

Age-Related Brain Cholinesterase Inhibition Kinetics following *In Vitro* Incubation with Chlorpyrifos-Oxon and Diazinon-Oxon

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Chlorpyrifos and diazinon are two commonly used organophosphorus insecticides (OPs), and their primary mechanism of action involves the inhibition of acetylcholinesterase by their metabolites chlorpyrifos-oxon (CPO) and diazinon-oxon (DZO), respectively. The study objectives were to assess the *in vitro* age-related inhibition kinetics of neonatal rat brain cholinesterase (ChE) for CPO and DZO by estimating the bimolecular inhibitory rate constant (k_i) values. Brain ChE inhibition and k_i values following CPO and DZO incubation with neonatal Sprague-Dawley rat brain homogenates were determined at postnatal day (PND) 5, 12, and 17 and compared with the corresponding inhibition and k_i values obtained in the adult rat. A modified Ellman method was utilized for measuring the ChE activity. CPO caused a greater ChE inhibition than DZO as evidenced from the estimated k_i values of both compounds. Neonatal brain ChE inhibition kinetics exhibited a marked age-related sensitivity to CPO, with the order of ChE inhibition being PND 5 > PND 7 > PND 17 with k_i values of 0.95, 0.50, and 0.22 nM⁻¹hr⁻¹, respectively. In contrast, DZO ChE inhibition was not age related in the neonatal brain, and the estimated k_i value at all PND ages was 0.02 nM⁻¹hr⁻¹. These results demonstrated an age- and OP-selective inhibition of rat brain ChE, which may be critically important in understanding the potential sensitivity of juveniles to specific OPs exposures.

Key Words: cholinesterase; organophosphate insecticide; chlorpyrifos-oxon; diazinon-oxon.

Chlorpyrifos (O,O-diethyl-O-[3,5,6-trichloro-2-pyridyl]-phosphorothioate) and diazinon (O,O-diethyl-O-[2-isopropyl-4-methyl-6-pyrimidinyl]-phosphorothioate) are two commonly used organophosphorus insecticides (OPs). The primary mechanism of action and the most acutely life-threatening effect of OPs' exposures result from the inhibition of acetylcholinesterase (AChE) mediated by their oxon metabolites (Milesen *et al.*, 1998). The oxons are also potent inhibitors of other nontarget B-esterases, such as butyrylcholinesterase (BuChE) and car-

boxylesterases (CaE) (Costa *et al.*, 1999). Interaction of the oxon with these esterases is considered a potential protective mechanism against AChE inhibition since BuChE and CaE stoichiometrically detoxify some of the oxon and prevent that fraction from inhibiting AChE (Yang and Dettbarn, 1998).

Exposure to insecticides may involve a large segment of the population. Children of agricultural families have a higher potential for OP exposures than children living in nonagricultural communities (Fenske *et al.*, 2002). There is currently a significant concern and focus over the potential increased sensitivity of infants and children to the toxic effects of chemicals. The importance of this issue is highlighted by the National Research Council's (NRC) report on *Pesticides in the Diets of Infants and Children* (NAS, 1993) and the passage of the *Food Quality Protection Act* (FQPA, 1996). In general, vulnerability to toxins is often found in early pregnancy and during postnatal development. In rodents, the early postnatal period (first 3 weeks) involves important development of the cholinergic system, increased neurochemical activity, and maturation of synaptic connections in the brain (Kiss and Patel, 1992). Therefore, it is possible that the anticholinesterase effects of OPs during this critical period may interfere with nervous system development, may disrupt the cellular processes of growth and differentiation, and could cause permanent adverse effects (Pope and Liu, 1997).

In general, toxicity data on mature animals may be insufficient for extrapolation to immature animals (NAS, 1993) since the mechanism responsible for age-related differences in toxicity has not been well characterized (Atterberry *et al.*, 1997). The greater neonatal/juvenile animal sensitivity to OPs has primarily been attributed to the lack of complete metabolic competence during development (Gagne and Brodeur, 1972; Murphy, 1982; Padilla *et al.*, 2004). Age-related differences may be associated with quantitative differences in the target tissues and the biological responses. Studies have demonstrated that juvenile animals are more vulnerable than adults to the acute toxic effects of some OPs (Moser and Padilla, 1998; Moser *et al.*, 1998; Pope and Liu, 1997). In contrast, a lack of juvenile animal sensitivity has also been reported for some other OPs, like methamidophos (Moser, 1999), suggesting

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chemical-specific differences. More recently, Pope *et al.* (2005) had evaluated CaE activity and their *in vitro* inhibition by chlorpyrifos-oxon (CPO) in human liver tissues and reported minimal differences of CaE expression or their sensitivity to CPO inhibition between infants and adults (3 months to 36 years). Interestingly, they also reported that CaE inhibition by CPO in the 2-month-old infants was significantly higher than that of the adults. In contrast, Karanth and Pope (2000) reported that the level of CaEs was significantly different between neonatal (7 days), juvenile (21 days), and adult (3 months) Sprague-Dawley rats' tissues (lung, plasma, and liver) and showed age-dependent increases of CaE activity in all evaluated tissues. However, Karanth and Pope (2000) reported no significant differences in the sensitivity of CaEs following CPO incubation in all evaluated tissues, which appears to be similar to the recent results obtained for human liver tissue's CaEs (Pope *et al.*, 2005). Consistent with these observations in juvenile animals, it has also been reported that newborn and young humans also have lower metabolic capacity for some key xenobiotic metabolism pathways compared to adults (Johnson, 2003; Mueller *et al.*, 1983); hence, the age-related phenomena observed in animals may also be of relevance to humans.

