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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[1]

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Chlorpyrifos (CPS; *O,O*-diethyl 3,5,6-trichloro-2-pyridyl phosphorothionate; Dursban) is a widely used broad-spectrum organophosphorus (OP) insecticide. Because some OP compounds can cause a sensory-motor distal axonopathy called OP compound-induced delayed neurotoxicity (OPIDN), CPS has been evaluated for this paralytic effect. Early studies of the neurotoxicity of CPS in young and adult hens reported reversible leg weakness but failed to detect OPIDN. More recently, a human case of mild OPIDN was reported to result from ingestion of a massive dose (about 300 mg/kg) in a suicide attempt. Subsequent experiments in adult hens (the currently accepted animal model of choice for studies of OPIDN) showed that doses of CPS in excess of the LD_{50} in atropine-treated animals inhibited brain neurotoxic esterase (NTE) and produced mild to moderate ataxia. Considering the extensive use of CPS and its demonstrated potential for causing OPIDN at supralethal doses, additional data are needed to enable quantitative estimates to be made of the neuropathic risk of this compound. Previous work has shown that the ability of OP insecticides to cause acute cholinergic toxicity versus OPIDN can be predicted from their relative tendency to inhibit the intended target, acetylcholinesterase (AChE), versus the putative neuropathic target, NTE, in brain tissue. The present study was designed to clarify the magnitude of neuropathic risk associated with CPS exposures by measuring hen brain AChE and NTE inhibition following dosing *in vivo* and determining the bimolecular rate constant of inhibition (k_i) for each enzyme by the active metabolite, CPS oxon (CPO), *in vitro*. CPS administered to atropine-treated adult hens at 0, 75, 150, and 300 mg/kg po in corn oil produced mean values for brain AChE inhibition 4 days after dosing of 0, 58, 75, and 86%, respectively, and mean values for brain NTE inhibition of 0, 21, 40, and 77%, respectively. Only the high dose (six times the unprotected LD_{50} in hens) produced NTE inhibition above the presumed threshold of 70%, and these animals were *in extremis* from cholinergic toxicity at the time of euthanization despite continual treatment with atropine. When 150 mg/kg CPS po in corn oil was given to atropine-treated hens on Day 0, inhibition on Days 1, 2, 4, 8, and 16 for brain AChE was 86, 82, 72, 44, and 29%, respectively, and for brain NTE was 30, 28, 38, 29, and 6%, respectively. No signs of OPIDN were observed in any of the animals during the 16-day study period. Kinetic studies of the inhibition of hen brain AChE and NTE by CPO *in vitro* demonstrated that CPO exhibits high potency and extraordinary selectivity for its intended target, AChE. The k_i values were 15.5 μM^{-1} min^{-1} for AChE and 0.145 μM^{-1} min^{-1} for NTE. The calculated fixed-time (20-min) I_{50} values were 2.24 nM for AChE and 239 nM for NTE, yielding an I_{50} ratio for NTE/AChE of 107. These results may be compared with data compiled for other OP compounds with respect to NTE/AChE I_{50} ratios and the corresponding doses required to produce OPIDN relative to the LD_{50}. In general, NTE/AChE I_{50} ratios greater than 1 indicate that the dose required to produce OPIDN is greater than the LD_{50}. Taken together, the results of this study indicate that acute exposures to CPS would not be expected to cause OPIDN except under extreme conditions such as attempted suicides involving medically assisted survival of doses considerably in excess of the LD_{50}.

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Chlorpyrifos (CPS; *O,O*-diethyl 3,5,6-trichloro-2-pyridyl phosphorothionate; Dursban) is a widely used broad-spectrum organophosphorus (OP) insecticide. The acute toxic-

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ity and intended insecticidal action of CPS requires its metabolic activation to CPS oxon (CPO), which inhibits acetylcholinesterase (AChE) (Eto, 1979; Chambers and Chambers, 1989). CPS has only moderate acute toxicity in mammals (oral LD_{50} of 118–245 mg/kg in rats) (McCollister *et al.*, 1974), which has been attributed to efficient detoxification of CPO by A-esterase hydrolysis to diethyl phosphate and 3,5,6-trichloro-2-pyridinol (Sultatos *et al.*, 1984, 1985; Costa *et al.*, 1990).

