FUNDAMENTAL AND APPLIED TOXICOLOGY 21, 89–96 (1993)

Chlorpyrifos: Assessment of Potential for Delayed Neurotoxicity by Repeated Dosing in Adult Hens with Monitoring of Brain Acetylcholinesterase, Brain and Lymphocyte Neurotoxic Esterase, and Plasma Butyrylcholinesterase Activities[1]

RUDY J. RICHARDSON,*,†,[2] THOMAS B. MOORE,[3] USAMAH S. KAYYALI,[4] AND JOSEPH C. RANDALL

*Neurotoxicology Research Laboratory, Toxicology Program, Department of Environmental & Industrial Health, School of Public Health; *Neuroscience Program; and †Department of Neurology, School of Medicine, The University of Michigan, Ann Arbor, Michigan 48109*

Received July 17, 1992; accepted February 8, 1993

Chlorpyrifos: Assessment of Potential for Delayed Neurotoxicity by Repeated Dosing in Adult Hens with Monitoring of Brain Acetylcholinesterase, Brain and Lymphocyte Neurotoxic Esterase, and Plasma Butyrylcholinesterase Activities. RICHARDSON, R. J., MOORE, T. B., KAYYALI, U. S., AND RANDALL, J. C. (1993). *Fundam. Appl. Toxicol.* **21**, 89–96.

Previous work has shown that acute exposures to chlorpyrifos (CPS; diethyl 3,5,6-trichloro-2-pyridyl phosphorothionate) cannot produce >70% inhibition of brain neurotoxic esterase (NTE) and cause organophosphorus compound-induced delayed neurotoxicity (OPIDN) unless the dose is well in excess of the LD50, necessitating aggressive therapy for cholinergic toxicity. The present study was carried out to determine if repeated doses of CPS at the maximum tolerated daily dose without prophylaxis against cholinergic toxicity could cause cumulative inhibition of NTE and OPIDN. Adult hens were dosed daily for 20 days with CPS (10 mg/kg/day po in 2 ml/kg corn oil) or corn oil (vehicle control) (2 ml/kg/day po) and observed for an additional 4 weeks. Brain acetylcholinesterase (AChE), brain and lymphocyte NTE, and plasma butyrylcholinesterase (BuChE) activities were assayed on Days 0 (control only), 4, 10, 15, 20, and 48. During Days 4–20, brain AChE and plasma BuChE activities from CPS-treated hens were inhibited 58–70% and 49–80% of contemporaneous controls, respectively. At 4 weeks after the end of dosing, brain AChE activity in treated birds had recovered to 86% of control and plasma BuChE activity was 134% of control. Brain and lymphocyte NTE activities of treated animals throughout the study were 82–99% and 85–128% of control, respectively. Neither brain nor lymphocyte NTE activities in treated hens exhibited cumulative inhibition. The 18% inhibition of brain NTE seen on days 10 and 20 was significant, but substantially below the putative threshold for OPIDN. Body weight of treated hens decreased 10–25% during Days 4–20 and recovered to 87% of control by the end of the study. Some treated hens developed a slight staggering gait during the first week of dosing, which disappeared by the second week. Throughout the 4-week observation period, all hens appeared normal and were able to perch on a horizontal rod. The results indicate that daily dosing with CPS at a level sufficient to cause significant loss of body weight as well as marked inhibition of brain AChE and plasma BuChE resulted in no significant change in lymphocyte NTE activity, a maximum inhibition of brain NTE of 18%, no cumulative inhibition of lymphocyte or brain NTE, and no clinical signs of OPIDN. © 1993 Society of Toxicology.

Chlorpyrifos (CPS; diethyl 3,5,6-trichloro-2-pyridyl phosphorothionate; Dursban) was developed in 1965 as a broad-spectrum organophosphorus (OP) insecticide (Eto, 1979). The parent compound is metabolically activated to CPS oxon (CPO) by oxidative desulfuration (Chambers and Chambers, 1989) and detoxified principally by A-esterase hydrolysis of CPO to diethyl phosphate and 3,5,6-trichloro-2-pyridinol (Sultatos *et al.*, 1984, 1985; Costa *et al.*, 1990). The insecticidal action of CPS stems from inhibition of acetylcholinesterase (AChE) by CPO, resulting in acute cholinergic toxicity (Eto, 1979). CPS is widely employed for a variety of agricultural and public health applications, and its use is likely to increase as a replacement for other insecticides that are either less effective for certain applications or no longer available due to regulatory action (Sultatos *et al.*, 1982; Jitsunari *et al.*, 1989; Barron *et al.*, 1991).

Sufficiently high acute or repeated doses of some OP compounds can cause a distal degeneration of sensory and

[1] Portions of this research were presented at the 30th annual meeting of the Society of Toxicology, Dallas, Texas, February 25–March 1, 1991.

[2] To whom correspondence should be addressed at Neurotoxicology Research Laboratory, The University of Michigan, 1420 Washington Heights, Ann Arbor, MI 48109-2029. Fax: (313) 764-9424.

[3] Current address: State of California Environmental Protection Agency, Department of Pesticide Regulation, Medical Toxicology Branch, Sacramento, CA 95814.

[4] *Current address:* Department of Neurobiology, Harvard Medical School, Boston, MA 02115.

0272-0590/93 $5.00

motor axons in peripheral nerves and spinal cord called OP compound-induced delayed neurotoxicity (OPIDN). Presumably because of spinal tract involvement, recovery from the resultant sensory loss and paresis in OPIDN is generally poor. This neurodegenerative disease is thought to be initiated by irreversible inhibition of a critical concentration of neurotoxic esterase (neuropathy target esterase, NTE) by neuropathic OP compounds in neural tissue (Davis and Richardson, 1980; Johnson, 1982). The approximate threshold of NTE inhibition in brain that correlates with initiation of OPIDN is >70% for acute exposures, but may be >50% for repeated exposures to some OP compounds (Davis *et al.*, 1985). Behavioral signs of ataxia and paresis typically appear following a delay of 8–18 days after threshold inhibition has been reached from either acute or repeated exposure to a neuropathic OP compound (Davis and Richardson, 1980; Davis *et al.*, 1985). Current guidelines for assessment of the neuropathic potential of OP compounds initially require acute dosing in adult chicken hens (female of *Gallus gallus domesticus*, >8 months of age at the beginning of the study). Specified endpoints include assays of brain AChE and NTE as well as behavioral observation of gait and histopathological examination of spinal cord and peripheral nerves. The guidelines also require repeated dosing with examination of the same endpoints stipulated for acute dosing if there have been positive or equivocal findings of OPIDN in acute studies (USEPA, 1991).

