was to have a balanced sex ratio for all litters (i.e., 5 males and 5 females). The pups were sexed and the litters with too few males or females were supplemented with pups from other litters (same treatment level) and then culled to a sex ratio of 5 males and 5 females. These supplemental pups were clearly identified, were for litter-size only, and were not used for any testing. On PND 11, the litters were randomly culled to eight pups (4 males and 4 females). All litters were weaned on PND 21.

Collection of Specimen

On GD 20 and PNDs 1, 5, and 11, samples of blood (dams and pups) were tested for CPF, CPF-oxon, and TCP, samples of milk for CPF and CPF-oxon, and samples for ChE activity were collected from plasma, RBC, heart, and two areas of brain (fore- and hindbrain, divided between the cerebellum and cerebral hemispheres). For each time period, 5 dams per dose group were assigned to maternal ChE analyses, and 5 to maternal CPF chemistry analyses. Fetuses (or pups) from each of the 10 dams per dose were assigned by sex to either ChE analyses or CPF chemistry so that, by counterbalancing, each litter provided either one male or one female for a particular analysis. As a result, the sample size for each test was 5 males and 5 females per dose level, each from separate litters. One fetus or pup was used for blood CPF/CPF-oxon analyses (minimum 0.1-ml blood), one for blood TCP analyses (minimum 0.1-ml blood), and one for ChE analyses (minimum 0.25-ml blood). When necessary on GD 20 and PND 1, the blood (for ChE analysis) of one or more male or female pups/fetuses of the same litter was pooled together to have the minimum of 0.25 ml of blood (only one pup was necessary on later test days). Pooled samples were considered as one male or one female sample per litter. If several pups/fetuses were used for blood collection, brain and heart samples were also pooled.

The timing of the samples was as follows: On GD 20, 4 h after gavage dosage, 10 dams were anesthetized with intraperitoneal (ip) ketamine (100 mg/kg) and xylazine (32 mg/kg), with heparin (2000 IU/kg). Blood samples were taken by cardiac puncture from the dams and by abdominal aortic puncture from the fetuses. Dams and fetuses were then killed and brain and heart tissue collected for ChE analysis. On PNDs 1 and 5, pups were separated from the dams 2-h post-dosing. Pups were anesthetized with ip ketamine (80 mg/kg) and xylazine (25 mg/kg), with heparin (2000 IU/kg). Blood samples were taken by abdominal aortic puncture. The pups were then killed and brain and heart tissue collected for ChE analysis (5 males and 5 females per dose level, one pup per litter). Dams were anesthetized 4-h post-dosing (and 2 h after pups had been removed to allow time for milk to accumulate). Blood and tissues were collected as on GD 20, except that dams first received an injection of oxytocin (2 IU), and milk was collected from the nipples by gently squeezing the gland and the nipple area. As much milk as feasible was collected from each nipple. A total of up to 0.5 ml of milk was collected per animal. Tap water was used as a lubricant to facilitate the procedure and to minimize bruising to the dam. Dams and pups were treated similarly on PND 11, except that dams were last dosed on PND 10. Therefore, on PND 11, the pups were separated from the dams and then anesthetized at 24-h post-dosing, and dams were anesthetized at 26-h post-dosing. Only ChE analyses were conducted on PND 22.