An untoward effect of some OP compounds is OP compound-induced delayed neurotoxicity (OPIDN), a sensorymotor distal axonopathy of spinal cord and peripheral nerves that is thought to result from the irreversible inhibition of a critical amount (>70%) of neurotoxic esterase (neuropathy target esterase, NTE) in neural tissue by OP compounds of the phosphate, phosphoramidate, or phosphonate classes (Davis *et al.*, 1985; Johnson, 1990; Lotti, 1992). Given that CPO belongs to the phosphate class, this compound would be expected to cause OPIDN if it could produce supracritical NTE inhibition without incurring lethal cholinergic toxicity due to excessive AChE inhibition (Richardson, 1992).

The adult chicken hen (*Gallus gallus domesticus,* female, >8 months of age at the beginning of the study) is the currently accepted animal model of choice for detection of OPIDN (U.S. EPA, 1991). Early published studies of CPS toxicity in adult (Gaines, 1969) and young (<8 months of age at the beginning of the study) (Francis *et al.*, 1985) hens did not find clinical signs consistent with OPIDN, but these investigations did not include histopathology or NTE assays that could have been used to detect subclinical neuropathic potential. Likewise, while corporate reports on CPS neurotoxicity studies in adult hens were negative for both clinical and histopathological signs of OPIDN (Rowe *et al.*, 1978; Barna-Lloyd *et al.*, 1986), NTE measurements were not carried out. In keeping with the negative findings on CPS in animal studies, an epidemiological study of 175 workers involved in the manufacture or formulation of this compound did not find any cases of OPIDN (Brenner *et al.*, 1989). Nevertheless, despite these indications of its apparent safety, CPS has been categorized as a delayed neurotoxicant capable of causing OPIDN (WHO, 1986; Lotti, 1992).

Concerns about the neurotoxicity of CPS began with the observation that atropine-treated hens dosed with >200 mg/kg CPS developed leg weakness with an onset 3–18 days postdosing that lasted 10–20 days, from which the animals recovered (Gaines, 1969). In a later review, Gaines' report of reversible leg weakness was interpreted as OPIDN (Hixson, 1983). Osterloh *et al.* (1983) then reported on a suicide case involving ingestion of a mixture of compounds, which included CPS. Postmortem peripheral nerve NTE activity was only 30% of normal, prompting further concern about the capability of CPS to produce OPIDN in humans. The final proof of this capability was furnished by the reports of Lotti *et al.* (1986a,b) describing a suicide attempt involving ingestion of an estimated 300 mg/kg CPS. Following prolonged and aggressive therapy for severe cholinergic toxicity, the patient's lymphocyte NTE activity was found to be 40% of normal on Day 30 postingestion, and a mild peripheral neuropathy developed during Days 43–62. More recently, Capodicasa *et al.* (1991) reported that CPS could cause mild to moderate OPIDN in hens. The minimal neuropathic dose was found to be 60–90 mg/kg (po in glycerol formal), which caused >70% inhibition of brain NTE after 5–6 days. It was noted that this dose was four to six times the estimated LD_{50} under the conditions of this experiment and that animals had to be given prophylaxis against acute cholinergic toxicity with both atropine and pralidoxime in order to survive the neuropathic dose.

Because of the extensive use of CPS and its recently demonstrated potential for causing OPIDN at supralethal doses, additional data are needed to enable quantitative estimates to be made of the neuropathic risk of this compound relative to its intended acute cholinergic action. One approach to this problem is to measure the relative inhibitory power of the active metabolite toward AChE and NTE *in vitro* using the NTE/AChE I_{50} ratio (Lotti and Johnson, 1978). However, because the I_{50} depends upon the preincubation time of the inhibitor with the enzyme and its determination does not take into account deviations from ideal kinetics or differences in kinetics of inhibition between different enzymes or inhibitors, a preferable approach is to use the bimolecular rate constant of inhibition (k_i) instead of the I_{50} as a measure of inhibitory potency (Richardson, 1992). Neuropathic potential is then estimated directly as the AChE/NTE k_i ratio (neuropathy target ratio, NTR), because the k_i and I_{50} are reciprocally related. In general, the higher the NTR, the greater the expected safety of the compound with respect to its neuropathic potential. In particular, it has been found that NTR values greater than 1 indicate that the dose required to produce OPIDN will be greater than the LD_{50} (Lotti and Johnson, 1978; Richardson, 1992).

Based on estimates of fixed-time I_{50} values, Capodicasa *et al.* (1991) concluded that hen brain AChE was about 25 times more sensitive than NTE to inhibition by CPO, which was consistent with their finding that doses in excess of the LD_{50} were required to produce mild to moderate neuropathy. However, the doses required to produce >70% inhibition of brain NTE in their report were substantially lower than those in our preliminary studies, and maximal inhibition was achieved after an unusually long period of 4–6 days. Furthermore, k_i values for inhibition of hen brain AChE and NTE by CPO were not derived in their study because of apparent nonlinear kinetics attributed to hydrolysis of inhibitor by A-esterases in the tissue.