Published studies on behavioral evaluation of gait after acute treatment of adult hens (Gaines, 1969) or subchronic treatment of young hens (<8 months of age at the beginning of the study) (Francis *et al.*, 1985) with CPS reported reversible leg weakness, which is inconsistent with the persistent paresis seen in OPIDN. Similarly, unpublished corporate reports were negative for both behavioral and histopathological signs of OPIDN following acute (Rowe *et al.*, 1978) or subchronic (Barna-Lloyd *et al.*, 1986) administration of CPS to adult hens. Likewise, other subchronic or chronic studies of CPS toxicity in fish, rats, or young chickens have shown no indications of OPIDN (Schlinke, 1970; Miyazaki and Hodgson, 1972; Sherman and Herrick, 1973; McCollister *et al.*, 1974; Jarvinen *et al.*, 1983; Ogawa *et al.*, 1988; and Corley *et al.*, 1989), but these experiments did not use the adult hen, which is the currently accepted animal model of choice for evaluation of OP compounds for their potential to produce OPIDN (Davis *et al.*, 1985; USEPA, 1991). Moreover, none of these studies included NTE assays, which would have enabled subthreshold neuropathic potential to have been detected (Davis *et al.*, 1985; Johnson, 1990; USEPA, 1991).

In apparent agreement with the earlier animal studies, an epidemiological study of 175 workers involved in the manufacture or formulation of CPS did not detect any cases of OPIDN (Brenner *et al.*, 1989). However, a human case of mild OPIDN resulting from ingestion of an estimated 300 mg/kg dose of CPS in a suicide attempt has been reported (Lotti *et al.*, 1986). This incident prompted the World Health Organization to categorize CPS as a delayed neurotoxicant capable of producing OPIDN (WHO, 1986) and triggered further investigations of the neuropathic potential of this compound. The most recent studies have shown that hen brain AChE is approximately 25–100 times more sensitive than NTE to inhibition by CPO *in vitro*, and that the acute dose of CPS required to reach the threshold for initiation of OPIDN in hens protected against acute cholinergic toxicity is several times the unprotected LD50 (Capodicasa *et al.*, 1991; Richardson *et al.*, 1993).

Although *in vitro* and single-dose studies have confirmed that the risk of developing OPIDN from acute environmental exposures to CPS is essentially nil, the fact that NTE inhibition and OPIDN can be produced at all means that repeated-dose studies with NTE assays are required to assess the possibility that cumulative inhibition of NTE beyond the threshold for initiation of OPIDN could occur during continuous low-level exposures (Johnson, 1990; USEPA, 1991). The need for such studies has been underscored further by recent preliminary case reports: Schaumburg *et al.* (1992) have found sensory peripheral neuropathy in 5 people who were environmentally exposed to CPS sprayed in confined areas, and the total number of such cases has increased to 11 in an updated study reported in abstract form (Kaplan *et al.*, 1992).

The present study was carried out in order to provide new data for use in the assessment of the neuropathic risk of repeated exposures to CPS. Adult hens were dosed with CPS for 20 days with the previously reported maximum tolerated daily dose without prophylaxis against cholinergic toxicity of 10 mg/kg/day po (Francis *et al.*, 1985; Barna-Lloyd *et al.*, 1986). During the dosing interval and subsequent 4-week observation period, general health and behavioral signs indicative of OPIDN were appraised by monitoring body weight, posture, gait, and perching ability. Cholinergic potency was evaluated by measuring brain AChE and plasma butyrylcholinesterase (BuChE) activities, and neuropathic tendency was evaluated by assaying brain and lymphocyte NTE activities. None of the previous repeated-dose studies of CPS toxicity included either brain or lymphocyte NTE assays, and the only other repeated-dose study of CPS in fully adult hens (Barna-Lloyd *et al.*, 1986) also omitted brain AChE or plasma BuChE assays. Brain AChE and NTE are now required by current neurotoxicity testing guidelines (USEPA, 1991), and plasma BuChE and lymphocyte NTE were included as accessible biomonitors of cholinergic or neuropathic OP compound exposures that are applicable to humans (Schwab and Richardson, 1986; Lotti *et al.*, 1986). A preliminary account of this work has been presented and published in abstract form (Richardson *et al.*, 1991).

METHODS

Chemicals. CPS, (100% pure, Lot No. AGR 220406) was kindly provided by The Dow Chemical Company (Midland, MI). Other chemicals were acquired from the following commercial sources: mipafox and phenyl valerate (Lark Enterprises, Webster, MA); acetylthiocholine iodide (ATCh) and paraoxon (Aldrich Chemical Co., Milwaukee, WI); butyrylthiocholine iodide (BuTCh) (Eastman–Kodak, Rochester, NY); 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and bovine serum albumin, fraction V (BSA-V) (Sigma Chemical Co., St. Louis, MO); 4-aminoantipyrine (Fisher Scientific, Livonia, MI); and Ficoll–Paque (Pharmacia, Piscataway, NJ). All other chemicals were reagent grade or the highest grade commercially available. Aqueous solutions were prepared in distilled-deionized water.

Animals and treatments. Adult white Leghorn laying hens (Omega Chicks, Haslett, MI) approximately 18 months of age and weighing 1.77 ± 0.02 kg (mean ± SE, n = 33) at the start of the study were used. These animals were housed in stainless steel cages with a maximum of four per cage, in AAALAC-approved facilities maintained at 21–23°C with a 12-hr light–dark cycle. Hens were given Layena feed (Ralston Purina, St. Louis, MO) and tap water *ad libitum* throughout the 1-week acclimation and 7-week study periods.

The starting flock was randomized into two groups of 18/group (corn oil control) and 15/group (CPS-treated). Surviving birds were dosed daily by gavage for 20 days (Study Days 0–19), with corn oil (2 ml/kg) or CPS (10 mg/kg/day po in 2 ml/kg corn oil). The CPS solution was prepared fresh daily just prior to dosing. The hens were weighed daily during Days 0–20 and weekly during the subsequent 4-week observation period. They were observed daily for signs of treatment effects throughout the study, with particular attention to changes in posture or gait indicative of OPIDN (Davis and Richardson, 1980). During the 4-week post-treatment observation period, the ability of the birds to maintain a perch was tested on Study Days 31, 34, 38, 41, 45, and 48 (i.e., starting on Day 12 and ending on Day 29 after the final daily dose) by placing them on a horizontal wooden rod (length, 100 cm; diameter, 2.4 cm) securely mounted 72 cm above the floor. Previous experience in our laboratory has shown that hens consistently fail the perch test beginning on Day 10–12 after receiving a sufficient dose of a neuropathic OP to produce OPIDN (Huggins, 1982).