Identification of the developmental physiological and pharmacodynamic differences is very important for understanding age-related sensitivity and the toxicity of xenobiotics in mature and young animals. Little is known about the impact of OPs on the *in vitro* age-dependent brain cholinesterase (ChE) inhibition kinetics. While the bimolecular inhibitory rate constant (k_i) values of several OPs have been estimated for the adult rodent brain ChE, no comparable evaluation has been done toward neonatal animals. This reflects the need to characterize the inhibition kinetics of OPs with neonatal brain ChE (target), which could offer (in addition to other pharmacokinetic factors) a better understanding of the age-related sensitivity following OPs' exposures. In the current study, CPO and diazinon-oxon (DZO) were selected based on similarities of their chemical structures, mechanism of action, and commonality of their molecular target (AChE). The study objectives were to assess the age-related inhibition kinetics of neonatal rat brain ChE following *in vitro* incubation with CPO and DZO.

MATERIALS AND METHODS

Chemicals

CPO and DZO were purchased from Chem Service, Inc. (West Chester, PA). Dithionitotriadenic acid was purchased from TCI America, Inc. (Portland, OR). 5,5'-Dithio-bis-2-nitrobenzoic acid (DTNB) and acetylthiocholine (ATC) were purchased from Sigma Chemical Company (St Louis, MO). The remaining chemicals used in this study were reagent grade or better and were purchased from Sigma Chemical Company.

Animals

Timed-pregnant female and adult male Sprague-Dawley rats (8–10 weeks) were purchased from Charles River Lab Inc. (Raleigh, NC). Prior to use, rats

were housed in solid-bottom cages with hardwood chips (laboratory-grade SANI chips; Teklad, Madison, WI) and given free access to water and food (PMI 5002, Certified Rodent Diet). All procedures involving animals were in accordance with protocols established in the National Institutes of Health/NRC *Guide and Use of Laboratory Animals* and were reviewed by the Institutional Animal Care and Use Committee at Battelle, Pacific Northwest Division.

Tissue Preparation

Groups of newborn rats were euthanized by CO₂ asphyxiation at postnatal day (PND) 5, 12, and 17 (five pups per age, mixed sex, i.e., gender was not considered as a study variable) and decapitated, and trunk blood from each pup was collected individually in heparinized glass vials and centrifuged for 15 min at 1000 × g to separate the plasma and RBCs. Immediately after blood collection, the brain was removed and dry blotted, and each pup brain was homogenized in nine volumes of 0.1M phosphate buffer (pH 7.4) using a polytron homogenizer (Brinkman Instruments, Westbury, NY). All tissues were stored at –80°C until the time of the experiments. For the adult studies, the rats were euthanized by CO₂ asphyxiation, blood was drawn from the posterior vena cava using a heparinized syringe, and brain was removed immediately after blood withdrawal and the tissues were prepared as described above.

Characterization of Tissue ChE Inhibition Kinetics

Determination of ChE activity. Methods to determine ChE activities have been reported previously (Kousba *et al.*, 2003, 2004). The optimal assay conditions for incubation time and tissue homogenate concentrations were determined in preliminary experiments (data not shown). No difference in the enzyme activities was detected in fresh or frozen homogenates of adult or neonatal animals provided that the homogenates were not kept frozen longer than 3 months (data not shown). The total brain ChE activity at PND 5 was approximately four times lower than adult brain ChE activity; therefore, a more concentrated neonatal brain homogenate was needed to achieve optimal experimental conditions (750× dilution for all neonatal experiments). Neonatal rat brain homogenate, plasma, and RBCs were thawed at room temperature and diluted in buffer 750, 110, and 250 times, respectively. Tissue dilutions were selected to place the absorbance in the linear range between 0.1 and 1 optical density (OD)/min. The age-related basal ChE activity was determined for plasma, RBCs, and brain, while ChE inhibition dynamics was evaluated only in the brain. The basal ChE activity was determined from samples incubated with phosphate buffer that did not include any CPO or DZO. No differences in results were observed in the estimated brain ChE activity with or without addition of 3mM EDTA to the buffer to inhibit A-esterases (PON-1) when the oxons were incubated with the brain homogenates, so EDTA was not routinely added (Sultatos, 1994). The lack of EDTA addition effects is consistent with Mortensen *et al.* (1998a), who reported that there was no detectable brain A-esterase activity in either the younger or the adult animal tissue's brain homogenates. EDTA was also not routinely added during the estimation of the age-related basal ChE activity for plasma and RBCs. In other words, the lack of EDTA effects on the measured brain ChE inhibition kinetics was due to the insignificant amount of A-esterases in the brain homogenates, and the lack of EDTA effects on the measured basal ChE activity in plasma and RBCs homogenates was due to the fact that no oxons were incubated with RBCs or plasma homogenates. Brain CaE's effects on the brain ChE inhibition kinetics were not evaluated since the current study evaluated utilized lower oxon concentrations than the concentrations needed to inhibit brain CaEs (see "Discussion" section for more information).

For the *in vitro* ChE inhibition kinetic studies, an aliquot of the brain homogenates was diluted in phosphate buffer, and 400 µl of diluted homogenates were incubated with 100 µl phosphate buffer containing different CPO (0.5–5nM) or DZO (31.5–250nM) concentrations in a shaker at room temperature for 0–1 h, and the reactions were terminated by the addition of excess buffer.

Plasma and brain ChE activities were determined using a modified Ellman method (Ellman *et al.*, 1961) using a 96-well automated microplate

spectrophotometer ELx808 equipped with a KC4 software package (Bio-Tek Instruments, Inc., Winooski, VT). Following reaction termination, a 250- μ l aliquot was transferred into a well and 25 μ l of DTNB and ATC were placed in each well, giving final concentrations of 0.1 and 0.4mM of DTNB and ATC, respectively, and a final volume of 300 μ l per well (Mortensen *et al.*, 1996). ChE activity was determined from the capacity of the enzyme to hydrolyze ATC since ATC is a good substrate for both AChE and BuChE (Dupree and Bigbee, 1994; Lassiter *et al.*, 1998). The rate of ATC hydrolysis was monitored by following the absorbance profile at 405 nm over 30–40 min, and the slope of the linear regression of that profile was used to measure the remaining enzyme activity. To determine RBC's ChE activity, 6,6'-dithionitonic acid was used as a coupling agent rather than the Ellman reagent (DTNB), and the absorbance was measured at 340 nm in order to avoid the hemoglobin interference at 405 nm wavelength (Hakathorn *et al.*, 1983).