The present study was carried out in order to provide additional and more definitive data for quantitatively assessing the neuropathic risk of CPS. This was done in part

by reevaluating the dose-response and time-course of inhibition of hen brain AChE and NTE by CPS *in vivo*. However, particular emphasis was placed on examining the kinetics of inhibition of hen brain AChE and NTE by CPO *in vitro* in sufficient detail to allow determinations of k_i values to be made. A preliminary account of this work has been presented and published in abstract form (Moore *et al.*, 1989).

METHODS

Chemicals. Chlorpyrifos, 100% pure, lot AGR 220406, and CPS oxon, 98.2% pure, lot AGR 203674, were kindly provided by The Dow Chemical Company (Midland, MI). Other chemicals were acquired from the following commercial sources: mipafox and phenyl valerate (PV) from Lark Enterprises (Webster, MA); acetylthiocholine iodide (ATCh) and paraoxon from Aldrich Chemical Co. (Milwaukee, WI); 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) from Sigma Chemical Co.; and 4-aminoantipyrine from Fisher Scientific (Livonia, MI). 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 were used in the *in vivo* experiments and as a source of brain tissue in the *in vitro* studies. Hens were housed in stainless steel cages with a maximum of four hens per cage in AAALAC-approved facilities maintained under temperature-controlled conditions (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 acclimation and study periods except as noted below.

Hens were euthanized by decapitation with a guillotine. At the time of euthanization, 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 1:50 (v/v) in either Tris buffer (NTE assay) or 20 mM Na phosphate buffer, pH 7.60 (AChE assay), to 2.0 mg tissue/ml.

In the dose–response study, 12 hens were randomized into four groups (3 per group). They were fasted overnight, dosed with CPS (0, 75, 150, or 300 mg/kg po in 2 ml/kg corn oil), and maintained for 4 days. Atropine sulfate (20 mg/kg sc in isotonic saline) was given to test birds at the time of CPS dosing and then continued as needed to minimize acute toxicity. No other prophylactic agent was used. Atropine was not given to the controls. Each hen in the 150 mg/kg group received 6 atropine doses during the first 35 hr, and 1 hen received a further 6 doses during the next 45 hr. Each hen in the 300 mg/kg group received 12 atropine doses during the first 70 hr; 2 hens received an additional 2 doses during the next 10 hr, and 1 hen received an additional dose (for a total of 15 doses) at 84 hr. The hens were euthanized by decapitation 4 days postdosing because maximal NTE inhibition was previously reported to occur 4 days postdosing with CPS (Lotti *et al.*, 1986a).

In the time-course study, 33 hens were randomized into two groups (18 control and 15 treated animals). Hens from both groups were fasted overnight prior to being dosed with either the vehicle alone (2 ml/kg po corn oil) or CPS (150 mg/kg po in 2 ml/kg corn oil). Atropine sulfate (20 mg/kg sc in isotonic saline) was given to all of the test hens at the time of CPS dosing and at 5 hr after dosing. Corn oil controls were not given atropine. Groups of 3 CPS-dosed hens and 3 control hens were euthanized on Study Days 1, 2, 4, 8, and 16, and a group of 3 control hens was euthanized on Day 0. CPS-dosed hens surviving after Day 1 received 2 additional atropine doses at 21 and 29 hr; hens surviving after Day 2 received 2 further atropine doses at 45 and 54 hr. No further atropine was given, nor were any other prophylactic agents used.