Analyses for CPF and CPF-Oxon

Chlorpyrifos, chlorpyrifos-oxon, 3,5,6-trichloro-2-pyridinol, and the internal standards (chlorpyrifos- 13 C 2 -15 N, chlorpyrifos-oxon- 13 C 2 -15 N, 3,5,6-tri...
chlooro-2-pyrindinol-\(^{13}\)C\(_2\) and 3,5,6-trichloro-2-pyrindinol-\(^{13}\)C\(_2\),\(^{15}\)N) were obtained from Dow AgroSciences (Indianapolis, IN). The stable isotopes were labeled in the 2-\(^{13}\)C, 1-\(^{15}\)N, and 6-\(^{13}\)C positions of the pyridinol ring for the internal standards. The silylation reagent (N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide, MTBSTFA) was obtained from Aldrich Chemical Company (Milwaukee, WI). Milk or blood was collected directly into a tared vial containing an acidified saturated salt solution to minimize degradation of the CPF-oxon (Brazak et al., 1998). The milk or blood specimen (0.1 to 0.5 ml) was mixed well on a vortex mixer and the vial re-weighed to obtain an accurate sample weight. An aliquot of an acetonitrile solution containing the CPF and CPF-oxon internal standards was added and the sample mixed briefly. The analytes were extracted into 0.25-ml methanol and 1.5-ml hexane by mixing for approximately 5 min on a vortex mixer, followed by centrifugation to separate layers. The hexane layer was transferred to a clean vial and centrifuged to dryness at room temperature with nitrogen. For milk, the residue was reconstituted in acetonitrile and transferred to a C\(_{18}\) solid-phase extraction column. The analytes were further eluted with an additional volume of acetonitrile. The total milk column eluent was collected and the acetonitrile was evaporated under nitrogen. For either milk or blood, the final residue was reconstituted in 50-\(\mu\)l toluene, for analysis by gas chromatography/mass spectrometry (GC/MS). Chromatographic separations were achieved on a DB-17 capillary column (J&W Scientific, Folsom, CA) or a ZB-50 capillary column (Phenomenex, Torrance, CA). The mass spectrometer was operated in the negative-ion chemical-ionization mode, monitoring selected ions for CPF, CPF-oxon, and the internal standards. Solvent standards were prepared in toluene and analyzed for quantitation of the analytes. Appropriate fortified samples were prepared in blood or milk from control animals to determine recoveries of the analytes. Blood was fortified with 0.9 to 126 ng/g CPF, and recoveries averaged 107% (range 102 to 110%). Blood was fortified with 0.9 to 125 ng/g CPF-oxon, and recoveries averaged 102% (range 95 to 104%). Milk was fortified with 0.9 to 252 ng/g CPF, and recoveries averaged 104% (range 101 to 106%). Milk was fortified with 0.9 or 4.6 ng/g CPF-oxon, and recoveries averaged 97% (range 72 to 109%). The limit of quantification was 0.7 ng/g blood or milk.

**CPF time-course data.** A separate experiment was conducted to determine the CPF time of peak concentration and exposure area under the curve (AUC) for a single gavage dose of CPF in corn oil to adult male Fischer 344 rats at dosage levels of 0, 0.5, 1.0, 5.0, or 10 mg/kg (\(n = 4\) per time point and dosage level). Blood was collected from anesthetized rats, by cardiac puncture, into heparinized syringes containing an acidic salt solution. Samples were taken post gavage at 10 and 20 min, and at 1, 3, 6, and 12 h. CPF analytical methods were the same as above.

**Analyses for TCP.** A blood sample (~0.1 ml) was placed into a tared vial containing an aliquot of a saturated salt solution and concentrated HCl. The solutions were mixed well by vortexing and the sample re-weighed to obtain an accurate sample weight. An aliquot of an acetonitrile solution containing the TCP internal standard was added and the solution mixed briefly. Extraction of the analytes was accomplished by addition of 2.5-ml ethyl acetate and mixing approximately 5 min on a vortex mixer, followed by centrifugation to separate layers. The hexane layer was transferred to a clean vial and centrifuged to dryness at room temperature with nitrogen. For milk, the residue was reconstituted in acetonitrile and transferred to a C\(_{18}\) solid-phase extraction column. The analytes were further eluted with an additional volume of acetonitrile. The total milk column eluent was collected and the acetonitrile was evaporated under nitrogen. For either milk or blood, the final residue was reconstituted in 50-\(\mu\)l toluene, for analysis by gas chromatography/mass spectrometry (GC/MS). Chromatographic separations were achieved on a DB-17 capillary column (J&W Scientific, Folsom, CA) or a ZB-50 capillary column (Phenomenex, Torrance, CA). The mass spectrometer was operated in the negative-ion chemical-ionization mode, monitoring selected ions for CPF, CPF-oxon, and the internal standards. Solvent standards were prepared in toluene and analyzed for quantitation of the analytes. Appropriate fortified samples were prepared in blood or milk from control animals to determine recoveries of the analytes. Blood was fortified with 0.9 to 126 ng/g CPF, and recoveries averaged 107% (range 102 to 110%). Blood was fortified with 0.9 to 125 ng/g CPF-oxon, and recoveries averaged 102% (range 95 to 104%). Milk was fortified with 0.9 to 252 ng/g CPF, and recoveries averaged 104% (range 101 to 106%). Milk was fortified with 0.9 or 4.6 ng/g CPF-oxon, and recoveries averaged 97% (range 72 to 109%). The limit of quantification was 0.7 ng/g blood or milk.