Brain ChE inhibitory rate constants. The apparent bimolecular inhibitory rate constant (k_i ; $\text{nM}^{-1}\text{hr}^{-1}$) of CPO and DZO toward rat brain ChE was calculated using the method of Main (1964). The oxon concentrations (CPO, 0.5–5nM, or DZO, 31.5–250nM) were selected to give a maximum ChE inhibition ranging from 10% to 90% over 5–60 min incubations. The log percentage activity was plotted against time, and the slopes of each log plot were calculated using a linear regression procedure applied to each set of data. These slopes were used for the final k_i calculation by double-reciprocal plot analysis.

RESULTS

The total body and brain weights of neonatal rats at PND 5, 12, and 17 were measured along with adult body and brain weights at PND 60 and 90. The age-dependent changes in brain and body growth and relative brain to total body weight from PND 5 to PND 90 are shown in Figure 1. The average neonatal body and brain weights at PND 5, 12, and 17 were 10.8 ± 1.5 , 26 ± 3 , and 39 ± 6 g, and 0.51 ± 0.07 , 1.13 ± 0.07 , and 1.45 ± 0.08 g, respectively. Although both body and brain weights increased with age, the increase in brain weight was faster. As illustrated in Figures 1B and 1A, at PND 17 the brain weight of the rat pup was ~73% of the adult brain weight, yet the pup's total body weight was only 10% of the adult's. Likewise, in very young animals, the brain represents a larger percentage of the overall body weight; however, with increasing age, the relative brain weight also decreases (Fig. 1C). These results imply that the growth rate of brain in neonatal rats is fast and that the brains of these very young animals represent a much larger percentage of the overall body weight than in adult animals.

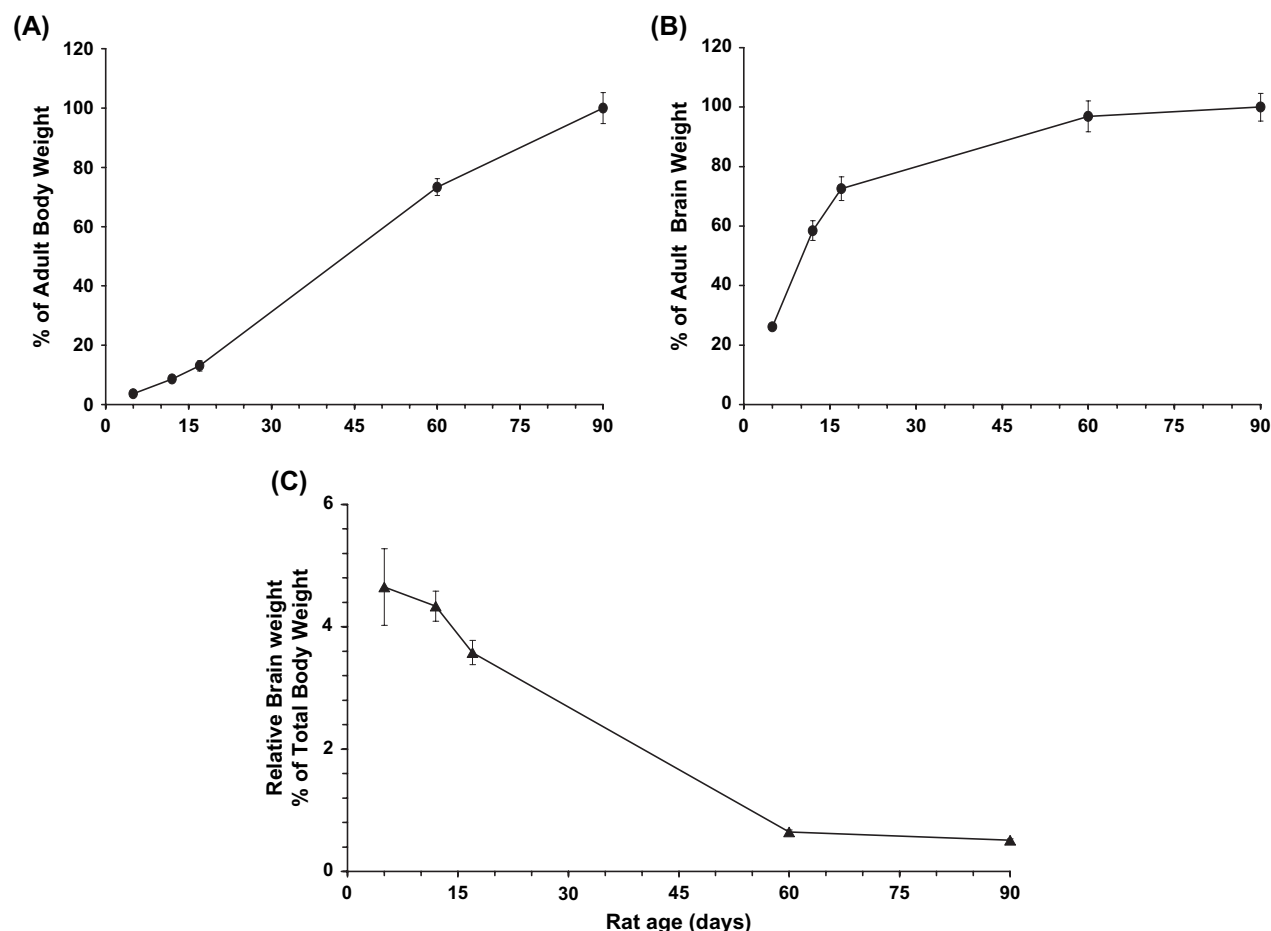


FIG. 1. (A) Body weight (percentage of total adult animal body weight), (B) brain weight (percentage of total adult animal brain weight), and (C) relative (%) brain to body weight plotted as a function of age. Each point represents the mean and SD of 30–40 rats per age group.