AChE assay and kinetics. The AChE assay was performed according to the modified colorimetric method of Ellman (Gorun *et al*, 1978). For the *in vivo* experiments, AChE activity was assayed by adding whole hen brain homogenates (200 μl of 2.0 mg tissue/ml in 20 mM Na phosphate buffer, pH 7.60) to prewarmed (37°C for 5 min) aliquots of ATCh (200 μl, 4.0 mM) and incubated at 37°C for 30 min. Enzyme activity was stopped and color developed by the addition of DTNB reagent in ethanol (3.60 ml). Absorbance was then measured at 412 nm. Activity was calculated using an ε value for the chromophore of 13,600 M^{-1} cm^{-1}. For the *in vitro* study, aliquots of buffer or CPO (2.0, 4.0, or 10.0 nM) (200 μl) in 20 mM Na phosphate buffer, pH 7.60, were warmed to 37°C for 5 min. Whole hen brain homogenate (2.0 mg tissue/ml) (200 μl) in phosphate buffer was added and the samples preincubated for timed intervals up to 20 min. After the addition of ATCh (1.0, 2.0, or 4.0 mM) (200 μl), the samples were incubated for 30 min. Enzyme activity was stopped and color developed by the addition of DTNB reagent in ethanol (3.40 ml); absorbance was read and activity calculated as stated above. Data were evaluated in terms of primary kinetic plots: log (% control activity) versus time of incubation with inhibitor for each inhibitor concentration and substrate concentration studied. The bimolecular rate constant of inhibition (k_i) was determined from the slope of the secondary plot of k' versus $[I]$, where $-k'/2.303$ is the slope of the primary kinetic plot and $[I]$ is the inhibitor concentration. The secondary plots were straight lines described by the relationship, $k' = k_i[I] + k_o$, where k_o is the intercept on the k' axis (Aldridge and Reiner, 1972; Wang and Murphy, 1982; Richardson, 1992). A fixed-time I_{50} was also determined by incubating hen brain homogenates (1.0 mg/ml final tissue concentration) for 20 min at 37°C with 0, 0.20, 0.50, 2.0, and 5.0 nM CPO in 20 mM Na phosphate buffer, pH 7.60. At the end of the preincubation interval, substrate was added and remaining enzyme activity determined. The I_{50} was calculated from the regression line of a plot of log (% activity) versus $[I]$.

NTE assay and kinetics. The colorimetric NTE assay was modified from Johnson (1977). For *in vivo* experiments, aliquots of paraoxon (400 μM, 0.25 ml) and Tris buffer (50 mM Tris/0.20 mM EDTA, pH 8.00 at 25°C, 0.25 ml) or paraoxon and mipafox (200 μM, 0.25 ml) in Tris buffer were warmed for 10 min at 37°C. Whole hen brain homogenate (2.0 mg tissue/ml, 0.50 ml) was added and samples preincubated for 20 min. PV substrate (5.30 mM, 1.00 ml) was then added and the samples incubated for 15 min. Enzyme activity was stopped by the addition of 0.025% (w/v) aminoantipyrine/1.0% (w/v) SDS (1.00 ml). Color was developed by adding 0.40% (w/v) $K_3Fe(CN)_6$ (0.50 ml) and absorbance measured at 510 nm. Activity was calculated based on the difference in absorbance between tubes with and without mipafox, using an ε value for the chromophore of 13,900 M^{-1} cm^{-1}. For the *in vitro* study, an aliquot of buffer or CPO (10, 30, or 60 μM, 10 μl) was added after the initial preincubation of tissue with paraoxon or paraoxon plus mipafox and the samples were incubated at timed intervals up to an additional 20 min. PV was then added (2.65, 5.30, or 10.6 mM, 1.00 ml) and the samples were incubated for 15 min. The reaction was stopped, color developed, and activity calculated as described for the *in vivo* study. Primary and secondary kinetic plots were used to determine the k_i for each substrate concentration as described above for AChE. The fixed-time I_{50} was determined by incubating hen brain homogenates (1.0 mg/ml tissue concentration) for 20 min at 37°C with 0, 10, 25, 100, 250, and 1000 nM CPO in 50 mM Tris/0.20 mM EDTA buffer, pH 8.00 (pH at 25°C). Substrate was then added and residual enzyme activity determined. The I_{50} was calculated from the regression line of a plot of log (% activity) versus $[I]$.

Statistical analyses. Unless otherwise indicated, data are reported as means ± SE from three animals or experiments. Significance of differences among several means was determined by one-way ANOVA ($p < 0.05$) and between pairs of means by an independent t test ($p < 0.05$). Primary and secondary kinetic plots as well as I_{50} plots were fitted by least-squares regression analysis. Calculations and analyses were carried out using SYSTAT statistical software (SYSTAT, 1992).

RESULTS

Increasing doses of CPS administered orally in corn oil to adult hens produced increasingly greater inhibition of hen

brain AChE and NTE activities 4 days after dosing (Fig. 1). The highest dose produced 76.6 ± 4.1% inhibition of NTE and 86.2 ± 7.3% inhibition of AChE. Acute cholinergic signs were observed at all doses, necessitating the use of atropine to ensure survival. Atropine was discontinued if the animal demonstrated signs of recovering from acute cholinergic effects. At the time of euthanization, both of the surviving hens in the 75 mg/kg group were fully recovered with respect to clinical signs (one hen died as the result of regurgitation and aspiration within 8 hr of CPS dosing); in the 150 mg/kg group, two of the three hens had fully recovered, but one animal was still weak with an unsteady gait; in the 300 mg/kg group, all animals appeared moribund.

