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SLOW ACCUMULATION OF ACETYLCHOLINESTERASE IN RAT BRAIN DURING ENZYME INHIBITION BY REPEATED DOSING WITH CHLORPYRIFOS

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Abstract—When given to rats, *O,O'*-diethyl-*O*-[3,5,6-trichloro-2-pyridyl]-phosphorothionate (chlorpyrifos), a common insecticide, causes an unusually lengthy dose-dependent fall in the activity of brain acetylcholinesterase (AChE; EC 3.1.1.7). To determine whether the slow recovery involves impaired AChE synthesis, experiments were designed to measure AChE activity, immunoreactive AChE protein (AChE-IR) and AChE mRNA. Male, Long–Evans rats, maintained at 350 ± 5 g, were dosed (s.c.) weekly for 4 weeks with 0, 15, 30, or 60 mg/kg chlorpyrifos in peanut oil. Brain tissue was harvested 1, 3, 5, 7 and 9 weeks after treatment began. AChE activity was measured by Ellman assay, and AChE-IR was estimated by two-site ELISA using monoclonal antibodies to rat brain AChE. While AChE activity fell significantly at all times and doses, AChE-IR increased at 3 and 5 weeks in the two higher dosage groups. Larger increases of AChE-IR were observed after chlorpyrifos was administered for 4 weeks by the oral route. Northern blots quantified with reference to cyclophilin were consistent with stable levels of AChE mRNA. Overall, it appears that chronically reduced brain AChE activity after chlorpyrifos reflects sustained enzyme inhibition, not loss of enzyme protein or suppression of AChE message.

Key words: chlorpyrifos; acetylcholinesterase gene expression; rat brain; organophosphate

Among the irreversible AChE§ (EC 3.1.1.7) inhibitors that must be bioactivated by cytochrome P450-dependent monooxygenases are the phosphorothionates [1]. The toxicology of these compounds offers several interesting features for study, such as the metabolic and kinetic factors that determine relative potency for acute toxicity and the duration of toxic effects *in vivo* [2–4]. In this regard, *O,O'*-diethyl-*O*-[3,5,6-trichloro-2-pyridyl]-phosphorothionate (chlorpyrifos) deserves special attention as an insecticide with a total U.S. usage of 10–20 million pounds in 1990–1991 [5].

Recovery from chlorpyrifos is unusually slow, much slower than from parathion and methyl parathion, related compounds that also undergo oxidative activation *in vivo* [6]. Animals exposed to chlorpyrifos exhibit a dose-dependent fall in brain AChE activity lasting for weeks after treatment ends [6–8]. The prolonged inhibition may reflect the compound's lipophilic nature, which would promote storage in lipid depots and delay bioactivation [4]. Since cholinesterase recovery presumably requires *de novo* enzyme synthesis, an alternative explanation for lengthy inhibition would be impaired AChE production in the brain. For example, chlorpyrifos might be directly toxic to central cholinergic neurons or might interfere with AChE gene expression. Such possibilities are best addressed by experiments measuring AChE mRNA, AChE protein, and AChE enzyme activity in a common set of samples. In the present study, we employed Northern blotting, immunoassay and enzyme assay to clarify the effects of chlorpyrifos on brain AChE.

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§ Abbreviations: AChE, acetylcholinesterase; AChE-IR, AChe-immunoreactivity; TBS, Tris-buffered saline; SSC, standard saline citrate.

MATERIALS AND METHODS

Toxicants, substrates, and anticholinesterases. Chlorpyrifos was obtained from the Chem Serv Co. (Westchester, PA). Chlorpyrifos oxon was supplied by the central repository of the U.S. EPA Facility, Research Triangle Park, NC. Echothiophate ([2-mercaptoethyl] trimethylammonium iodide *O,O'*-diethylphosphorothioate) was obtained from Ayerst Laboratories, Inc. (New York, NY). Acetylthiocholine iodide and ethopropazine (10-[2-diethylaminopropyl]-phenothiazine hydrochloride) were purchased from the Sigma Chemical Co., St. Louis, MO. BW 284C51 (1:5-bis [4-allyldimethylammoniumphenyl]-pentan-3-one dibromide) was purchased from Burroughs Wellcome Inc. (Research Triangle Park, NC).

Animals. All animal procedures were in strict conformance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of the Health Effects Laboratory of the U.S. Environmental Protection Agency. Male, Long–Evans rats (Charles River Breeding Laboratories, Raleigh, NC), 60 to 70-days-old, were housed individually in suspended plastic cages and allowed free access to water.