**Cholinesterase Determination**

A total of 0.25 ml of blood was collected from pups and 0.5 ml from adults into heparin-treated tubes. Plasma was separated from RBCs by centrifugation as soon as possible after blood collection. The anesthetized rats were then decapitated. The heart (atria and ventricles) and brain were removed, and the brain was dissected into fore- and hindbrain. Each sample was weighed, homogenized with a Polytron (Lucerne, Switzerland), and frozen at –80°C until analyzed. ChE assays were conducted using a Hitachi 911 Automatic Analyzer (Boehringer Mannheim, Indianapolis, IN). All tissues were homogenized and diluted to 1:10 in 0.1 M sodium phosphate buffer (pH 8) with 1% Triton X-100 added, except for plasma which was undiluted. Further dilution of some tissue was necessary for analysis on the automated analyzer using the original 1:10 tissue homogenate in 0.1 M sodium phosphate buffer (pH 8) to yield 1:25 or 1:50 final dilutions. All ChE determinations were made using the modified method described in Hunter et al. (1997).

**Statistics**

ChE absorbance values were evaluated statistically. A multivariate analysis of variance (MANOVA) was used for the dams’ brain and blood ChE data, and had one factor (dosage) and 2 sets of dependent variables (forebrain and hindbrain ChE, or plasma and RBC ChE), and were performed at each time point. The dams’ heart data were analyzed by analysis of variance (ANOVA) at each time point. The Pillai trace statistic was used to determine the statistical significance of the MANOVAs. Pups’ data were statistically analyzed in the same manner as dams’ data with the exception that sex was an additional factor. The minimum number of derived \(p\) values was greater than 150. The acceptable type I error rate for statistical significance was set at 0.02 per MANOVA or ANOVA. Means (±SD), but no inferential statistics, were calculated for CPF and CPF-oxon concentrations in blood and milk, or for TCP in blood. Means (±SD) were calculated when at least 3 of the 5 values were above the limit of quantification (loq). Because values below the loq are progressively unreliable, a value of one-half the loq (0.4 ng/g for CPF/CPF-oxon; 5 ng/g for TCP) was assigned instead of the measured value.

**RESULTS**

**Clinical Observations**

Dams were examined just before daily gavage and did not show clinical evidence of toxicity. High-dose dams had lower weight gain for a few days after birth (18% less gain when compared to controls). Pup deaths were few on PND 0 (control 0.6%, high-dose 0.7%). On PNDs 1 to 4, there were more deaths among pups of high-dose dams than among controls (per day: control range 0.3% to 1.1%; high-dose range 2.2% to 4.5%). There were no deaths in high-dose pups on PND 5, and only a few random deaths thereafter.

**Blood and milk CPF and CPF-oxon concentrations (Fig. 1).** Blood and milk levels of CPF and CPF-oxon were assayed on GD 20, on PNDs 1 and 5 in dams 4 h post-dosing, and on PND 11 approximately 26 h post-dosing. CPF was readily detected in the blood of high-dosage dams when measured 4 h after each dose, but not 26 h post exposure on PND 11 (Fig. 1). CPF was detected in the blood of mid-dosage dams only on GD 20 (Fig. 1), and at no time point in low-dosage dams. The dose-response pattern was disproportional. The mid- to high-dosage ratio was 5, while the blood CPF ratio was 43 at these dosages. CPF-oxon was not detected in blood of dams at any dosage level. There was no attempt to measure CPF or CPF-oxon in the brain.

CPF, but not CPF-oxon, was also detected in the milk of the dams (Fig. 1), and CPF in milk showed a strongly dispropro-
tional increase with dosage. Therefore, nursing pups had a measurable exposure to CPF via the milk.