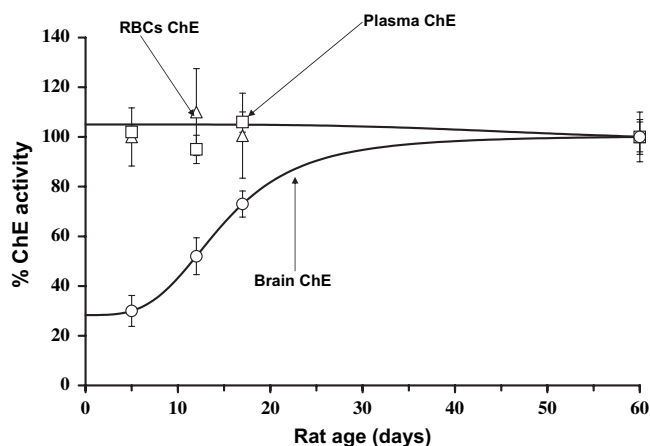


FIG. 2. Age-dependent (PND 5, 12, and 17) ChE activity determined in naive rat brain, plasma, and RBCs, presented as a percentage of adult ChE activity. Total ChE activity was determined from the rate of ATC substrate hydrolysis (milli-optical density/min). The plasma and RBC ChE activities are represented by the open squares and triangles, respectively, while brain ChE activity is represented by open circles. The symbols represent the experimental data, where each data point represents the mean and SD for five individual animals determined in triplicates.

The total ChE activity in brain, plasma, and RBC from naive rats determined at PND 5, 12, and 17 and expressed as percentage of adult ChE activity is illustrated in Figure 2. For each tissue, the ChE activities were calculated relative to adults based on OD comparisons and normalization by the dilution factors (see the “Materials and Methods” section). Among the three tissues, only brain ChE activity demonstrated an age-related difference with PND 5, 12, and 17 being ~25%, 53%, and 75% of the adult activity, respectively. Similar findings have been reported previously (Lassiter *et al.*, 1998; Mortensen *et al.*, 1998b). Based upon the brain tissue homogenate dilutions used in the current assay, these enzyme activities represent actual brain ChE concentrations of 0.03, 0.07, and 0.09 nmol per brain for PND 5, 12, and 17 rats, respectively. Since the plasma and RBCs demonstrated no age-related changes in ChE activity relative to adults, the final tissue dilutions for optimal experimental conditions were similar to those previously used with adult tissues (Kousba *et al.*, 2004).

The *in vitro* age-related ChE inhibition kinetics at PND 5, 12, and 17 following incubation of DZO or CPO with brain ChE are presented in Figures 3–6. ChE inhibition was linear at all incubated DZO or CPO concentrations, as illustrated in Figures 3 and 5. The rate of ChE inhibition as described by the bimolecular inhibitory rate constant (k_i) is the rate of ChE phosphorylation and is used to quantify OPs’ inhibitory capacity (Carr and Chambers, 1996; Kousba *et al.*, 2003; Main, 1964; Rosenfeld *et al.*, 2001). In the current study, the inhibition was determined according to the method of Main (1964), assuming that the *in vitro* interaction between ChE and OPs approximates first-order conditions with respect to un-

inhibited enzyme concentration. This method utilizes OPs’ concentrations many times higher than the tissue homogenate ChE concentration, yielding a single k_i estimate of ChE enzyme inhibition. Our original focus was using the traditional approach for the k_i estimation (Main, 1964), which has been used for decades as a proof of concept realizing that should k_i differences be found, a more comprehensive kinetic analysis over a wide range of oxon concentrations will be followed up for further evaluation. As is illustrated in Figure 3, increasing DZO concentrations resulted in a linear dose-dependent inhibition of brain ChE activity in all postnatal ages. The estimated DZO k_i values toward ChE at PND 5, 12, and 17 were $0.02\text{ nM}^{-1}\text{ hr}^{-1}$ for all postnatal ages (Fig. 4). Likewise, in the case of CPO, increasing concentrations also resulted in a linear dose-response inhibition of brain ChE at all ages (Fig. 5); however, unlike with DZO, the estimated CPO k_i toward brain ChE showed age-related changes. For example, Figure 6 shows CPO k_i values toward brain ChE at PND 5, 12, and 17 of 0.95 , 0.50 , and $0.22\text{ nM}^{-1}\text{ hr}^{-1}$, respectively (Fig. 6). These findings are particularly interesting and suggest a chemical-specific difference in the age-related ChE inhibition dynamics since the DZO k_i was not impacted by age, whereas CPO showed a marked age-related decrease.

Figure 7 compares the k_i values of CPO (average of two determinants) and DZO (single determination) toward brain ChE at PND 5, 12, and 17 and PND 60 and 90 (adults). The k_i values following CPO or DZO for adult brain ChE (PND 60 and 90) were determined by the method of Main (1964) and according to our previously described procedure (Kousba *et al.*, 2003, 2004). Characterization of the relationship between the resultant k_i and rat age indicated that the CPO k_i decreased as a function of age, approaching adult values by PND 17. Based on the nonlinear regression analysis, the theoretical maximum k_i at birth is $\sim 1.4\text{ nM}^{-1}\text{ hr}^{-1}$ and declines to a minimum of $\sim 0.21\text{ nM}^{-1}\text{ hr}^{-1}$ from PND 17 through PND 90. The k_i results for CPO ChE inhibition in adult rat brain ChE are consistent with our previously reported values from this group (Kousba *et al.*, 2004) and with other values reported in the literature (Atteberry *et al.*, 1997; Carr and Chambers, 1996). In contrast, the k_i for DZO do not demonstrate any age-related change with all estimates ranging from 0.016 to $0.02\text{ nM}^{-1}\text{ hr}^{-1}$. Overall, these results suggest an interesting age-related sensitivity that may be associated with specific OPs.