Tissue collection. Three hens per group were sacrificed by decapitation on Study Days 0 (control only), 4, 10, 15, 20, and 48. During the treatment period (Days 0–19), animals were not dosed on the day they were sacrificed. Whole blood from each animal (20–30 ml) was collected by exsanguination into a siliconized Erlenmeyer flask containing 0.3 ml heparin (10,000 units/ml). The brain was quickly removed, placed in ice-cold Tris buffer (50 mM Tris/0.2 mM EDTA, pH 8.00, at 25°C), stripped of superficial blood vessels and meninges, blotted, and weighed. Brains were homogenized in Tris buffer (10%, w/v) and further diluted 1:25 (v/v) in Tris buffer to 4.0 mg/ml for the NTE assay, or 1:50 in phosphate buffer (20 mM Na phosphate, pH 7.60) to 2.0 mg/ml for the AChE assay.

Preparation of plasma and lymphocyte samples. A 5.0-ml aliquot of the heparinized blood was centrifuged at 1600g in a Beckman J-6 centrifuge for 10 min at room temperature. The plasma supernatant was diluted 1:50 (v/v) in 20 mM Na phosphate buffer, pH 7.60. Lymphocytes were isolated by a modification of the method reported by Schwab and Richardson (1986). Whole blood was diluted 1:1 (v/v) with a balanced salt solution (BSS) (14.5 mM Tris, 0.01% (w/v) D-glucose, 0.005 mM $CaCl_2$, 0.098 mM $MgCl_2$, 0.54 mM KCl, 126 mM NaCl, pH 7.60); 8.0 ml of this preparation was then layered onto 7.0 ml of Ficoll–Paque. The layered samples were centrifuged at 500g for 30 min at 20°C in siliconized test tubes. The lymphocytes were recovered from the interface between the plasma and the Ficoll–Paque. The volume of lymphocytes collected was diluted 1:1 with BSS and centrifuged at 1000g for 10 min at 20°C. The pellet was resuspended in 10 ml of BSS and recentrifuged. The washed pellet was resuspended in 50 mM Tris/0.2 mM EDTA, (pH 8.0 at 25°C) to a volume equal to that of the original diluted blood volume. Prior to assaying for NTE activity, the samples were sonicated for 3 × 10 sec at a power output of 50 W with a Branson cell disrupter (Branson Sonic Power, Danbury, CT) fitted with a microtip which was immersed halfway into the suspension.

AChE and BuChE assays. The AChE and BuChE assays were performed according to a modification (Gorun *et al.*, 1978) of the colorimetric method of Ellman *et al.* (1961). Whole hen brain homogenate (2.0 mg tissue/ml) or plasma (20 μl plasma/ml) (200 μl) was prewarmed (37°C) for 5 min, ATCh or BuTCh (4.0 mM) (200 μl) was added, and the samples were incubated for 30 min. Enzyme activity was stopped by the addition of DTNB reagent in ethanol (3.60 ml). Absorbance of the 5-thio-2-nitrobenzoate chromophore formed from the reaction of thiocholine with DTNB was measured at 412 nm and activity was calculated using an ϵ value of 13,600 M^{-1} cm^{-1}. Activities were expressed per gram of tissue for AChE and per milliliter of plasma for BuChE.

NTE assay. The NTE assay was modified from that of Johnson (1977). Aliquots of paraoxon (400 μM, 0.25 ml) and buffer (0.25 ml) or paraoxon and mipafox (200 μM, 0.25 ml) in 50 mM Tris/0.2 mM EDTA, pH 8.00 (pH at 25°C), were prewarmed (37°C) for 5 min. After the addition of whole hen brain homogenate (0.50 ml of 4 mg tissue/ml) or lymphocyte sonicate, samples were preincubated for 20 min to effect differential inhibition of NTE between samples incubated only with paraoxon (to inhibit esterases other than NTE) or with paraoxon plus mipafox (to inhibit esterases including NTE). Phenyl valerate substrate (1.00 ml of 5.30 mM) was added and the samples incubated for 15 min. Enzyme activity (hydrolysis of phenyl valerate to phenol and valeric acid) was stopped by the addition of 1.00 ml of 1.0% (w/v) sodium dodecyl sulfate containing 0.025% (w/v) of the color reagent, 4-aminoantipyrine. $K_3Fe(CN)_6$ (0.50 ml of 0.40%, w/v) was added to develop the phenol chromophore and its absorbance was measured at 510 nm. Activity was calculated based on the difference in absorbance between tubes with or without mipafox, using an ϵ value of 13,900 M^{-1} cm^{-1}. Activities were expressed per gram of tissue for brain and per milligram of protein for lymphocytes. Protein concentration in lymphocyte samples was measured by the colorimetric method of Bradford (1976), using BSA-V as the reference standard.

Statistical analyses. All statistical calculations and analyses were carried out using SYSTAT statistical software (SYSTAT, 1992). Homogeneity of variances was evaluated by Bartlett's test ($p < 0.05$). Body weight data represent mean values of all remaining animals at each time point in the control and treated groups; because the number of animals in each group decreased over time, differences between control and treated mean values at each time point were determined by an independent t test using the Bonferroni correction for six comparisons (critical value for significance, α, = 0.05). Brain AChE, brain NTE, and lymphocyte NTE data were evaluated by two-way ANOVA; significance of differences between control and treated mean values at each time point were determined by post hoc pairwise comparisons using the Bonferroni test adjusted for five comparisons ($p < 0.05$). Plasma BuChE data had heterogeneous variances; therefore, differences between control and treated mean values at each time point were determined by an independent t test using separate variances and the Bonferroni correction for five comparisons (α = 0.05).