In fetuses and pups, blood levels of CPF and CPF-oxon were assayed on GD 20 at 4-h post-dosing (contemporaneous with their dam’s), on PNDs 1 and 5 at 2-h post-dosing, and on PND 11 approximately 24-h post-dosing. CPF levels (ng/g blood) in fetuses and pups are shown in Figure 1 (males and females combined). CPF was detected in the blood of mid- and high-dosage-group fetuses on GD 20, with a mean ± SD for high-dosage males = 53 ± 25; high-dosage females = 39 ± 13; mid-dosage males = 1.0 ± 0.4; and mid-dosage females = 1.2 ± 0.3. All CPF levels measured at GD 20 at 1 and 5 mg/kg/day were above the limit of quantification except for one male fetus at 1 mg/kg/day. On PND 1, only the high-dosage group had quantifiable levels of CPF (males = 18 ± 25; females = 6.6 ± 8.0). CPF was not quantifiable in any dosage group on PNDs 5 or 11.

CPF-oxon (ng/g blood) was quantifiable in GD-20 fetal blood in the high-dosage group (males = 1.0 ± 0.6; females = 0.9 ± 0.4). No CPF-oxon was quantifiable at any other time point or dosage level (limit of quantification of 0.7 ng/g).

ChE inhibition data (Fig. 2). ChE was profoundly inhibited in all tissues in high-dosage dams, and clear differential inhibition of tissues occurred in mid-dosage dams with RBC = plasma ≥ heart > brain (least inhibited). Low-dosage dams had significant inhibition of only plasma and RBC ChE. Data from male and female pups were combined for illustration purposes, because of the lack of sex-by-treatment statistical interactions at any time point. There were no treatment-versus-control differences on PND 65, and these data are not reported. ChE inhibition only occurred in high-dosage fetuses and pups, which showed rapid recovery toward control values by PND 5. By PND 11, only RBC ChE was significantly inhibited. The differential inhibition of ChE appeared to be RBC = plasma ≥ heart > brain (least inhibited). ChE inhibition clearly was greater in dams than in fetuses or pups (Fig. 2), and the pup no-observed-effect level was 1 mg/kg/day of maternal exposure.

TCP (ng/g) was present in a dose-proportional manner in the blood of all dosage groups on GD 20 and PNDs 1 and 5. Because TCP levels in dams were similar on these test days, the values were averaged for presentation. Fetal and pup TCP values decreased with time, but male and female fetal and pup TCP values were comparable at each time point and were averaged for presentation in Table 1. In dams, TCP levels on PND 11 (which was about 26 h after the last treatment on PND 10) were much lower than during treatment. Thus, TCP was cleared rapidly from blood. There was no attempt to measure TCP in the milk or in the brain. Blood TCP levels in fetuses and pups were proportional to dosage. On GD 20, fetal TCP levels were 92 to 76% of the dam’s at all dosage levels. Blood TCP levels were much less than dam’s during nursing.
**FIG. 2.** ChE activity in dams, fetuses and pups (% of control activity). Male and female fetal and pup data were combined. ChE was inhibited in a pattern RBC ≈ plasma ≈ heart > brain (least inhibited). Inhibition was greater in dams than in fetuses or pups. The no-observed-effect level for pups was 1 mg/kg/day maternal exposure. *Indicates p values <0.02.

**CPF time-course data.** The data from adult Fischer 344 rats are plotted in Figure 3. The limit of quantification was 0.7 ng/g blood. The time of peak CPF concentration was 3 h at all dosage levels where quantification was possible. No CPF was detectable at any time in rats given 0.5 mg/kg, and only at 3 h in rats given 1 mg/kg. In rats given 5 mg/kg, CPF was detected only at 1, 3, and 6 h; CPF was non-quantifiable at 10 min and 20 min. In rats given 10 mg/kg, CPF was not quantifiable at 10 or 20 min, but was quantifiable at all time points thereafter. The estimated area under the curve (AUC) at 5 mg/kg, from 0 to 12 h, was 153 ng h/ml, or an average of 12.75 ng/ml for 12 h. It was assumed that the quantity at 12-h post gavage was one-half the quantification limit (i.e., 0.35 ng/g). A similar 12-h value would be acquired by following the rate of decline from 3 to 12 h for the 10 mg/kg dosage level, adjusted to the 5-mg/kg data. The 0- to 12-h AUC for 10-mg/kg dosage level was 375-ng h/ml.