In the time-course study of brain AChE and NTE inhibition following a single dose of CPS (150 mg/kg) (Fig. 2), apparent maximal AChE inhibition (86.4 ± 3.5%) was seen at 24 hr, the earliest time point measured. AChE inhibition showed an apparent decline between 1 and 4 days and had fallen to 43.9 ± 2.1% by Day 8. Apparent maximal NTE inhibition (37.8 ± 9.3%) occurred 4 days after dosing, although there were no significant differences between NTE inhibition values during Days 1–8 (one-way ANOVA, $p > 0.05$). The surviving treated hens exhibited recovery from the acute effects of the dosing 72 to 120 hr after dosing. No signs of OPIDN were seen in any of the animals during the course of the experiment.

Primary kinetic plots for the inhibition of hen brain AChE by CPO *in vitro* are shown in Fig. 3. The lines did not extrapolate precisely through the origin (log 100 = 2), indicating that there was a small amount of zero-time inhibition. Increasing the substrate concentration did not shift the intercepts toward the origin, suggesting the presence of a non-Michaelis complex (Aldridge and Reiner, 1972; Richardson, 1992). Nevertheless, nearly ideal kinetics were obtained: the rates were first order, and the slopes of the primary plot lines, $-k'/2.303$, were linearly related to the inhibitor concentration, $[I]$, for each substrate concentration used. The k_i values derived from the relationship, $k' = k_i[I] + k_o$, as described under Methods, are listed in Table 1. Because the mean k_i values obtained from three experiments for each substrate concentration were not significantly different from each other (one-way ANOVA, $p > 0.05$), they were pooled to give an overall mean value across the three substrate concentrations of 15.5 ± 0.2 μM^{-1} min^{-1}. The corresponding fixed-time (20-min) I_{50} value calculated from the relationship, $I_{50} = 0.693/k_i t$, where t = preincubation time with inhibitor, was 2.24 ± 0.03 nM. The I_{50} determined by fixed-time preincubation was 2.16 ± 0.13 (mean value from five experiments), which was not significantly different from the value derived from the k_i (independent t test, $p > 0.05$).

Figure 4 shows the primary plots for inhibition of hen brain NTE by CPO *in vitro*. There was a pronounced zero-

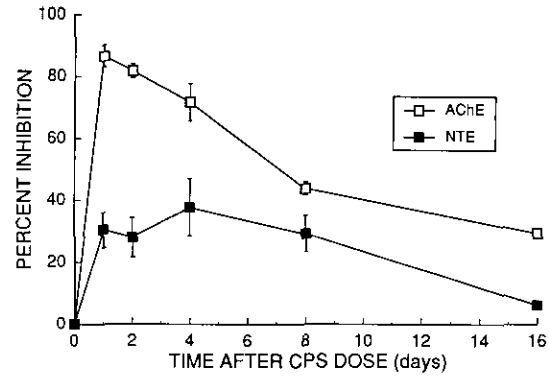

FIG. 2. Time-course (percentage of contemporaneous control) of hen brain AChE and NTE inhibition after a single dose (150 mg/kg po in 2 ml/kg corn oil) of CPS. Each point represents the mean ± SE of duplicate determinations from three animals. The range of mean control activities over the time-course of the experiment for AChE and NTE was 16.3–19.1 and 2.96–3.44 μmol/min/g wet weight tissue, respectively.

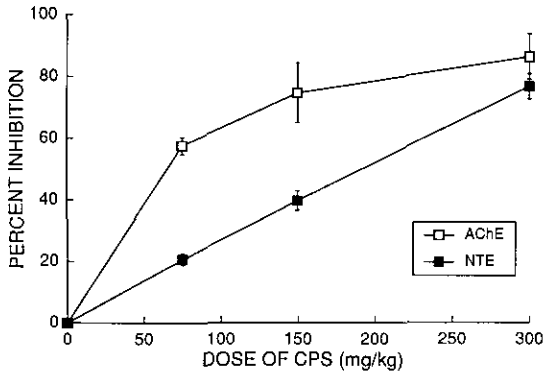

FIG. 1. Dose–response (percentage of control) of hen brain AChE and NTE inhibition 4 days after dosing with CPS (po in 2 ml/kg corn oil). Each point represents the mean ± SE of duplicate determinations from three animals, except for the 75 mg/kg point, which was determined for two animals. Mean ± SE control activities for AChE and NTE were 16.5 ± 0.44 and 2.82 ± 0.06 μmol/min/g wet weight tissue, respectively.