RESULTS

Daily oral dosing of CPS at the rate of 10 mg/kg/day for 20 days (Days 0–19) resulted in an apparent decrease in body weight in CPS-treated hens during Days 4–20, which was statistically significant except for Day 15 (Fig. 1). The mean body weight of the CPS-treated hens was 75% of the contemporaneous control value on Day 20. Despite the weight loss, these birds were active and alert and appeared to feed well. Several of the birds in the treated group exhib-

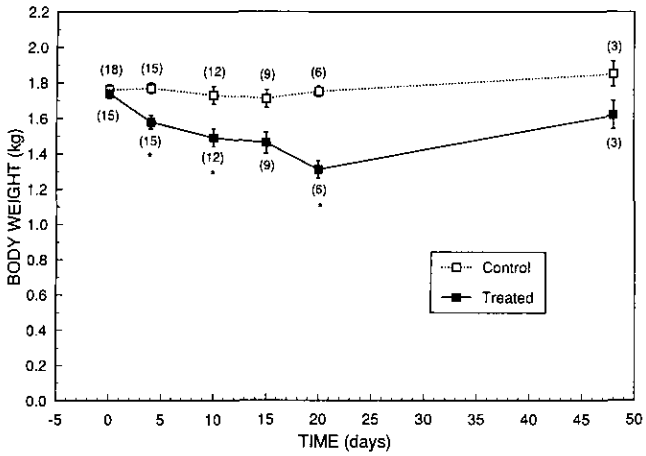

FIG. 1. Time course of body weight (mean ± SE) of control and CPS-treated hens. CPS (10 mg/kg/day po in 2.0 ml/kg corn oil) was given on Days 0–19; control hens received corn oil (2.0 ml/kg/day). The number of animals for each time point is shown in parentheses; three animals were sacrificed from each group on Days 0 (control only), 4, 10, 15, 20, and 48 for biochemical measurements. Significance of differences between CPS-treated and contemporaneous control values: $*\alpha < 0.05$, independent t test with Bonferroni correction for six comparisons.

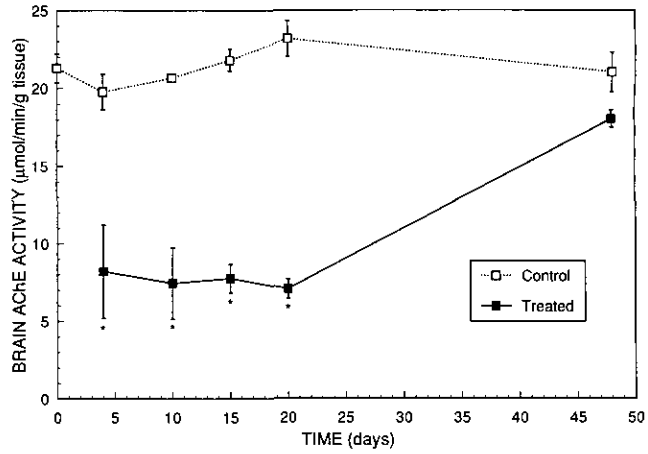

FIG. 2. Time course of brain AChE activity (mean ± SE, $n = 3$) in control and CPS-treated hens. CPS (10 mg/kg/day po in 2.0 ml/kg corn oil) was given on Days 0–19; control hens received corn oil (2.0 ml/kg/day). Significance of differences between CPS-treated and contemporaneous control values: $*p < 0.05$, two-way ANOVA with post hoc Bonferroni test adjusted for five comparisons.

ited a slight staggering gait and suffered from diarrhea during the first week of the study. These are expected cholinergic signs, although some of the control animals also had diarrhea, suggesting that this response may have been due in part to daily treatment with the corn oil vehicle. Any signs of leg weakness or abnormal gait in the treated birds disappeared by the second week of dosing. At the end of the 4-week post-treatment observation period, the mean body weight of the surviving treated hens was 87% of control, which was not statistically significant. Throughout the 4-week observation period, the birds in both groups appeared behaviorally normal and were able to maintain their balance on a perch (horizontal wooden rod). Perching ability was assessed on Study Days 31, 34, 38, 41, 45, and 48, which encompassed Days 12–29 after the last daily dose.

As expected for a cholinesterase inhibitor given at its maximum tolerated daily dose, marked inhibition of both brain AChE and plasma BuChE activity was observed (Figs. 2 and 3). Brain AChE activity was inhibited 58–70% during Days 4–20; inhibition was statistically significant for every time point during this interval, but the small apparent increase in inhibition over time was not significant (Fig. 2). By 4 weeks after the end of the dosing period, brain AChE activity had recovered to 86% of control, which was not significantly different from control (Fig. 2). Plasma BuChE activity in treated hens was inhibited 49–80% during Days 4–20 with no evident pattern of increasing or decreasing inhibition over time; even though apparent inhibition was a substantial 66–67% on Days 15 and 20, these values were not statistically significant due to heterogeneity of variances. At the completion of the study, plasma BuChE activity in treated animals had recovered to 134% of control, which was not significantly different from control (Fig. 3).

In contrast to the high levels of inhibition of brain AChE and plasma BuChE activities produced by CPS treatment, brain NTE activities in treated hens were 82–99% of contemporaneous control values and lymphocyte NTE activities were 85–128% of contemporaneous control values throughout the study. There were no statistically significant differences observed between treated and control values at any time point for lymphocyte NTE, but the 18% inhibition

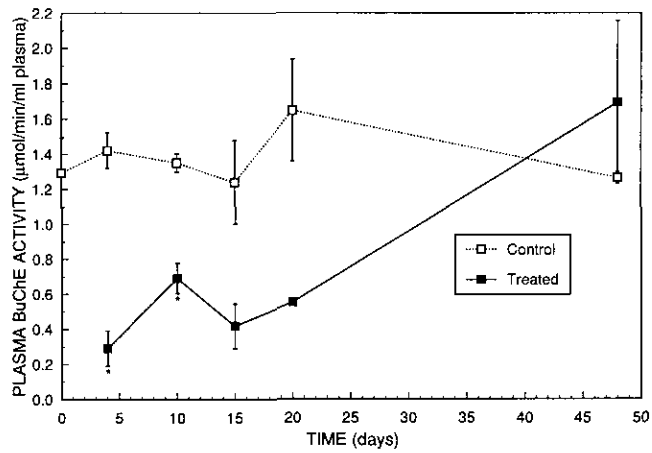

FIG. 3. Time course of plasma BuChE activity (mean ± SE, $n = 3$) in control and CPS-treated hens. CPS (10 mg/kg/day po in 2.0 ml/kg corn oil) was given on Days 0–19; control hens received corn oil (2.0 ml/kg/day). Significance of differences between CPS-treated and contemporaneous control values: $*\alpha < 0.05$, independent t test with separate variances and Bonferroni correction for five comparisons.

seen in brain NTE on Days 10 and 20 was statistically significant. Neither lymphocyte nor brain NTE showed cumulative inhibition during Days 4–20 (Figs. 4 and 5).