Treatment regimens. Rats were dosed with chlorpyrifos according to one of two regimens. One set of animals received s.c. injections of chlorpyrifos in peanut oil. These animals were weighed daily and each was maintained at 350 ± 5 g body weight by scheduled, weighed feedings [9]. Dosages were 60 ("high dose"), 30, or 15 mg/kg, or vehicle only (control). Injections were given in a volume of 2 mL/kg at weekly intervals for 4 weeks (i.e. up to five doses). Animals were killed, with no further injection, at 1, 3, and 5 weeks after starting treatment (dosing phase) and also at 7 and 9 weeks (recovery phase). The experiment was repeated with a second set of controls and "high dose" rats which were killed at 3 and 5 weeks.

In a separate experiment intended to produce maximal AChE inhibition, ten rats with free access to food received chlorpyrifos in corn oil by oral gavage at a constant volume of 1 mL/kg. During week 1, toxicant was given at 100 mg/kg for 3 days. This level of dosage was not well tolerated, so after allowing a day for recovery, a reduced dose of 75 mg/kg was given. For weeks 2–4, the rats received 75 mg/kg each day, Monday through Friday. Only four rats survived until tissues were harvested at 4 weeks, approximately 24 hr after the last dosing. Vehicle-treated rats were not available as they were needed for ongoing behavioral studies in a concurrent experiment, but animals of the same age and weight were used as controls.

Preparation of tissues from treated rats. At varying times after dosage, rats were euthanized by carbon dioxide anesthesia followed by decapitation. Brains were quickly removed and dissected with guidance from a standard atlas [10]. In selected cases, cerebellum, hippocampus and frontal regions forward of the optic chiasm were sampled; in general, however, these areas were reserved for unrelated studies. Remaining parts of the telencephalon, diencephalon and brainstem were collected from all rats. Care was taken to ensure that these "global brain samples" had a consistent composition with closely comparable weights and AChE activities in all samples from a given experimental group. Diaphragm, femoral biceps muscle, and liver were collected from a few rats. Immediately after dissection, tissue pieces (divided at the midline for separate analyses of enzyme and mRNA) were frozen on dry ice for storage at −80°.

In vitro *exposure.* Brains were removed from rats overdosed with ether. Cubes (1 mm) of cerebellum were cut with a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.) and distributed as rapidly as possible into vials with 10 mL of glucose-containing, bicarbonate-buffered physiologic saline solution [11]. Less than 10 min elapsed between removal of the brain and transfer of the tissue cubes, which were maintained at 37° and continuously gassed with 95% O_2–5% CO_2. Chlorpyrifos oxon or echothiophate was added at a final concentration of 10^{-7} M; after 20 min, the solutions were decanted and the cubes were rinsed with 15 mL of drug-free saline. Rinsing was repeated every 20 min until 1 hr after the onset of exposure. The vials were then chilled to ice temperature and rinsed at 10-min intervals for an additional half-hour.

For experiments on the rate of AChE inhibition *in vitro*, brain extracts (see below) were prepared from the forebrain of control rats or rats treated for 4 weeks with the high oral dose of chlorpyrifos. Because AChE in the latter was inhibited approximately 90%, the control samples were diluted 1:10 (v/v) with homogenization buffer to yield approximately the same final enzyme activity (70–90 mOD/min). At time-zero, chlorpyrifos oxon (0.0625, 0.125 or 0.25 μM, final concentration in 0.25% acetone) or the acetone vehicle was added together with acetylthiocholine iodide (0.6 mM final concentration), according to the protocol of Kemp and Wallace [12]. Reactions were maintained at 26°, and $\varepsilon_{412\,nm}$ was recorded every 2 min. AChE activity in each sample exposed to oxon *in vitro* was compared with the activity in the same sample exposed to vehicle alone.

Tissue extraction for ELISA and AChE assay. All procedures were performed on ice. Frozen tissue samples were thawed and homogenized in 10 vol. of 10 mM Tris buffer, pH 7.4, containing 0.9% (w/v) NaCl, 1 mM EDTA, 0.05% (v/v) Triton X-100 and 1% (w/v) BSA (Sigma). Muscles were chopped before homogenization; NaCl in the buffer was increased to 0.5 M for muscle extractions. Fresh brain cubes were homogenized in groups of 4 in 1.5 mL of 50 mM sodium phosphate, pH 7.4, 0.5% Triton X-100, 0.1% BSA. Extracts were centrifuged at 10,000 *g* for 15 min at 4°, and the supernatants were retained for assay.