**DISCUSSION**

When clinical examinations were conducted 3 to 4 h post-exposure in the developmental neurotoxicity study (Hoberman, 1998), dams gavaged at 5 mg/kg/day showed clear evidence of maternal toxicity at the end of gestation and the first few days after birth (fasciculations, hyperpnea, hyper-reactivity). In our study, dams were examined just before gavage and did not show clinical evidence of toxicity. The clinical findings of the two studies cannot be directly compared because of the different timing of the examinations, but finding clinical effects at 3 to 4 h post-exposure would be consistent with expected peak levels of blood CPF (Fig. 3) and time of maximal inhibition of ChE as seen in Lassiter et al. (1998). High-dose dams in our study had lower weight gain for a few days after birth (18% less gain when compared to controls). A similarly diminished weight gain was also noted in the developmental neurotoxicity
study of Hoberman (1998). This author also reported increased mortality, between PND 1 and 5, in pups from the 5-mg/kg/day dose level; this was interpreted as likely being due to maternal toxicity.

It can be readily seen in Figure 2 that ChE inhibition in all tissues was greater in dams than in fetuses or pups. Dams had plasma and RBC ChE inhibition at 0.3 mg/kg/day, and ChE inhibition in all tissue at 1 and 5 mg/kg/day, while fetuses and pups had inhibition of ChE only at 5.0 mg/kg/day. The day of maximal effects in offspring, at 5-mg/kg/day maternal dosage, occurred during gestation (GD 20). Dams had ChE inhibition at >3× lower dose than fetuses (0.3 mg/kg/day was an effect level for dams and there was no ChE inhibition in fetuses from dams given 1 mg/kg/day). Similar findings have been reported for repeated maternal dosing by Chanda and Pope (1996) and Lassiter et al. (1998). ChE inhibition followed the order of RBC > plasma > heart > brain (least inhibited), and this order of inhibition is most apparent in dams when the data are viewed across dose levels in Figure 2. In 5 mg/kg/day offspring, the high level of ChE inhibition obscured the differences between plasma, RBC and heart, but the lesser ChE inhibition of the brain was apparent.

Dams clearly had more inhibition of ChE than fetuses, and because so little CPF-oxon was found, both ChE inhibition and blood CPF concentrations were used as surrogates of oxon exposure. Some of the maternal:fetal difference in ChE inhibition may have been due to pharmacokinetics. At 5 mg/kg/day, 4-h post-exposure on GD 20, CPF blood levels in dams were about 110 ng/g, and in fetuses were about 40 to 50 ng/g (Fig. 1). At 1 mg/kg/day, CPF levels in dams were about 2.5 ng/g, and in fetuses about 1.1 ng/g. CPF vs. time data (Fig. 3) indicated that 4-h post-exposure blood content should be near the maximal level for gavage exposure to CPF. In support of this timing, a rat gestational exposure study by Lassiter et al. (1998) showed maximal inhibition of both dam and fetal brain ChE at about 5 h post-exposure. Consequently, 4-h post-exposure should be about the time of peak CPF levels, and at this time the dams’ blood CPF levels were about twice as high as that of the fetuses.

The measurement of lower CPF levels in the blood of fetuses may be germane to conclusions about maternal protection (reduced exposure to fetuses) of Lassiter et al. (1998). They presented maternal and fetal brain ChE inhibition data (their Fig. 8) after dams were dosed by gavage on GD 18, and tissues

### TABLE 1

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<td>nq</td>
<td>10 ± 5</td>
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<td>nq</td>
<td>45 ± 13</td>
</tr>
</tbody>
</table>

*Note.* nq = non-quantifiable, limit of quantification 10 ng/g.

![FIG. 3. Time course of blood CPF in adult male Fischer 344 rats given a single gavage dose of CPF in oil. Time of peak concentration was 3 h. CPF was non-quantifiable (nq) at 10 min and 20 min at all dosage levels. The area under the curve (AUC) for a 5 mg/kg dose was 153 ng h/ml, or an average of 12.75 ng/ml for 12 h. The AUC for 10 mg/kg was 375 ng h/ml.](image-url)
were sampled 5 h later, at the time of peak ChE inhibition and before appreciable ChE re-synthesis could occur. At 7 mg/kg, dam brain ChE was inhibited about 32% and fetuses about 19%, and at 10 mg/kg, the dam’s ChE was inhibited about 51% and fetuses about 37% (ChE values estimated visually from Fig. 8). Although dam and fetal brain ChE inhibition were not statistically different, the lesser inhibition in fetuses was repeatable across doses and fetal inhibition at 10 mg/kg was a close match to maternal inhibition at 7 mg/kg. The authors, however, stated that both the dams and fetuses were comparably sensitive to ChE inhibition from a single maternal dose of CPF. It was concluded that the lesser inhibition of ChE in fetuses in repeated-dose studies was due to the high rates of re-synthesis of fetal ChE that resulted in less net inhibition of fetal ChE. Although high rates of ChE synthesis are important, the blood CPF data presented above suggest that fetuses also have a lower exposure than their dams, since blood CPF levels were about 2× higher in dams than fetuses (Fig. 1).