DISCUSSION

This paper describes the first repeated-dose study of CPS in adult hens to include assays for target enzymes in brain (AChE and NTE) as stipulated by current guidelines for neurotoxicity testing of OP compounds (USEPA, 1991). In addition, determinations of biomarker enzymes in blood (plasma BuChE and lymphocyte NTE) that could be monitored in human exposures were also carried out (Lotti *et al.*, 1983; Richardson and Dudek, 1983; USEPA, 1992). Although erythrocyte AChE can be a useful index of OP compound exposure in humans (Sanz *et al.*, 1991), this parameter could not be used in the present study because hen erythrocytes do not contain this enzyme (Pickering and Pickering, 1977). This work was completed using a 20-day dosing interval before the neurotoxicity guidelines recommending a 28-day period were published (USEPA, 1991). Nevertheless, 20 days at the high dose rate that was used were sufficient to show that there was no cumulative inhibition of any of the esterases assayed, even though pronounced inhibition of brain AChE and plasma BuChE had occurred by Day 4. Moreover, the present study used a 4-week postexposure observation period rather than the 21-day period recommended in the guidelines, thus providing additional time for signs of OPIDN to be detected.

The most consistent biochemical indicator of exposure to CPS in this study was the marked decrease in brain AChE activity, reflecting its inhibition by CPO. Substantial depression of brain AChE was expected in view of the high inhibitory potency of CPO for this enzyme (Capodicasa *et al.*, 1991; Richardson *et al.*, 1993) and the fact that the dose used has been reported to be the maximum tolerated daily dose without using prophylactic agents against cholinergic toxicity (Francis *et al.*, 1985; Barna-Lloyd *et al.*, 1986). Plasma BuChE activity was also assayed in order to provide information on an accessible biomarker of anticholinesterase action. While pronounced inhibition of plasma BuChE activity was seen, there was also a high degree of variability in this activity, both in control and CPS-treated animals. Large variances in plasma BuChE determinations during repeated-dose studies of OP compounds are not uncommon (USEPA, 1992). Such variability may be due in part to the fact that activity measurements are not yet routinely corrected for plasma concentrations of the enzyme, which are known to fluctuate in response to physiological changes that can be unrelated to anticholinesterase exposure (Wills, 1972; Sanz, 1991; USEPA, 1992).

Reversible decreases in body weight along with transient effects on gait and apparent leg strength as seen in the present study have also been observed in other repeated-dose studies of 10 mg/kg/day CPS in young (Francis *et al.*, 1985) and adult (Barna-Lloyd *et al.*, 1986) hens and are most likely to be the result of cholinergic hyperstimulation (Murphy, 1986). It is important to note that the ostensibly cholinergic effects of unsteady gait and leg weakness disappeared by the second week of our study, even though CPS treatment and concomitant brain AChE inhibition continued. This apparent development of tolerance to the behavioral effects of repeated exposures to CPS is similar to what has been described for a variety of anticholinesterase com-

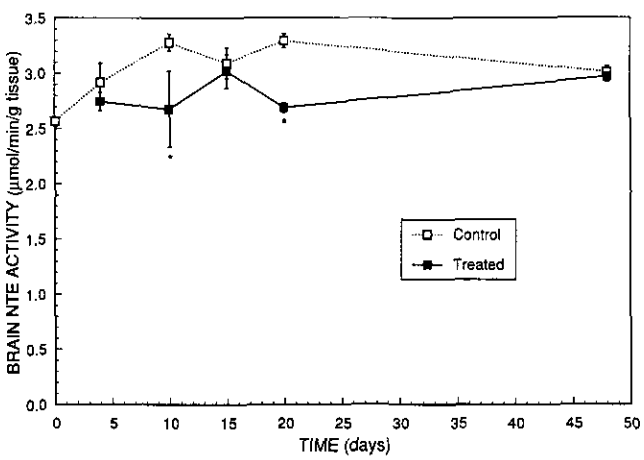

FIG. 4. Time course of brain NTE activity (mean ± SE, $n = 3$) in control and CPS-treated hens. CPS (10 mg/kg/day po in 2.0 ml/kg corn oil) was given on Days 0–19; control hens received corn oil (2.0 ml/kg/day). Significance of differences between CPS-treated and contemporaneous control values: $*p < 0.05$, two-way ANOVA with post hoc Bonferroni test adjusted for five comparisons.

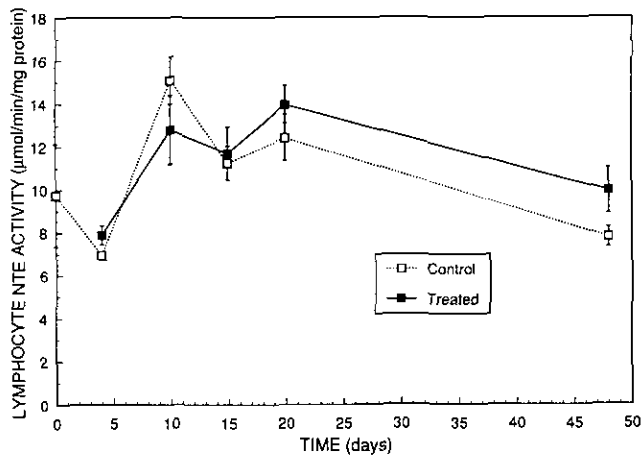

FIG. 5. Time course of lymphocyte NTE activity (mean ± SE, $n = 3$) in control and CPS-treated hens. CPS (10 mg/kg/day po in 2.0 ml/kg corn oil) was given on Days 0–19; control hens received corn oil (2.0 ml/kg/day). There were no significant differences between CPS-treated and contemporaneous control values at any time point (two-way ANOVA with post hoc Bonferroni test adjusted for five comparisons).

pounds (Costa *et al.*, 1982a). Tolerance has been attributed to an attenuation of cholinergic responsiveness arising from a postsynaptic reduction in the densities of muscarinic (Costa *et al.*, 1982b) and nicotinic (Costa and Murphy, 1983) receptors, although presynaptic mechanisms may also be involved (Lim *et al.*, 1987). For acute doses in naive animals, the relative tendency for a given OP compound to inhibit AChE versus NTE determines whether the compound can be tolerated in sufficient quantity to cause suprathreshold NTE inhibition in target neural tissue and precipitate behavioral signs of OPIDN (Richardson, 1992). However, for repeated doses of OP compounds, the development of tolerance to cholinergic effects would enable higher doses to be survived than would be the case in naive animals (Schwab and Murphy, 1981), thus increasing the likelihood that NTE inhibition could accumulate to threshold levels even in the face of high AChE inhibition.