AChE immunoassay. AChE-IR was determined by a two-site ELISA using an adaptation of published procedures [13, 14]. The ELISA was based on two monoclonal antibodies to rat brain AChE [15]: a primary antibody, ZR 6, chosen to optimize specific antigen binding, and a second antibody, ZR3, biotinylated as detailed earlier [13].

Primary antibody, 10^{-8} M in 0.1 M sodium carbonate buffer, pH 9.6, was adsorbed overnight at 4° to polystyrene microtiter wells (Immulon II, Dynatech, Chantilly, VA). Unbound antibody was washed away and residual binding sites were blocked ≥ 2 hr by 3% (w/v) BSA in carbonate buffer. Two rinses with TBS (10 mM Tris–HCl, pH 7.4, 0.9% NaCl) preceded the addition of AChE samples for overnight incubation at 4°. Following three rinses with TBS, incubation was continued for 2 hr at room temperature with a second antibody, 10^{-8} M in TBS/Tween (TBS, 0.05% Tween 20) plus 0.1% (w/v) BSA. Three rinses with TBS/Tween preceded the addition of alkaline-phosphatase-conjugated avidin (Boehringer Mannheim) diluted in TBS/Tween/BSA. After a final 2-hr incubation at room temperature, the wells were washed four times with TBS/Tween, and bound phosphatase was detected with *p*-nitrophenyl-phosphate (Sigma) in a kinetic microtiter plate reader.

Considerable effort was made to minimize and compensate for inter-assay and inter-plate variation in the ELISA. Assays were always run in triplicate, with experimental and control samples arranged together on a single microtiter plate. Plates were often replicated in reverse order for repeat assay.

Under these conditions, intra-assay coefficients of variation ranged from 7 to 9%. Absolute levels of AChE protein could not be obtained, in the absence of a pure internal standard. Actual assay signals, representing the activity of bound alkaline phosphatase, varied from day to day and plate to plate. Therefore, to facilitate comparison between runs, all ELISA data are expressed as percentages of the mean value from the concurrently assayed controls.

Estimation of enzyme activity. AChE activity was determined in triplicate by a spectrophotometric method [16] with 1 mM acetylthiocholine as substrate. Butyrylcholinesterase (EC 3.1.1.8) was inhibited by ethopropazine at a final concentration of 10^{-4} M. Blanks typically consisted of extraction buffer in place of sample. Controls with tissue extract and ethopropazine (10^{-4} M) plus BW 284C51 (10^{-6} M) demonstrated that thiocholine hydrolysis under standard conditions was attributable to AChE.

Preparation of RNA. Using a standard protocol (InVitrogen, San Diego, CA), poly-A^+-RNA was extracted from brain tissue by lysis in SDS-based buffer containing RNAse inhibitor. Lysates were incubated at 45° and applied to oligo-(dT) cellulose columns. Differential salt-elution was used to isolate the mRNA, whose concentration was determined by ε_{260} readings.

Northern blotting. Probes were prepared from plasmids containing a 590 bp mouse AChE cDNA cloned into pBLUESCRIPT II KS- [17], supplied by Dr. Palmer Taylor (UCSD, LaJolla, CA), or a 680 bp cDNA for rat cyclophilin, p1b15 [18]. Fragments of the expected sizes were isolated from XI1 Blue *Escherichia coli* lysates by endonuclease digestion and agarose gel electrophoresis. Isolated probes were random prime labeled with [α-^{32}P]dCTP to a specific radioactivity of 1–3 × 10^8 cpm/μg DNA.

Poly-A^+-mRNA was denatured in 50% (v/v) formamide at 65° for 10 min and electrophoresed in 1.2% (w/v) agarose gels containing 6% formaldehyde. After electrophoresis, RNA was blotted onto nylon membranes, which were dried at room temperature and baked at 80° for 2 hr. Dried blots were treated for 4 hr at 42° with 25 mL of 50% formamide—10× Denhardt's solution—5× SSC (1× SSC = 0.15 M sodium chloride and 0.015 M sodium citrate) containing 250 μg/mL denatured herring sperm DNA, 50 μg/mL poly-A, and 0.1% SDS. Blots were then hybridized in fresh buffer with random prime-labeled cDNA for AChE (≈ 5 × 10^6 cpm/mL; 36–40 hr at 42°). Hybridized blots were washed with 1× SSC (two times at room temperature, two more times at 45°), air dried and exposed to Kodak XAR-5 film for up to 10 days at −80°. Measurements of AChE mRNA in different samples were standardized with reference to cyclophilin mRNA. For this purpose, gels were stripped by boiling in low salt buffer and rehybridized with cyclophilin cDNA (same conditions as for AChE hybridization). To produce bands of density similar to those obtained with the AChE probe, the second exposure was reduced to 6–12 hr. Relative band densities were determined with a scanning densitometer. Since intensities were highly variable from gel to gel, experiments were designed so that comparisons were confined to lanes on a single gel.