For GD 20, when one compares ChE inhibition with blood-CPF levels across dosage levels, dams given 0.3 mg CPF/kg/day had a blood-CPF concentration of less than 0.7 ng/g (limit of quantification) and fetuses of dams given 1 mg/kg/day had a higher blood-CPF concentration of 1.1 ng/g. Although fetuses had a higher blood-CPF concentration, no inhibition of ChE was noted in any tissue. In contrast, dams with lower blood-CPF concentrations had clear inhibition of RBC ChE. These data are aggregated in Figure 4 to make the exposure-response relationships more clear. It appears that, at the blood-CPF concentrations measured at 4 h post-exposure, the fetuses were not more sensitive to ChE inhibition than dams, and may have been somewhat less sensitive. It is unlikely that quantifiable levels of CPF would have been found at any time in dams given 0.3 mg/kg/day, since adult male rats given 0.5 mg/kg did not have detectable blood levels of CPF at any time (Fig. 3). Therefore, dams had inhibition of plasma and RBC ChE at blood-CPF levels that caused no inhibition of ChE in fetuses.

In contrast to lower blood-CPF concentrations in fetuses, CPF-oxon (ng/g blood) was quantifiable in GD-20 fetal blood in the high-dosage group (males = 1.0 ± 0.6; females = 0.9 ± 0.4) but not in dams or nursing pups at any other time point or dosage level (limit of quantification 0.7 ng/g). The presence of oxon in high-dosage GD-20 fetuses but not dams may be related to slightly different times of maximal concentration with age, since the limit of quantification (0.7 ng/g) was close to the measured values in fetuses. High-dose GD-20 fetuses had about half the blood CPF of their dams, but had comparable or more CPF-oxon. This pattern would be consistent with lower oxon detoxification capacity of fetuses (Atterberry et al., 1997; Lassiter et al., 1998; Mortensen et al., 1996; Moser et al., 1998). However, the higher levels of oxon in high-dose fetuses did not translate into lower levels of brain ChE activity when compared to their dams (Fig. 2).

The data in Figures 2 and 4 indicate that low- and mid-dose fetuses were not exposed to sufficient levels of CPF-oxon to cause inhibition of RBC ChE or ChE of other tissues. While dams had inhibition of RBC ChE at 0.3 and 1 mg/kg/day, their GD-20 fetuses did not. RBC ChE was selected for this illustration because of its considerable sensitivity to inhibition from CPF-oxon, and because it typically is the slowest to recover from inhibition (our Fig. 2; Pope et al., 1991; Moser and Padilla, 1998). Although some masking of CPF-oxon inhibition of brain ChE might occur from the high rate of brain ChE synthesis of fetuses and neonates (Lassiter et al., 1998; Pope et al., 1991; Pope and Liu, 1997), the slower recovery of RBC ChE compared to brain ChE should mitigate this masking.

While the lower levels of ChE inhibition in fetuses are not readily explained by current knowledge about enzymes important to CPF activation and CPF/CPF-oxon detoxification, there are other poorly understood factors which may be important. One might readily expect differences in maternal and/or fetal protein binding of CPF, and pregnancy-associated hyperlipidemia, which might affect the distribution and metabolism of CPF and CPF-oxon. Overall, however, given that ChE activity is a balance between rates of loss (whether natural or induced) and rates of synthesis, the lesser inhibition of brain ChE in high-dose GD-20 fetuses than in their dams may indicate that fetuses have a net ability to sustain ChE activity during repeated CPF exposure that is comparable to their dams.