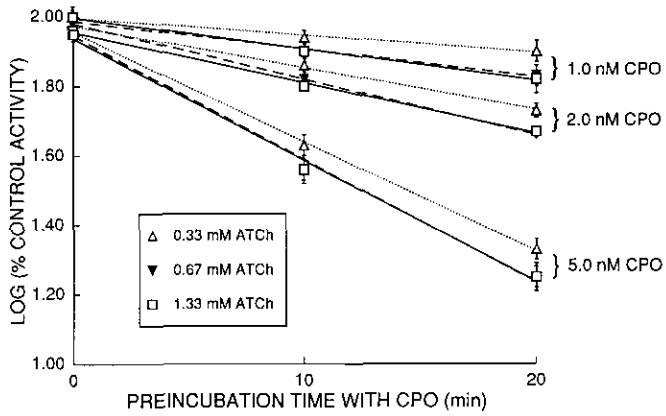

FIG. 3. Kinetics of hen brain AChE inhibition *in vitro* by 1.0, 2.0, or 5.0 nM CPO. Preincubation with inhibitor for 0, 10, or 20 min was followed by a 30-min incubation with 0.33, 0.67, or 1.33 mM substrate (ATCh) at 37°C, pH 7.60 Na phosphate. Each point represents the mean ± SE from three experiments.

TABLE 1
Bimolecular Rate Constants of Inhibition of AChE and NTE by CPO[a]

AChE		NTE	
[ATCh] (mM)	k_i (μM^{-1} min^{-1})	[PV] (mM)	k_i (μM^{-1} min^{-1})
0.33	15.6 ± 0.24	1.33	0.134 ± 0.008
0.67	15.8 ± 0.50	2.65	0.154 ± 0.012
1.33	15.1 ± 0.64	5.30	0.146 ± 0.008

[a] k_i values were determined as described under Methods from the slopes of secondary plots of k' versus [CPO], where $-k'/2.303$ is the slope of the primary kinetic plot (see Figs. 3 and 4) for a given [CPO] and substrate concentration. Data are means ± SE from three experiments.

time inhibition at the two highest CPO concentrations which was reversed by increasing the substrate concentration, indicating the presence of a Michaelis-type complex between NTE and CPO (Aldridge and Reiner, 1972). The primary plots were first order, with slopes linearly related to inhibitor concentration for each substrate concentration. The k_i values derived from these plots as described under Methods are given in Table 1. The k_i values obtained from three experiments for each substrate concentration were statistically indistinguishable from each other (one-way ANOVA, $p > 0.05$). Therefore, the three k_i values were pooled to give an overall mean k_i of 0.145 ± 0.010 μM^{-1} min^{-1}, corresponding to a derived fixed-time (20-min) I_{50} of 239 ± 10 nM. The I_{50} determined by fixed-time (20-min) preincubation was 206 ± 1.6 nM (mean value from four experiments), which was significantly different from the value derived from the k_i (independent t test, $p < 0.05$).

DISCUSSION

The acute effects of CPS *in vivo* in the dose–response study were similar to those documented in a corporate report by Rowe *et al.* (1978), who found that the LD_{50} of CPS in hens without atropine treatment was 50 mg/kg (po, gelatin capsules) and that animals given 100 mg/kg with atropine treatment required 79–127 hr to recover from acute cholinergic effects. They also noted that in preliminary trials, hens given atropine could consistently survive 100 mg/kg CPS, but doses of 200 mg/kg were not consistently survived. Johnson (1982) reported an approximate LD_{50} value of 100 mg/kg for CPS in hens. He found that this dose produced a brain NTE inhibition of 51% 1–2 days after dosing and that there were no subsequent clinical signs of OPIDN. Capodicasa *et al.* (1991) estimated the unprotected LD_{50} of 99% pure CPS administered po in glycerol formal to be only 15 mg/kg in hens, but that six times this amount was required to produce 80% brain NTE inhibition on Day 6 and mild clinical ataxia (a mean ataxia score of 1.1 on a scale of 0.0–4.0) on Day 25 after dosing. In order to survive a dose of six times the LD_{50}, Capodicasa *et al.* (1991) found that prophylaxis with atropine alone was insufficient and that pralidoxime was also required. Likewise, we found that the dose of CPS required to produce inhibition of brain NTE above the putative threshold for neuropathy on Day 4 (300 mg/kg) produced severe cholinergic toxicity in hens even with aggressive atropine treatment; this dose was 3–20 times the LD_{50}, depending upon the estimate used.