Statistical calculations. Data were examined for significant differences using analysis of variance (ANOVA) and the unpaired Student's *t*-test. Individual data were normalized by expression as percentages of the mean control value in a given assay with a given tissue. The criterion for significance was adjusted to allow for effects of multiple comparisons and to keep the overall probability of type I error below 5%. Regression analysis was used to ascertain whether a dose–response relationship existed.

RESULTS

In vitro *tests of chlorpyrifos oxon.* Initially, to determine whether organophosphate interfered with the AChE ELISA, whole brain extracts were incubated for 1 hr at 37° with chlorpyrifos oxon, 10^{-6} M in 1% ethanol. AChE enzyme activity in these samples was only 2% of that in extracts treated similarly but without organophosphate (three independent determinations). In contrast, immunoassay signals from the oxon-treated samples averaged 97 ± 4% of the control values (four independent trials). Thus, the AChE ELISA was not affected by AChE inhibition or by the presence of the organophosphate.

Another *in vitro* experiment was performed to determine whether chlorpyrifos oxon could gain access to all AChE, including enzyme located in intracellular compartments of the brain. Pieces of rat cerebellum (1 mm^3) were incubated at 37° in oxygenated physiological saline with 10^{-7} M concentrations of chlorpyrifos oxon or the non-permeant organophosphorus compound, echothiophate (see Materials and Methods). For comparison, rat brain homogenates were exposed to the same agents under similar conditions. Echothiophate was restricted from some tissue compartments as it inhibited 99% of the AChE activity in homogenates but only 73% of the activity in "intact" brain cubes. On the other hand, chlorpyrifos oxon exhibited ready access to all tissue AChE, inhibiting the enzyme nearly equally in both types of sample (99% in homogenates, 96% in brain cubes).

The rate of AChE inhibition when chlorpyrifos oxon was added to brain homogenates *in vitro* was strongly dose dependent (Fig. 1). However, this rate was essentially identical in homogenates from control rats and homogenates from rats given repeated high doses of chlorpyrifos (see below). Thus, prior *in vivo* exposure to chlorpyrifos did not alter inherent sensitivity to the organophosphate.

AChE activity and AChE-IR after weekly s.c. chlorpyrifos. The course of AChE inhibition by chlorpyrifos *in vivo* was slowest in the samples from the lowest dosage group (15 mg/kg per week), in which the level of inhibition increased from approximately 15% at 1 week to 50% at 5 weeks, the end of the dosing phase (Fig. 2). Higher doses caused faster and greater inhibition. At each of the three time points during dosing, there was a significant correlation between the dose of chlorpyrifos and the degree of AChE inhibition ($P < 0.001$). Inhibition in global brain samples never

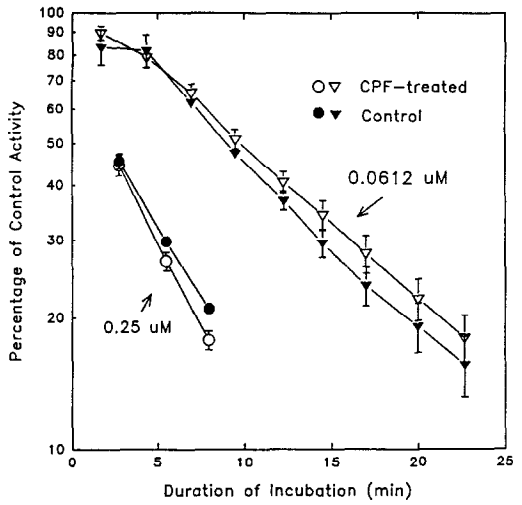

Fig. 1. Rate of AChE inhibition by chlorpyrifos oxon (CPF) *in vitro*. Extracts of forebrain were prepared from control rats and from rats treated for 4 weeks with the high oral dose of chlorpyrifos. Control extracts were diluted 10-fold to equalize AChE activity with the pretreated samples. Chlorpyrifos, in 0.25% acetone, was then added in the indicated final concentrations. For each sample, AChE activity is expressed as a percentage of activity in aliquots incubated with vehicle alone (no change with time). Means (± SEM) of 4 determinations are shown (100% = 10.9 and 1.0 μmol/min/g for control and test forebrain, respectively; see Table 3).