Blood CPF levels in dams were more than 7-fold greater just before birth (GD 20) than just after birth (PND 1 or 5) (Fig. 1). The reason for these high levels of blood CPF levels just before...
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birth is unknown, but they may be at least partly related to the high lipophilicity of CPF and the hyperlipidemia that occurs during pregnancy (Herrera et al., 1988, 1994). During lactation, the dam’s blood CPF levels were similar to but slightly lower than in comparably dosed male Fischer 344 rats (Fig. 3). The slightly lower values in lactating dams than in males would be consistent with additional loss of CPF into milk fat. There also was a large amount of disproportionality between administered dose and blood levels of CPF. On GD 20, blood CPF concentrations were more than 40 times greater in dams given 5 mg/kg/day (108.8 ng/g blood) than those dams given 1 mg/kg/day (2.55 ng/g blood), a ratio of 40:5 (Fig. 1). Blood CPF levels were much lower during lactation, but the high-dose:mid-dose blood CPF ratio was still at least 15 (15 ng/g divided by the limit of detection).

CPF was present in the milk of dams at all dosage levels (Fig. 1), and again showed disproportionate increases with dosage. On PND 1, for a high:middle dosage ratio of 5 the milk CPF ratio was 22; for a middle:low dosage ratio of 3, the milk CPF ratio was 6.8. Ratios in milk were very similar on PND 5. CPF was concentrated in the milk, and milk:blood ratios were 208 on PND 1 and 104 on PND 5; the differences in milk:blood partitioning between PNDs 1 and 5 were attributed to decreases in milk fat. Nicholas and Hartman (1991) reported Wistar lactation day 0 (our PND 0) milk fat to be about 23%, and about 0.5 mg CPF/kg/day. For the more refined estimate, it was assumed that milk CPF concentration was constant for 24 h. On postnatal day 1, milk from high-dose dams contained about 3 μg/ml of CPF and no measurable CPF-oxon (limit of quantification 0.7 ng/g). Milk consumption is a function of body weight and rate of growth (6.1 g + 0.6 g/d), which was taken from the developmental neurotoxicity study of Hoberman (1998). Using published equations from Sampson and Jansen (1984), a newborn pup would drink about 1 ml of milk per day. Under these conditions, a nursing pup’s exposure would be about 0.5 mg CPF/kg/day.

Data that increase the confidence in this estimate are: peak blood CPF occurred at 3 h postexposure in the kinetic study (Fig. 3), and peak inhibition of brain ChE occurred after 2 h and probably nearer to 5 h post-exposure in both dams and fetuses in the study by Lassiter et al. (1998). Blood and milk samples were taken 4 h post-exposure in this study. At 4 h, maternal blood concentrations were about 60% of those in the kinetic study in male rats, which appears reasonable since milk fat becomes an additional sink for lactating females.

The threshold for plasma ChE inhibition in adult rats is about 0.1 mg/kg/day (Breslin et al., 1996), and consistent with these earlier results, dams in the current study had clear plasma and RBC ChE inhibition at 0.3 mg/kg/day (Fig. 2). We estimated that high-dose nursing pups ingested about 0.1 mg/kg/day, which would cause ChE inhibition if pups were more sensitive than dams. Instead, pups’ ChE activity returned to, or nearly to, control values while nursing (Fig. 2). Based on the lesser ChE inhibition in fetuses, and on estimates of CPF consumption in milk, neither fetuses nor neonates demonstrated greater sensitivity to ChE inhibition than their dams. These data are consistent with the observations of Pope and Chakraborti (1992), Zheng et al. (1999), and Stiles and Pope (1999) that differential sensitivity of pups to ChE inhibition from repeated doses of CPF disappears as doses approach the adult no-observed-effect level.

ACKNOWLEDGMENTS

The authors wish to thank, from the U.S. EPA, Stephanie Padilla for her contributions to the design of this study and for her offer to analyze ChE samples; as well as Renée S. Marshall for performing the very large number of ChE activity assays. The authors also wish to thank, from The Dow Chemical Company, Tony K. Jeffries for collecting milk and blood samples from the rats, and for computerizing the test schedule; Carol L. Zablotny for managing the test; Jane W. Lacher for the anesthesia and care of the rats; Alan L. Mendrala for providing the time course data of blood CPF in adult rats; as well as the staffs of the Pathology group, the Toxicology Analytical group, the Neurotoxicology group, and the Developmental and Reproductive Toxicology group.

REFERENCES