The time-course of hen brain NTE inhibition produced by 150 mg/kg CPS in our study was in agreement with that reported by Capodicasa *et al.* (1991), but in their study this dose was over twice as potent at inhibiting NTE, producing 92% inhibition on Day 4 and moderate clinical ataxia (mean ataxia score of 2.5 on a scale of 0.0–4.0) on Day 25 postdosing. AChE inhibition following 150 mg/kg in our study reached its highest value of 86% on Day 1, whereas this dose appeared to saturate AChE in the other study, so that inhibition was 95–100% for Days 1–4. Possible reasons for the apparent discrepancy between the two studies with respect to the potency and time-course of inhibition of brain AChE and NTE by CPS *in vivo* include the use of different CPS purities and dosing vehicles. For the 150 mg/kg dose, Capodicasa *et al.* (1991) used commercial formulations containing 40–60% CPS in methylene chloride rather than pure CPS, and their dosing vehicle was glycerol formal rather than corn oil. However, with 99% pure CPS delivered in glycerol formal, they obtained 37% inhibition of brain NTE and 82% inhibition of brain AChE 4 days after dosing with 90 mg/kg; this compares with 38% inhibition of brain NTE and 72% inhibition of brain AChE 4 days after dosing with 150 mg/kg in our study. Thus, even though there is a difference of approximately twofold in absolute inhibitory potency of CPS *in vivo* between the two studies, both studies indicate a similar degree of relative selectivity for AChE over NTE.

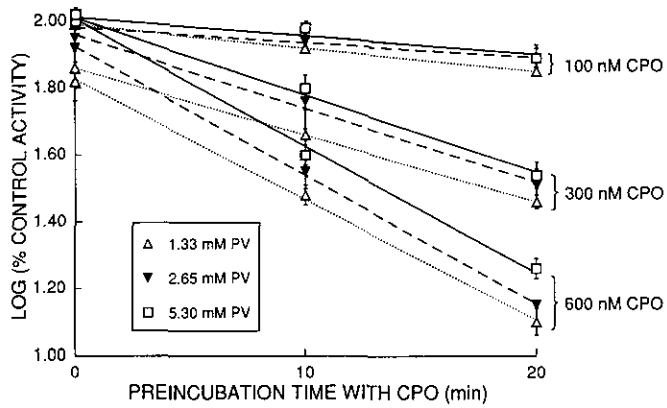

FIG. 4. Kinetics of hen brain NTE inhibition by 100, 300, or 600 nM CPO. Preincubation with inhibitor for 0, 10, or 20 min was followed by a 15-min incubation with 1.33, 2.65, or 5.30 mM substrate (PV) at 37°C, pH 8.00 Tris (pH at 25°C). Each point represents the mean ± SE from three experiments.

Our kinetics studies *in vitro* indicate that CPO, the active metabolite of CPS, has extraordinary potency for AChE inhibition, comparable to that of paraoxon and only about 10-fold less than that of the nerve agent, soman. The k_i for inhibition of hen brain AChE by CPO is 15.5 μM^{-1} min^{-1}, corresponding to a fixed-time (20-min) I_{50} of 2.24 nM (the relationship $I_{50} = 0.693/k_i t$ may be used to calculate a fixed-time I_{50} when ideal kinetics are obtained). Capodicasa *et al.* (1991) reported nonlinear kinetics of hen brain AChE inhibition by CPO from which no k_i could be calculated, but they determined a 20-min I_{50} of 6 nM. Sultatos *et al.* (1982) reported a 60-min I_{50} of 3.6 nM for inhibition of mouse brain AChE by CPO determined at 27°C; this value would be expected to be about 5.4 nM for 20 min and 37°C (Aldridge and Reiner, 1972). Wang and Murphy (1982) compared the sensitivity of brain AChE from various species to inhibition by different OP oxons at 22°C and found that hen brain AChE was the most sensitive to paraoxon, with a k_i of 3.98 μM^{-1} min^{-1}; this value was 3.3 times the value found for monkey brain and approaches our value for CPO when adjusted for temperature. Soman was reported to have a k_i of 90 μM^{-1} min^{-1} for inhibition of rabbit brain AChE at pH 7.0 and 25°C (Gray and Dawson, 1987); this value would be expected to increase about 2-fold at 37°C to a value only about 10-fold more potent than that of CPO. These results indicate that CPO is among the most potent OP inhibitors of AChE known. Thus, once it reaches its AChE target, CPO would be expected to be unusually effective at producing its intended toxicological action. The fact that CPS has comparatively moderate acute toxicity to nontarget organisms underscores the existence of efficient detoxification mechanisms for this compound, particularly in mammals (Eto, 1979; Sultatos *et al.*, 1984, 1985; Costa *et al.*, 1990).