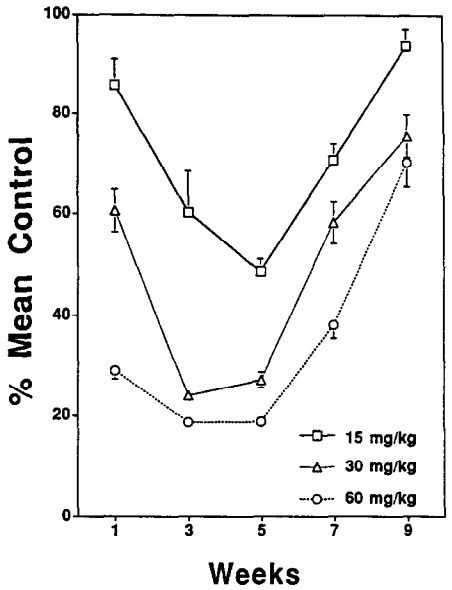

Fig. 2. Time- and dose-dependent loss and recovery of AChE enzyme activity during and after treatment with chlorpyrifos. Rats were given weekly s.c. injections of chlorpyrifos or vehicle at the indicated dosages for up to 4 weeks. Brains were obtained after 1, 3 and 5 weeks (intoxication phase) and 7 and 9 weeks (recovery phase). One week had elapsed between the last injection and the time of collection of samples. Mean values (± SEM) of AChE activity in global samples (hindbrain plus portions of diencephalon and telencephalon) are represented as percentages of the concurrently assayed control mean values (100% = 4.9 μmol/min/g). The number of rats per group ranged from 6 to 18; all experimental groups had significantly less AChE activity than the controls ($P < 0.01$), except the low dose group at week 9.

exceeded 80%, even after 4 weeks of treatment at the 60 mg/kg level. The distribution of inhibition in other samples was characteristic: greatest in forebrain, somewhat less in hippocampus, and substantially less in cerebellum, diaphragm, and liver (Fig. 3).

Mixing experiments were performed to determine whether free AChE inhibitor could be detected in brain samples obtained during the dosing phase. AChE activity was measured in extracts from the control and high-dose groups assayed separately and after mixing in equal parts. At all three time points (1, 3 and 5 weeks), activities in the mixtures were within 5% of the component sums. In other words, we could not detect any inhibitory effects attributable to the presence of chlorpyrifos oxon or other active metabolites *in vitro*.

During the recovery phase, brain AChE activity returned slowly but steadily. At 9 weeks, the lowest dosage group was no longer affected significantly, but the rats in the higher dosage groups still exhibited activities 25–30% below control. Absolute rate of recovery, however, appeared to be independent of dose (Fig. 2).

The effect of chlorpyrifos on AChE protein was determined by ELISA for AChE-IR in aliquots of the same brain extracts used for enzyme assay (Table 1). In contrast to enzyme activity, AChE-IR was not decreased by any dosage at any time. Instead, a modest *increase* developed over time. No change occurred after treatment with 15 mg/kg, but, compared with vehicle-treated controls, AChE-IR in global brain samples increased after chlorpyrifos in doses of 30 mg/kg (5 weeks) and 60 mg/kg (3 and 5 weeks). The increases were small, at most 15%, but they were clearly significant (Table 1). Furthermore, at both 3 and 5 weeks, regression analyses showed a statistically significant relationship between the dose of chlorpyrifos and the level of AChE-IR in the global brain samples ($P < 0.01$). On the other hand, no significant changes in AChE-IR were seen elsewhere in the brain (forebrain, hippocampus, cerebellum) or in diaphragm or liver (Table 2).

AChE activity and AChE-IR after daily oral chlorpyrifos. Chlorpyrifos given orally was also effective in decreasing AChE enzyme activity (Table 3). Control brains exhibited large regional variations in AChE activity, each area differing significantly from each other in its mean "starting" activity. After oral chlorpyrifos for 4 weeks, AChE activity dropped sharply throughout the brain, but percentage reduction was greatest where activity was normally highest. Consequently, residual AChE activity after chlorpyrifos was distributed more uniformly across the brain.