DISCUSSION

OPs have been routinely used to control pests in virtually all crop and commercial applications; therefore, it is entirely feasible to anticipate the potential for exposure in the general population, including children. In this regard, children of agricultural families and families that live in proximity to farms and orchards may be at greater risk from OPs’ exposure. Simcox *et al.* (1995) noted the presence of azinphos-methyl,

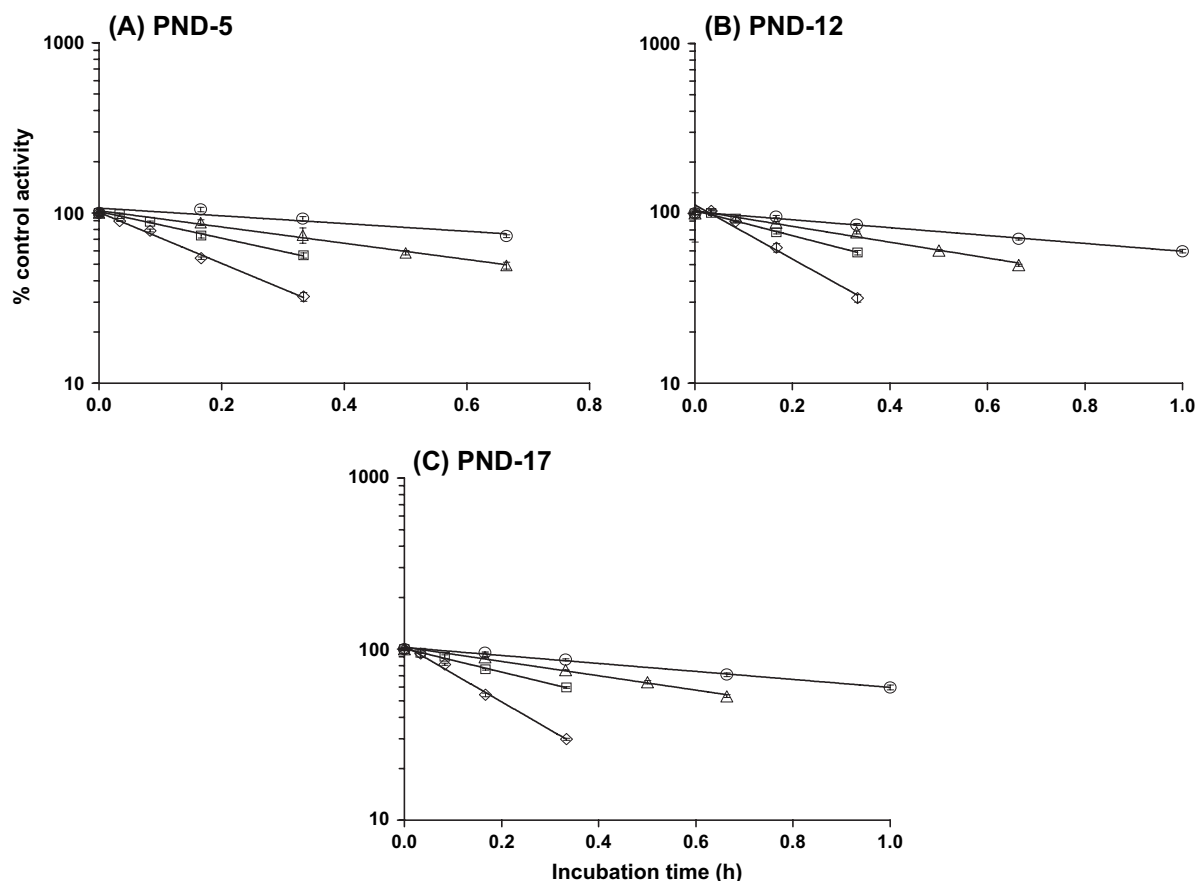


FIG. 3. *In vitro* neonatal rat brain ChE activity as determined from the rate of ATC substrate hydrolysis (mOD/min). The data are expressed as percentage of total ChE activity at PND 5 (A), PND 12 (B), and PND 17 (C), following different DZO concentrations for different incubation periods. The symbols represent the experimental data where each data point represents mean and SD of three determinations. In all panels, circles represent DZO concentration of 31.5nM; triangles, 62.5nM; rectangles, 125nM; and diamonds, 250nM. The lines represent the best fit of a log-linear regression analysis for each data set, and the slopes obtained from regression analysis showed a correlation > 0.95 and were used for the final k_i calculation as shown in Figure 4.