In order to evaluate the relative potency of OPs to cause acute and delayed neurotoxic effects, Lotti and Johnson (1978) compared NTE/AChE I_{50} ratios for a variety of OPs with the corresponding NPD/LD_{50} ratios, where NPD is the neuropathic dose, i.e., the minimal dose required to produce frank clinical neuropathy. In general, they noted that I_{50} ratios greater than 1 correlated with NPDs greater than the LD_{50}. For example, in the homologous series of dialkyl 2,2-dichlorovinyl phosphates (dichlorvos homologs), the I_{50} ratios are 46, 6.2, 0.37, and 0.03 for the dimethyl, diethyl, di-*n*-propyl, and di-*n*-pentyl derivatives, respectively. The corresponding NPD/LD_{50} ratios are 18, 6, 0.2, and 0.08. Here we have an example of a complete spectrum of acute and delayed neurotoxic effects within a single family of compounds. The dipentyl derivative is potently neuropathic in hens and produces no signs of acute cholinergic toxicity, whereas the dimethyl compound is potently cholinergic and requires massive prophylaxis with atropine in order to survive the multiple LD_{50} dose needed to produce a neuropathic effect. Capodicasa *et al.* (1991) determined fixed-time (20-min) I_{50} values of 150 and 6 nM for CPO inhibition of hen brain NTE and AChE, respectively, yielding an I_{50} ratio of 25. This is a large ratio that falls between the values obtained for dimethyl and diethyl dichlorvos, consistent with their finding that the minimal neuropathic dose of CPS was 4–6 times the LD_{50}.

A principal contribution of the present study is the determination of the k_i values for inhibition of hen brain AChE and NTE by CPO. These rate constants may be used to improve the quantitative estimation of the neuropathic risk of CPS because the AChE/NTE k_i ratio is a more generally applicable comparison of relative inhibitory potency than the reciprocally related NTE/AChE I_{50} ratio. Unlike the I_{50}, the k_i represents an intrinsic measure of inhibitory power that is independent of the time of incubation and that takes into account deviations from ideal kinetics or differences in the kinetics of inhibition between the two enzymes (Clothier *et al.*, 1981; Richardson, 1992). To the best of our knowledge, this paper is the first to report k_i values for inhibition of hen brain AChE and NTE by CPO, and only one other study (Capodicasa *et al.*, 1991) has reported an I_{50} value for inhibition of hen brain NTE by CPO.

Because of the nearly ideal characteristics of the kinetics of inhibition of AChE and NTE by CPO, we found reasonably good agreement between I_{50} values calculated from the k_i and our fixed-time I_{50} values. However, the presence of zero-time inhibition resulted in a small but significant decrease in the I_{50} for NTE when this parameter was determined by fixed-time rather than kinetic means. Accordingly, the AChE/NTE I_{50} ratio was 107 when calculated from kinetic experiments and 86 when calculated from fixed-time experiments. These results may be compared with the previously determined fixed-time AChE/NTE I_{50} ratios of 25 for CPO, which corresponded to a dose of CPS of 4–6 times the LD_{50} to produce neuropathy (Capodicasa *et al.*, 1991), and 46 for dimethyl dichlorvos, which required a dose 18 times the LD_{50} to produce neuropathy (Lotti and Johnson, 1978; Richardson, 1992). If the correlation between the AChE/NTE I_{50} ratio and the neuropathic dose holds, our results with a kinetically determined I_{50} ratio suggest that the neuropathic dose of CPS is even higher than the previous estimate, a number that would be impractical to obtain *in vivo*. Certainly, our results confirm that *in vitro* indicators of relative inhibitory potency toward AChE versus NTE have predictive value in estimating the neuropathic risk of OP compounds and indicate that acute exposures to CPS would not be expected to result in OPIDN except under extreme conditions such as attempted suicides involving medically assisted survival of doses considerably in excess of the LD_{50}. Moreover, because many OP compounds do not exhibit ideal kinetic behavior in their inhibition of target esterases (Clothier *et al.*, 1981), we suggest using kinetically determined k_i values in preference to fixed-time I_{50} values as the criterion of relative inhibitory potency when making quantitative assessments of the neuropathic risk of these materials.

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