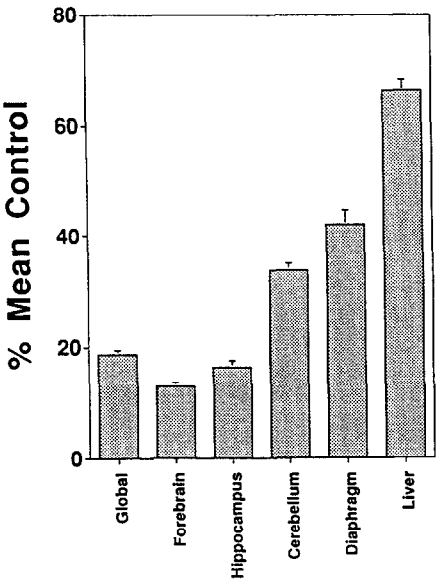

Fig. 3. Regional variation of AChE inhibition. Brain samples were obtained 3 weeks after beginning once per week s.c. injections of chlorpyrifos, 60 mg/kg (11–17 rats). One week had elapsed between the last injection and the time of collection of samples. AChE activity is expressed as a percentage of the mean activity (± SEM) from corresponding samples from control rats treated with vehicle alone (for absolute values see Table 2). All experimental values were significantly less than controls ($P < 0.0001$).

Like the larger s.c. injections, high oral doses of chlorpyrifos for 4 weeks caused increases in brain AChE-IR. These increases were greater than those obtained in the earlier experiment, up to 56% above control levels in global brain samples. The effects were statistically significant in all brain areas examined (Table 3).

AChE mRNA. Northern blotting with a ^{32}P-labelled cDNA probe for murine AChE served to quantitate AChE message levels in contralateral samples for comparison with enzyme activity and immunoreactivity. In all cases, a major band was observed with an apparent size of 2.4 kb (Fig. 4). The intensity of this band, relative to the 0.9 kb band labeled by a cDNA probe for cyclophilin, was taken to reflect the abundance of AChE mRNA. In global brain extracts from rats given weekly injections of chlorpyrifos at a dosage of 60 mg/kg, s.c., there were no statistically significant changes in AChE message levels, either during dosing or during recovery (Table 4). Message level did appear to increase by 87% after 4 weeks of high oral dosage. Because of the need to combine samples for adequate sensitivity, however, this determination was based on a single pool of four brains. Overall, therefore, the data fail to prove that AChE mRNA increased in response to treatment with chlorpyrifos, though they clearly show that message levels did not *decrease*.

DISCUSSION

AChE protein levels and toxicological implications. Chlorpyrifos-induced increases in brain AChE-IR were modest, but it is surprising that enzyme content rose at all. Nonetheless, we are unable to identify experimental artifacts that would imply a spurious result. It is unlikely on *a priori* grounds that chlorpyrifos would nonspecifically enhance the signal from an AChE ELISA. In any case, the study with chlorpyrifos oxon *in vitro* showed that neither the presence of active organophosphate nor the phosphorylation of AChE had appreciable effects on the immunoassay. Experimental groups were interlaced rigorously on a single assay plate in order to reduce inter-plate and inter-assay variation as

Table 1. AChE-IR in brain after s.c. chlorpyrifos (time–course and dose–response)

	AChE-IR (% of mean control)				
	Intoxication phase			Recovery phase	
Dosage	Week 1	Week 3	Week 5	Week 7	Week 9
Vehicle control	100 ± 4.6 (6)	100 ± 2.2 (17)	100 ± 3.3 (18)	100 ± 6.0 (5)	100 ± 12 (6)
15 mg/kg	101 ± 4.6 (6)	106 ± 9.7 (6)	107 ± 5.9 (6)	103 ± 4.1 (6)	106 ± 14 (6)
30 mg/kg	108 ± 7.6 (6)	106 ± 7.4 (6)	113 ± 6.7* (6)	103 ± 6.6 (6)	104 ± 14 (6)
60 mg/kg	104 ± 7.5 (6)	115 ± 3.5† (17)	115 ± 4.0† (17)	107 ± 7.0 (6)	105 ± 16 (6)

Immunoreactivity in global brain samples expressed as percentages (±SEM) of the mean control value (average across all experiments, 11.7 ± 2.1 mOD/min/mg wet weight). Dosage was by weekly s.c. injection of chlorpyrifos or vehicle, ending after 4 weeks (number of rats in parentheses). Samples were obtained 1 week after injection during the intoxication phase.

* $P < 0.05$.

† $P < 0.001$.