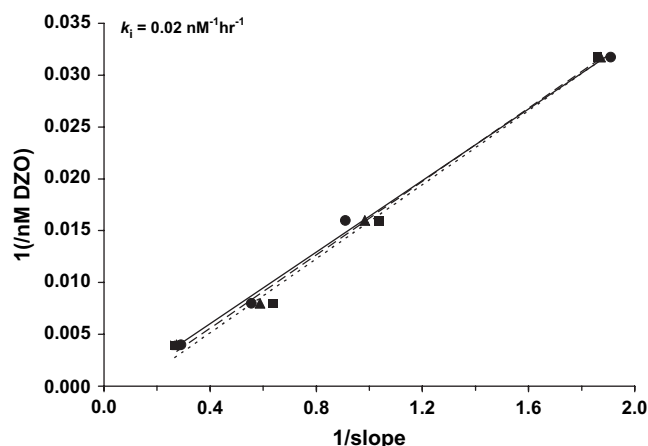


FIG. 4. Inhibitory constant (k_i) determination plot for neonatal rat brain ChE inhibition by DZO. Each symbol represents a specific slope obtained from each data set (see Fig. 3) at a given DZO concentration. The straight lines represent the best fit by regression analysis, and their slopes equal the k_i of DZO toward rat brain ChE as determined using the Main (1964) approach. The k_i estimated values for ChE inhibition by DZO were 0.017, 0.018, and 0.018 $\text{nM}^{-1}\text{hr}^{-1}$ for PND 5, 12, and 17, respectively.

chlorpyrifos, parathion, and phosmet in 62% of dust samples collected from the households of children of agricultural workers, suggesting the potential for nonoccupational exposure to these insecticides. Therefore, it is important to understand the toxicological implications of pesticide exposures for children, yet little is understood about the definitive mechanisms which lead to age-related differences in sensitivity in either animal model systems or humans following chlorpyrifos and diazinon exposures.

Although k_i values of several OPs have been estimated for brain ChE in adult rodents, we are unaware of any reports in the literature for neonatal/juvenile rats. The determination of a k_i for an OP is of particular importance since it is a measure of the extent of oxon binding with ChE and subsequent potency for enzyme inhibition. Hence, we have characterized the inhibition kinetics for CPO and DZO with neonatal brain ChE, which could offer a better understanding of the age-related sensitivity.

The measured changes in rat brain growth in relationship to total body weight (Fig. 1) are comparable with the overall age-related growth profiles of brain in different species (Dobbing

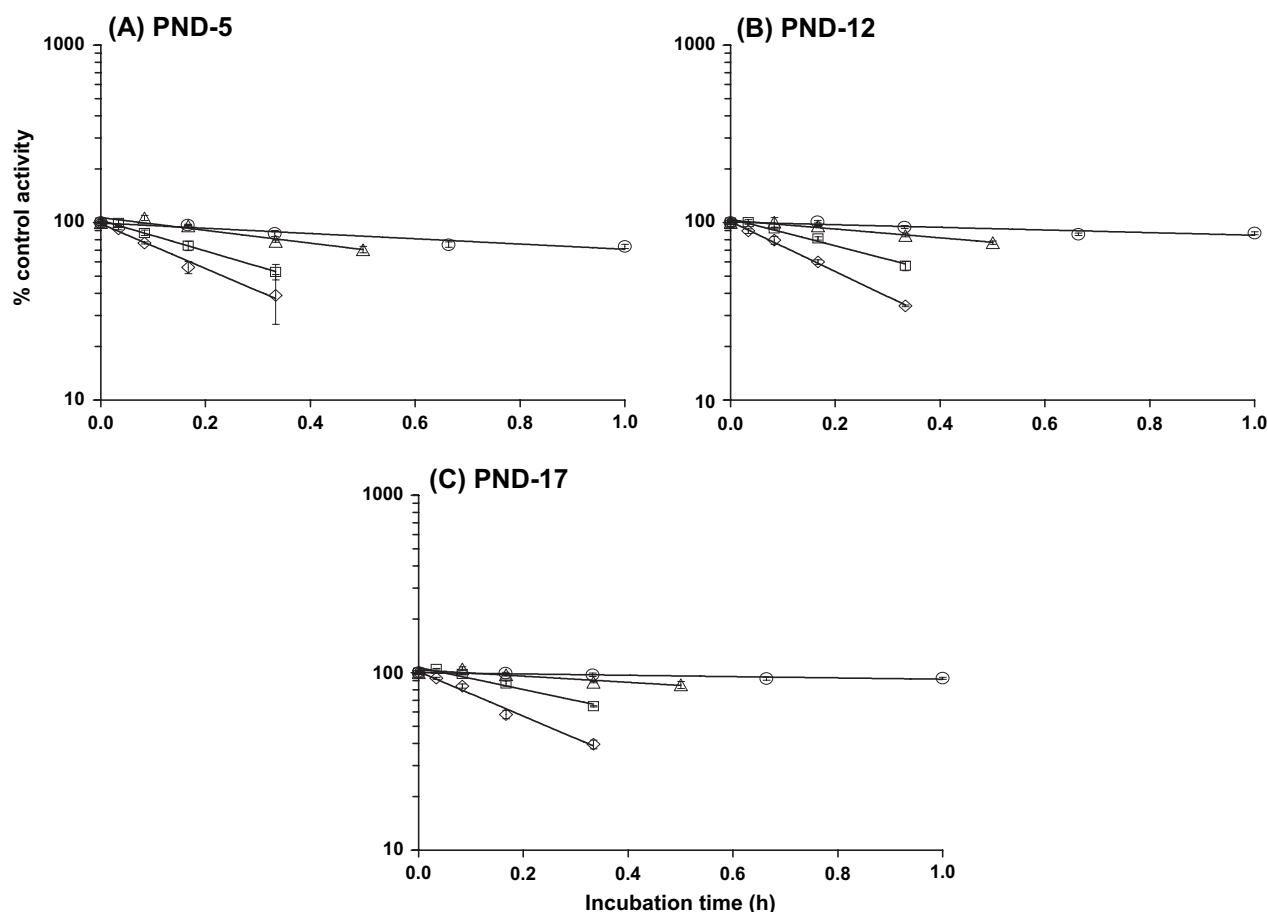


FIG. 5. *In vitro* neonatal rat brain ChE activity as determined from the rate of ATC substrate hydrolysis (mOD/min). The data are expressed as percentage of total ChE activity at PND 5 (A), PND 12 (B), and PND 17 (C) following different CPO concentrations for different incubation periods. The symbols represent the experimental data, where each data point represents mean and SD of three determinations. In all panels, circles represent CPO concentration of 0.5nM; triangles, 1nM; rectangles, 2.5nM; and diamonds represent 5nM. The lines represent the best fit from log-linear regression analysis for each data set, and the slopes obtained from regression analysis showed a correlation > 0.95 and were used for the final k_i calculation as shown in Figure 6.