There is some question about the threshold level of NTE inhibition in neural tissue required to precipitate OPIDN, both in acute and repeated exposures (Davis *et al.*, 1985; Schwab and Richardson, 1986). Because brain NTE inhibition in hens either parallels or exceeds that in spinal cord or peripheral nerve following dosing with neuropathic OP compounds, brain NTE is used as a convenient index of inhibition throughout the nervous system (Davis and Richardson, 1980; Johnson, 1982). For acute exposures, the threshold for initiation of OPIDN corresponds to a brain NTE inhibition of about 70%, although some experiments suggest that the actual threshold is considerably higher (Lotti, 1992). For repeated exposures, comparatively little work has been done to assess the steady-state brain NTE inhibition needed to initiate OPIDN, but experiments with mono-2-cresyl diphenyl phosphate (MOCP) in hens indicate that a high-point of inhibition similar to that required in acute exposures must be reached (Johnson and Lotti, 1980; Lotti and Johnson, 1980). However, other repeated-dose experiments with di-2-propylphosphorofluoridate in hens (Sprague *et al.*, 1981) as well as ethyl 4-nitrophenyl phenylphosphonothionate or 4-bromo-2,5-dichlorophenyl methyl phenylphosphonothionate (leptophos) in mallards (Hoffman *et al.*, 1984) suggest that the threshold brain NTE inhibition for OPIDN from repeated dosing may be as low as 50%. In the present study, brain NTE inhibition during the dosing interval fluctuated between approximately 2 and 18%. Even though the 18% level attained on Days 5 and 20 was statistically significant, there was no apparent trend of increasing inhibition over time, and the maximum level attained was well below the suggested minimum threshold of 50% required to cause OPIDN by repeated dosing. This biochemical result is consistent with the observation that all hens in the present study were able to maintain a perch on a wooden rod and exhibited normal posture and gait throughout the postexposure observation period (Days 12–29 after the last daily dose), indicating that no functional or behavioral signs of OPIDN had developed during this time.

Since its discovery in our laboratory, lymphocyte NTE has been proposed for use as an accessible biomarker of exposure to neuropathic OP compounds (Dudek and Richardson, 1982). Early work demonstrated that there was an excellent correlation between brain and lymphocyte NTE inhibition within 4 hr of dosing with seven different NTE inhibitors (Richardson and Dudek, 1983). Further study of three neuropathic OP esters showed that brain and lymphocyte NTE inhibition were well correlated within 24 hr of an acute exposure, but the correlation was poor or not significant 48 hr after exposure (Schwab and Richardson, 1986). Repeated daily dosing of MOCP at a level that produced a steady-state inhibition of brain NTE of about 60% within 2–3 weeks and no signs of OPIDN for up to 8 additional weeks resulted in no consistent effects on lymphocyte NTE activity throughout the experiment (Lotti and Johnson, 1980). Taken together, the foregoing results suggest that lymphocyte NTE might serve as a good biomarker if assayed within 24 hr of an acute exposure but that it would not be useful if assayed at longer times or during repeated exposures. However, a massive acute ingestion of CPS in a suicide attempt produced 60% inhibition of lymphocyte NTE 30 days after exposure (Lotti *et al.*, 1986), and a supralethal neuropathic dose of 300 mg/kg CPS administered to adult cats caused 46% inhibition of lymphocyte NTE 7 days after dosing (Fikes *et al.*, 1992). Moreover, prolonged occupational exposure (approximately 4 weeks) of humans to tributylphosphorothioate and tributylphosphorothioite (merphos) resulted in 40–60% inhibition of lymphocyte NTE activity (Lotti *et al.*, 1983). Therefore, while the lack of inhibition of lymphocyte NTE seen in the present study would appear to be consistent with the slight (2–18%) inhibition observed in brain, further work is needed to assess properly the value of lymphocyte NTE assays as a biomarker of neuropathic OP exposures, particularly in repeated-dose situations.

In conclusion, the results of the present study indicate that daily oral dosing of adult hens with 10 mg/kg/day CPS for 20 days caused significant loss of body weight as well as marked inhibition of brain AChE and plasma BuChE. However, this dosing regimen produced no significant change in lymphocyte NTE activity, a maximum noncumulative inhibition of brain NTE activity of approximately 18%, and no behavioral signs of OPIDN during the dosing interval or subsequent 4-week observation period. Moreover, the decreases in body weight and esterase activities observed during the dosing interval recovered to levels that were not significantly different from control values by the end of the 4-week postexposure observation period. These results provide new biochemical data along with confirmatory behavioral observations that are in accord with those of Barna-Lloyd *et al.* (1986), who found that daily oral dosing of

adult hens with CPS up to the maximum tolerated rate of 10 mg/kg/day for 91 days failed to produce any behavioral signs or histopathological lesions characteristic of OPIDN. These negative findings with respect to the development of OPIDN from repeated exposures to CPS would appear to be inconsistent with recent preliminary reports of sensory neuropathy occurring in humans environmentally exposed to high levels of this insecticide (Schaumburg *et al.*, 1992; Kaplan *et al.*, 1992). It is not clear whether this sensory neuropathy, which is completely reversible after cessation of exposure (Schaumburg, 1992), represents a peripheral sensory component of OPIDN or an entirely new phenomenon, but further research is needed to characterize this effect and to assess the risk of its occurrence.

ACKNOWLEDGMENTS

This work was supported in part by NIH Training Grant ES07062, NIAAA Center Grant AA07378, research grants from the American Diabetes Association and Mobil Oil Corporation, and donations in support of research from The Dow Chemical Company and DowElanco. Statistical consulting was provided by K. E. Guire and K. B. Welch, Center for Statistical Consultation and Research, The University of Michigan.

REFERENCES

Barna-Lloyd, T., Szabo, J. R., and Young, J. T. (1986). Chlorpyrifos: Subchronic organophosphate-induced delayed-neurotoxicity (OPIDN) study in laying chicken hens. Report TXT:K-044793-064. Dow Chemical Co., Freeport, TX.