Table 2. Regional distribution of AChE-IR and AChE activity after s.c. chlorpyrifos

Sample	AChE-IR (% of mean control)		AChE activity (μmol/min/g)	
	Control	Test	Control	Test
Global brain	100 ± 2.2 (17)	115 ± 3.5* (17)	5.4 ± 0.17	1.0 ± 0.04*
Forebrain	100 ± 3.5 (12)	116 ± 6.6 (11)	9.9 ± 0.27	1.3 ± 0.07*
Hippocampus	100 ± 2.0 (12)	100 ± 3.9 (11)	4.7 ± 0.17	0.8 ± 0.06*
Cerebellum	100 ± 2.1 (11)	101 ± 2.3 (11)	3.7 ± 0.10	1.2 ± 0.05*
Diaphragm	100 ± 4.5 (12)	107 ± 5.4 (11)	0.9 ± 0.04	0.4 ± 0.02*
Liver	100 ± 4.0 (10)	99 ± 3.3 (11)	0.4 ± 0.02	0.3 ± 0.01*

Treatment by weekly s.c. injection of 60 mg/kg chlorpyrifos or vehicle (number of rats in parentheses). Tissues were harvested at 3 weeks, 1 week after the last injection. Results are given as means ± SEM. AChE-IR is acetylcholinesterase immunoreactivity expressed as a percentage of mean control values (average across all experiments, 31.7 ± 5.3 mOD/min/mg wet weight). Two-way analysis of variance of data from all brain regions showed a highly significant overall treatment effect on AChE immunoreactivity, $F = 8.34$, $P < 0.005$.

* $P < 0.001$ vs control, by individual t-test.

Table 3. AChE-IR and AChE activity after oral chlorpyrifos

Sample	AChE-IR (% of mean control)		AChE activity (μmol/min/g)	
	Control	Test	Control	Test
Global brain	100 ± 9.1	156 ± 14.3*	5.9 ± 0.22	0.9 ± 0.14†
Forebrain	100 ± 6.9	129 ± 6.9*	10.9 ± 0.10	1.0 ± 0.18†
Hippocampus	100 ± 3.0	126 ± 8.3*	4.2 ± 0.06	0.6 ± 0.11†
Cerebellum	100 ± 3.9	126 ± 8.2*	3.3 ± 0.06	0.9 ± 0.06†

Treatment by daily oral gavage with chlorpyrifos ≈ 75 mg/kg, 5 days per week (4 rats per group). Tissues were harvested at 4 weeks, approximately 24 hr after the last dose. Results are given as means ± SEM. AChE-IR is acetylcholinesterase immunoreactivity expressed as a percentage of mean control values (average across all experiments, 11.4 ± 1.0 mOD/min/mg wet weight). Two-way analysis of variance showed a highly significant overall treatment effect on AChE immunoreactivity, $F = 34.5$, $P < 0.0001$.

* $P < 0.05$ vs control, by individual t-test.

† $P < 0.0001$ vs control, by individual t-test.

sources of systematic error. The statistical analyses argue strongly against random error. Finally, the time–course and dose–relationship both point to a genuine result: namely, that chlorpyrifos treatment leads to a very gradual, dose-dependent accumulation of AChE protein in the brain.

A clear implication of the persistence and increase of AChE-IR is that chlorpyrifos does not induce the sort of neurological damage that would cause outright loss of AChE or AChE-bearing neurons in the rat brain. Specific mRNA was not suppressed, and AChE protein levels actually rose during dosing. In view of these findings, depot storage and gradual bioactivation must be the basis for prolonged enzyme inhibition after exposure to chlorpyrifos. This conclusion is in agreement with that of Chambers and Carr [4].

Mechanism of AChE accumulation. One obvious way to explain the accumulation of AChE-IR after treatment with chlorpyrifos is to postulate a toxicant-induced increase in AChE synthesis. The idea that organophosphate treatment might up-regulate the production of neural AChE is not altogether new. Some earlier observations on the recovery of enzyme activity in cultured muscle cells suggested that AChE synthesis is transiently accelerated after enzyme inhibition [19, 20]. Since those initial reports, however, a solid body of evidence supporting feedback regulation of AChE has never materialized; therefore, our observations of AChE accumulation do not fit into an established context.

We could not demonstrate significant elevation of AChE mRNA after chlorpyrifos administration. Nonetheless, our data do not rule out some increase of the message level, or a post-translational effect leading to greater production of brain AChE. Another possible mechanism for AChE accumulation would be a reduced rate of degradation secondary to inhibition of protease activity in the brain. Blockade of serine proteases, to be expected from most organophosphates [21–23], might well prolong the life span of intracellular and extracellular proteins. Experiments on AChE turnover in brain should be carried out to clarify these issues.