and Sands, 1979). Likewise, the observed age-related increase in brain ChE in the current study (Fig. 2) is very consistent to previous reports in both rats and mice (Atterberry *et al.*, 1997; Kristt, 1983; Mortensen *et al.*, 1998b; Timchalk *et al.*, 2006). Theoretically, in young animals, and presumably in young children, an initial lower brain ChE activity and larger brain volume (relative to total body weight) might suggest age-related differences in both the disposition and biological effect of chemicals like OPs that target the brain and inhibit ChE. However, it must be noted that brain development is only one of many age-related modifications that take place in young animals and children, and other changes associated with enzyme maturation and metabolic clearance may play a more important role (Ginsberg *et al.*, 2004).

The diethylphosphorothionate insecticides chlorpyrifos and diazinon and their respective oxon metabolites, CPO and DZO, are structurally similar and share common metabolic pathways and molecular targets (Milesen *et al.*, 1998; Poet *et al.*, 2003; Sams *et al.*, 2000). So, it was initially hypothesized that both

oxons would exhibit similar ChE inhibition potencies and comparable age-related inhibition kinetics for brain ChE. However, at equimolar concentrations of the oxons, CPO exhibited markedly higher inhibitory potency for neonatal brain ChE than DZO, as evidenced from the estimated CPO k_i values being 15–50 times higher. The basis of this difference is not fully understood particularly since CPO and DZO share a common diethyl phosphate side chain that is responsible for binding and inactivation of ChE; hence, the enzyme inhibition complex for the two substrates are chemically similar (Poet *et al.*, 2003; Sams *et al.*, 2000). In this regard, Carr and Chambers (1996) suggested that the potency difference among OPs may be attributed to the differences in the interaction of the leaving group with the active site, whereby the association of the leaving group moiety with the anionic site may produce an environment that affects the rate of phosphorylation of the active site by inducing conformational changes in the tertiary structure of the enzyme. Secondly, we have previously proposed (Kousba *et al.*, 2004) that the potency difference among

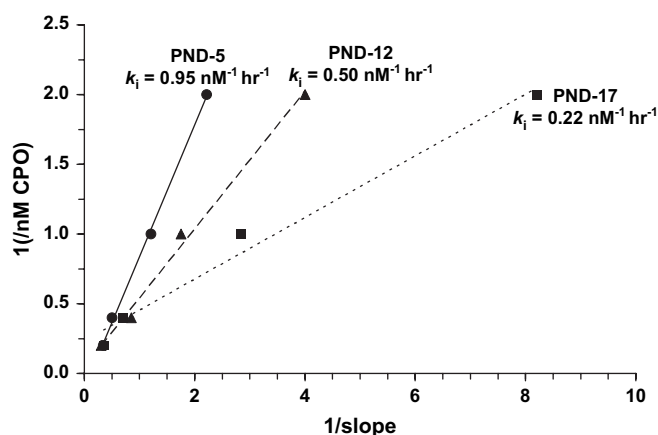


FIG. 6. Inhibitory constant (k_i) determination plot for neonatal rat brain ChE inhibition by CPO at PND 5 (circles), PND 12 (triangles), and PND 17 (squares). Each symbol represents a specific slope obtained from each data set (see Fig. 5) at a given CPO concentration. The straight lines represent the best fit by regression analysis, and their slopes equal the k_i of CPO toward rat brain ChE at each specific age as determined using the Main (1964) approach. The k_i estimated values for ChE inhibition by CPO were 0.95, 0.50, and 0.22 $\text{nM}^{-1}\text{hr}^{-1}$ for PND 5, 12, and 17, respectively.

OPs may also be attributed to the differences in their interaction with a proposed peripheral binding site on the ChE molecule, which when occupied, reduces the capacity of additional oxon molecules to phosphorylate the active site. We reported differing k_i values as a result of changing the oxon concentrations within the *in vitro* incubations, where the k_i values estimated at a low oxon concentration (1 μM) were

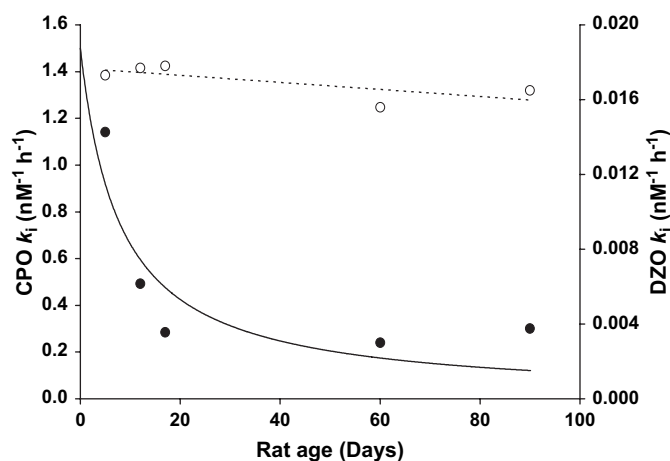


FIG. 7. Inhibitory constant (k_i) estimates for CPO (left y-axis; filled circles; solid line) or DZO (right y-axis; open circles; dotted line) toward rat brain ChE plotted as a function of age. The data are the experimentally determined k_i values, and each point represents the single k_i estimate per age group for DZO or an average of two determinations (i.e., $n = 2$) for CPO. The estimated average CPO k_i toward brain ChE at PND 5, 12, 17, 60, and 90 were 1.142, 0.493, 0.285, 0.240, and 0.305 $\text{nM}^{-1}\text{hr}^{-1}$, respectively. The estimated DZO k_i toward brain ChE at PND 5, 12, 17, 60, and 90 were 0.017, 0.018, 0.018, 0.0165, and 0.0157 $\text{nM}^{-1}\text{hr}^{-1}$, respectively.