Barron, M. G., Plakas, S. M., and Wilga, P. C. (1991). Chlorpyrifos pharmacokinetics and metabolism following intravascular and dietary administration in channel catfish. *Toxicol. Appl. Pharmacol.* **108,** 474–482.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

Brenner, F. E., Bond, G. G., McLaren, E. A., and Cook, R. R. (1989). Morbidity among employees engaged in the manufacture or formulation of chlorpyrifos. *Br. J. Ind. Med.* **46,** 133–137.

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

Chambers, F. E., and Chambers, H. W. (1989). Oxidative desulfuration of chlorpyrifos, chlorpyrifos-methyl, and leptophos by rat brain and liver. *J. Biochem. Toxicol.* **4,** 201–203.

Corley, R. A., Calhoun, L. L., Dittenber, D. A., Lomax, L. G., and Landry, T. D. (1989). Chlorpyrifos: a 13-week nose-only vapor inhalation study in Fisher 344 rats. *Fundam. Appl. Toxicol.* **13,** 616–618.

Costa, L. G., Schwab, B. W., and Murphy, S. D. (1982a). Tolerance to anticholinesterase compounds in mammals. *Toxicology* **25,** 79–97.

Costa, L. G., Schwab, B. W., and Murphy, S. D. (1982b). Differential alterations of cholinergic muscarinic receptors in chronic and acute tolerance to organophosphorus insecticides. *Biochem. Pharmacol.* **31,** 3407–3413.

Costa, L. G., McDonald, B. E., Murphy, S. D., Omenn, G. S., Richter, R. J., Motulsky, A. G., and Furlong, C. E. (1990). Serum paraoxonase and its influence on paraoxon and chlorpyrifos-oxon toxicity in rats. *Toxicol. Appl. Pharmacol.* **103,** 66–76.

Davis, C. S., and Richardson, R. J. (1980). Organophosphorus compounds. In *Experimental and Clinical Neurotoxicology* (P. S. Spencer and H. H. Schaumburg, Eds.), pp. 527–544. Williams and Wilkins, Baltimore.

Davis, C. S., Johnson, M. K., and Richardson, R. J. (1985). Organophosphorus compounds. In *Neurotoxicity of Industrial and Commercial Chemicals* (J. L. O'Donoghue, Ed.), Vol. II, pp. 1–23. CRC Press, Boca Raton.

Dudek, B. R., and Richardson, R. J. (1982). Evidence for the existence of neurotoxic esterase in neural and lymphatic tissues of the adult hen. *Biochem. Pharmacol.* **31,** 1117–1121.

Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7,** 88–95.

Eto, M. (1979). *Organophosphorus Pesticides: Organic and Biological Chemistry*, p. 248. CRC Press, Boca Raton.

Fikes, J. D., Zachary, J. F., Parker, A. J., and Beasley, V. R. (1992). Clinical, biochemical, electrophysiologic, and histologic assessment of chlorpyrifos induced delayed neuropathy in the cat. *Neurotoxicology* **13,** 663–678.

Francis, B. M., Metcalf, R. L., and Hansen, L. G. (1985). Toxicity of organophosphorus esters to laying hens after oral and dermal administration. *J. Environ. Sci. Health* **B20,** 73–95.

Gaines, T. B. (1969). Acute toxicity of pesticides. *Toxicol. Appl. Pharmacol.* **14,** 515–534.

Gorun, V., Proinov, I., Baltescu, V., Balaban, G., and Barzu, O. (1978). Modified Ellman procedure for assay of cholinesterase in crude enzymatic preparations. *Anal. Biochem.* **86,** 324–326.

Hoffman, D. J., Sileo, L., and Murray, H. C. (1984). Subchronic organophosphorus ester-induced delayed neurotoxicity in mallards. *Toxicol. Appl. Pharmacol.* **75,** 128–136.

Huggins, D. J. (1982). *Central Nervous System Endogenous Phosphorylation in Organophosphorus Delayed Neuropathy.* Ph.D. Thesis, The University of Michigan, pp. 37–40. University Microfilms International, Ann Arbor, MI.

Jarvinen, A. W., Nordling, B. R., and Henry, M. E. (1983). Chronic toxicity of Dursban (chlorpyrifos) to the fathead minnow (*Pimephales promelas*) and the resultant acetylcholinesterase inhibition. *Ecotoxicol. Environ. Safety* **7,** 423–434.

Jitsunari, F., Asakawa, F., Nakajima, T., Shimada, J., and Ogata, M. (1989). Determination of 3,5,6-trichloro-2-pyridinol levels in the urine of termite control workers using chlorpyrifos. *Acta Med. Okayama* **43,** 299–306.

Johnson, M. K. (1977). Improved assay of neurotoxic esterase for screening organophosphates for delayed neurotoxicity potential. *Arch. Toxicol.* **37,** 113–115.

Johnson, M. K. (1982). The target for initiation of delayed neurotoxicity by organophosphorus esters: Biochemical studies and toxicological applications. *Rev. Biochem. Toxicol.* **4,** 141–212.

Johnson, M. K. (1990). Organophosphates and delayed neuropathy—Is NTE alive and well? *Toxicol. Appl. Pharmacol.* **102,** 385–399.

Johnson, M. K., and Lotti, M. (1980). Delayed neurotoxicity caused by chronic feeding of organophosphates requires a high-point of inhibition of neurotoxic esterase. *Toxicol. Lett.* **5,** 99–102.

Kaplan, J. G., Rosenberg, N., Kessler, J., and Schaumburg, H. (1992). Chlorpyrifos (Dursban) causes peripheral neuropathy and cognitive dysfunction. *Neurology* **42** (Suppl. 3), 269–270. [Abstract 454P]

Lim, D. K., Hoskins, B., and Ho, I. K. (1987). Evidence for the involvement of presynaptic cholinergic functions in tolerance to diisopropylfluorophosphate. *Toxicol. Appl. Pharmacol.* **90,** 465–476.

Lotti, M. (1992). The pathogenesis of organophosphate polyneuropathy. *Crit. Revs. Toxicol.* **21,** 465–487.

Lotti, M., and Johnson, M. K. (1980). Repeated small doses of a neurotoxic organophosphate: Monitoring of neurotoxic esterase in brain and spinal cord. *Arch. Toxicol.* **45,** 263–271.

Lotti, M., Becker, C. E., Aminoff, M. J., Woodrow, J. E., Seiber, J. N., Talcott, R. E., and Richardson, R. J. (1983). Occupational exposure to the cotton defoliants DEF and merphos: A rational approach to monitoring organophosphorous-induced delayed neurotoxicity. *J. Occup. Med.* **25,** 517–522.