~1000 times higher than those determined at high concentrations (nM). In addition, at low oxon concentrations, the k_i estimates were similar for both CPO and paraoxon and both oxons exhibited similar inhibitory potency in contrast to the marked difference (15 \times) exhibited at higher oxon concentrations.

The pharmacokinetics of chlorpyrifos, the major metabolites, and the extent of ChE inhibition as a function of preweanling age suggest that the extent of metabolism and ChE inhibition were age dependent and were also consistent with observed age-related susceptibility (Timchalk *et al.*, 2006). In the current study, CPO but not DZO demonstrated an age-related inhibition of neonatal rat brain ChE. In the case of CPO, the k_i value was approximately four times higher for the PND 5 than PND 17 rats, which was approximately equal to the k_i estimates determined for adult rat brain. Of particular interest are the findings with DZO, which did not show any age-related differences in the k_i estimates, suggesting that age-related changes in oxon binding/inhibition may be oxon specific.

Previous studies have reported that the greater toxicity in young animals to some OPs is not due to greater age-related sensitivity of AChE to inhibition (Karanth and Pope, 2003; Mortensen *et al.*, 1996, 1998a). However, the current study does suggest that age-related changes in k_i , specifically for CPO binding with brain ChE, may result in a greater sensitivity of AChE in very young animals exposed to this chemical. Several plausible explanations may be considered, including (1) the potential chemical instability of CPO within the *in vitro* incubation system (2) the presence of additional proteins in the brain tissue homogenates that can function to modify the *in vitro* binding affinity of CPO with AChE, and (3) age-related changes in the AChE peripheral binding site. The potential *in vitro* instability of CPO during the incubation, although plausible, seems unlikely since within a given PND age group the dose-response is very linear (Fig. 5), and a good linear response over a range of concentrations would be unlikely if the oxons were undergoing chemical degradation. Secondly, if chemical or enzymatic degradation were occurring, one would expect to see a similar response with DZO, which did not happen in the current study. The potential for CPO interacting with secondary proteins that modify binding affinity of CPO with AChE is feasible, and in this regard, Murphy (1982) suggested that a lower toxicity in adult rats may in part be due to a higher binding of the oxon to noncritical tissues in the brain, in addition to the differences in age-related metabolic detoxification. Likewise, Mortensen *et al.* (1998b) showed that the brain protein concentration increases from 7% to 11.5% from PND 4 to 90. The presence of other proteins could function as an alternative chemical-specific (CPO vs. DZO) binding site. Finally, the observed differences with CPO may be related to the modification of the peripheral binding site on the ChE molecule since this site has been shown to modify ChE inhibition as a function of CPO concentrations (Kousba *et al.*, 2004). Although studies have been conducted to evaluate the

potential role of a peripheral binding site with paraoxon inhibition of AChE, similar evaluations have yet to be conducted with CPO or DZO; hence, it is not yet possible to confirm if binding differences between these oxons and a peripheral binding site would account for the observed age-related k_i estimates for CPO and DZO. Therefore, more mechanistic studies are necessary for exploring the underlying mechanism of the observed age-related changes in CPO inhibition kinetics versus DZO. In order to explore the role of the peripheral site on ChE molecule in the k_i changes, *in vitro* studies that evaluate the age-related k_i changes as a function of CPO and DZO concentration over a wider range of oxon concentrations (pM–nM range) similar to the studies performed by Kousba *et al.* (2004) are also needed. It should be noted that the brain ChE kinetics determined *in vitro* in the current study are unlikely to be affected by the contents of CaEs in brain tissue homogenates due to the extremely lower inhibition sensitivity of brain CaEs relative to ChE by the OPs. Chanda *et al.* (1997) reported that brain CaEs were ~25 times less sensitive than brain ChE to the *in vitro* inhibition by CPO, with estimated 50% inhibitory concentration values of ~80 and 3nM, respectively. Brain ChE inhibition kinetics determined in the current study utilize very low oxon concentration range relative to the oxon concentration required to cause significant inhibition of brain CaEs.

It is very clear that the qualitative and quantitative evaluation of the interaction of DZO with ChE in adult or neonates is very limited compared with CPO. We are only familiar with a single study that evaluated the *in vivo* age-related diazinon toxicity in rats (Padilla *et al.*, 2004). They reported more brain ChE inhibition in younger animals, which was potentially attributed to lower detoxification by CaE and/or A-esterase in young animals. Hence, additional toxicity, pharmacokinetic, and pharmacodynamic evaluation of diazinon or DZO will be particularly helpful.

The observed *in vitro* age-related differences in inhibition kinetics following CPO and DZO incubation make it difficult to predict chemical-specific differences in susceptibility following OPs' exposures. In general, the effects of chemicals on the central nervous system are often unpredictable, and there are no systematic ways to predict age-related toxicity differences. However, the development of age-dependent pharmacokinetic models offers great promise to better identify "early life stage"-related sensitivity (Clewell *et al.*, 2004). In this regard, the physiologically based pharmacokinetic and pharmacodynamic models for chlorpyrifos and diazinon reported previously (Poet *et al.*, 2004; Timchalk *et al.*, 2002) are currently being modified and utilize those k_i estimates to address age-related changes in pharmacokinetics and pharmacodynamics. In summary, we conclude that the observed age-related inhibition kinetics following CPO, but not DZO, exposure suggest that brain ChE inhibition may be OP specific. These differences along with other pharmacokinetic and pharmacodynamic, anatomical, and physiological factors collectively may contribute to age-related OP sensitivity.

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