Lotti, M., Moretto, A., Zoppellari, R., Dainese, R., Rizzuto, N., and Barusco, G. (1986). Inhibition of lymphocytic neuropathy target esterase predicts the development of organophosphate-induced polyneuropathy. *Arch. Toxicol.* **59,** 176–179.

McCollister, S. B., Kociba, R. J., Humiston, C. G., McCollister, D. D., and Gehring, P. J. (1974). Studies of the acute and long-term toxicity of chlorpyrifos (*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate). *Food Cosmet. Toxicol.* **12,** 46–61.

Miyazaki, S., and Hodgson, G. C. (1972). Chronic toxicity of Dursban and its metabolite, 3,5,6-trichloro-2-pyridinol, in chickens. *Toxicol. Appl. Pharmacol.* **23,** 391–398.

Murphy, S. D. (1986). Toxic effects of pesticides. In *Casarett and Doull's Toxicology: The Basic Science of Poisons* (C. D. Klaassen, M. O. Amdur, and J. Doull, Eds.), 3rd ed., pp. 519–581. Macmillan, New York.

Ogawa, Y., Suzuki, S., Uchida, O., Kamata, E., Saito, M., Umemura, T., Wakana, M., Kaneko, T., Kurokawa, Y., and Tobe, M. (1988). 28-day repeated dose toxicity test for chlorpyrifos in Wistar rat. *Eisei Shikenjo Hokoko* **106,** 48–54.

Pickering, C. E., and Pickering, R. G. (1977). The interference by erythrocyte "acetylcholinesterase" in the estimation of the blood cholinesterase activity of the chicken. *Toxicol. Appl. Pharmacol.* **39,** 229–237.

Richardson, R. J. (1992). Interactions of organophosphorus compounds with neurotoxic esterase. In *Organophosphates: Chemistry, Fate, and Effects.* (J. E. Chambers and P. E. Levi, Eds.), pp. 299–323. Academic Press, San Diego.

Richardson, R. J., and Dudek, B. R. (1983). Neurotoxic esterase: Characterization and potential for a predictive screen for exposure to neuropathic organophosphates. In *Pesticide Chemistry: Human Welfare and the Environment* (J. Miyamoto and P. C. Kearney, Eds.), Vol. 3, pp. 491–495. Pergamon, Oxford.

Richardson, R. J., Moore, T. B., Kayyali, U. S., and Randall, J. C. (1991). Chlorpyrifos: Daily dosing for 20 days in hens with monitoring of brain, plasma, and lymphocyte esterases. *The Toxicologist* **11,** 304.

Richardson, R. J., Moore, T. B., Kayyali, U. S., Fowke, J. H., and Randall, J. C. (1993). Inhibition of hen brain acetylcholinesterase and neurotoxic esterase by chlorpyrifos *in vivo* and kinetics of inhibition by chlorpyrifos oxon *in vitro:* Application to assessment of neuropathic risk. *Fundam. Appl. Toxicol.* **20,** 273–279.

Rowe, L. D., Warner, S. D., and Johnston, R. V. (1978). Acute delayed neurotoxicologic evaluation of chlorpyrifos in white Leghorn hens. Report TA-609. Dow Chemical Co., Lake Jackson, TX.

Sanz, P., Rodriguez-Vicente, M. C., Diaz, D., Repetto, J., and Repetto, M. (1991). Red blood cell and total acetylcholinesterase and plasma pseudocholinesterase in humans: Observed variances. *J. Toxicol. Clin. Toxicol.* **29,** 81–90.

Schaumburg, H. H. (1992). Personal communication.

Schaumburg, H. H., Berger, A. R., and Thomas, P. K. (1992). *Disorders of Peripheral Nerves,* 2nd ed., p. 315. Davis, Philadelphia.

Schlinke, J. C. (1970). Chronic toxicity of Dursban in chickens, 1969. *J. Econ. Entomol.* **63,** 319.

Schwab, B. W., and Murphy, S. D. (1981). Induction of anticholinesterase tolerance in rats with doses of disulfoton that produce no cholinergic signs. *J. Toxicol. Environ. Health* **8,** 199–204.

Schwab, B. W., and Richardson, R. J. (1986). Lymphocyte and brain neurotoxic esterase: Dose and time dependence of inhibition in the hen examined with three organophosphorus esters. *Toxicol. Appl. Pharmacol.* **83,** 1–9.

Sherman, M., and Herrick, R. B. (1973). Fly control and chronic toxicity from feeding Dursban® (*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate) to laying hens. *Poultry Sci.* **52,** 741–747.

Sprague, G. L., Sandvik, L. L., and Bickford, A. A. (1981). Time course for neurotoxic esterase inhibition in hens given multiple diisopropyl fluorophosphate injections. *Neurotoxicol.* **2,** 523–532.

Sultatos, L. G., Costa, L. G., and Murphy, S. D. (1982). Factors involved in the differential acute toxicity of the insecticides chlorpyrifos and methyl chlorpyrifos in mice. *Toxicol. Appl. Pharmacol.* **65,** 144–152.

Sultatos, L. G., Shao, M., and Murphy, S. D. (1984). The role of hepatic biotransformation in mediating the acute toxicity of the phosphorothionate insecticide chlorpyrifos. *Toxicol. Appl. Pharmacol.* **73,** 60–68.

Sultatos, L. G., Minor, L. D., and Murphy, S. D. (1985). Metabolic activation of phosphorothioate pesticides: Role of the liver. *J. Pharmacol. Exp. Ther.* **232,** 624–628.

SYSTAT (1992). *SYSTAT: Statistics, Version 5.2.* SYSTAT, Inc., Evanston, IL.

USEPA (1991). Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Human and Domestic Animals, Addendum 10, *Neurotoxicity,* Series 81, 82, and 83. EPA 540/09-91-123, PB 91-154617.

USEPA (1992). Proceedings of the USEPA Workshop on Cholinesterase Methodologies, Arlington, VA, December 4–5, 1991. Office of Pesticide Programs, USEPA.

WHO (1986). Organophosphorus Insecticides: A General Introduction. *Environ. Health Crit.* **63.**

Wills, J. H. (1972). The measurement and significance of changes in the cholinesterase of erythrocytes and plasma in man and animals. *CRC Crit. Rev. Toxicol.* **1,** 153–202.