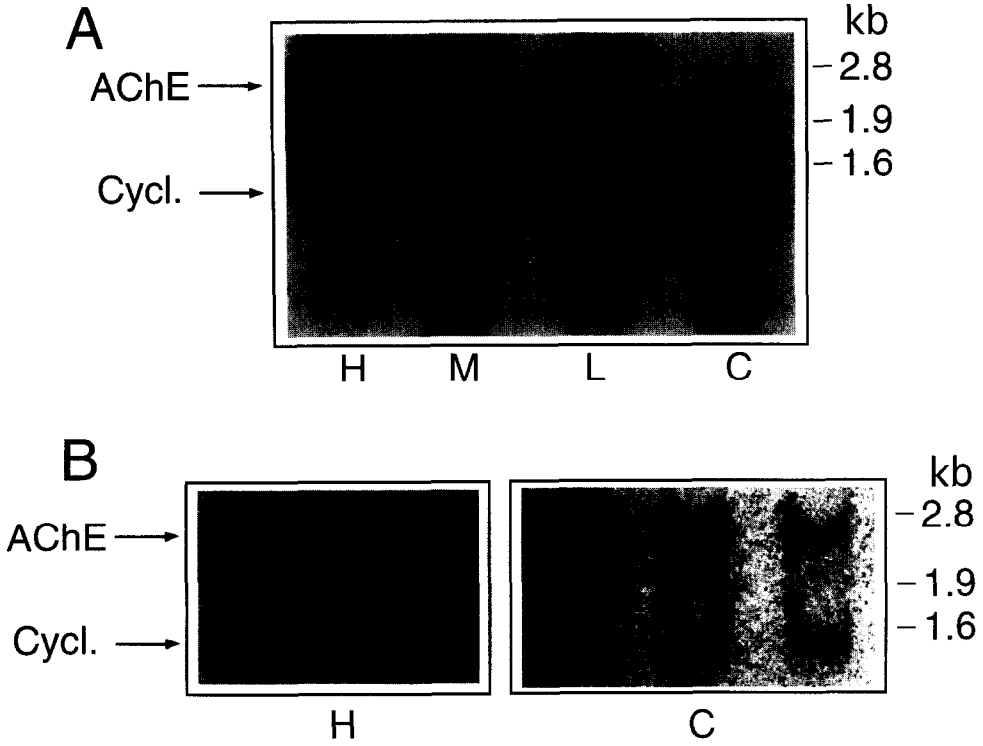

Fig. 4. Expression of AChE mRNA in rat brain 3 weeks after beginning weekly s.c. injections of chlorpyrifos. Contralateral tissue from the brains sampled for determinations of AChE-IR and AChE activity was used to prepare mRNA for Northern blotting (see Materials and Methods). AChE bands were quantitated by densitometry and normalized with reference to cyclophilin ("Cycl."; see Table 4 for quantitative data). Each lane represents a pooled sample from a different set of 3–6 brains. (A) Dose–response comparison: H = high dose (60 mg/kg); M = middle dose (30 mg/kg); L = low dose (15 mg/kg); C = control (vehicle alone). (B) Multiple examples of high dose and control groups.

Table 4. AChE mRNA levels in global brain samples after chlorpyrifos

	AChE mRNA levels (% of mean control)				
	Subcutaneous administration*			Recovery	Oral†
Treatment	Week 1	Week 3	Week 5	Week 7	Week 4
Control	100 (1)	100 ± 18 (6)	100 ± 18 (7)	100 ± 6.0 (1)	100 (1)
Chlorpyrifos	100 (1)	131 ± 28 (6)	98 ± 19 (7)	107 ± 7.0 (1)	187 (1)

mRNA levels, expressed as a percentage (±SEM) of mean control values, were measured as the ratio of band densities for AChE and cyclophilin obtained in separate film exposures (control average, 100% = 1.1 ± 0.35). Each sample represents a pool of 2–4 different brains; the number of separate pools is in parentheses.

* Weekly s.c. injection, 60 mg/kg, ending after 4 weeks.

† Daily gavage (≈75 mg/kg, 5 days per week).

Significance of residual AChE activity. A curious aspect of chlorpyrifos *in vivo* was its tendency to reduce AChE activity more in regions with high activity than in regions with low activity. *In vitro*, the active metabolite, chlorpyrifos oxon, reduced AChE enzyme activity to near zero, both when added to brain homogenates and when incubated under physiologic conditions with "intact" pieces. In contrast, residual activity was always demonstrable in rat brain after systemic administration of the parent compound. Residual, active AChE is a common finding when organophosphorus compounds are administered *in vivo* [24, 25, *]. However, given the wide regional variation in controls, it was

* Padilla S, unpublished observations.

surprising to find such similar residual activities in different regions of the brains from dosed rats (Tables 2 and 3).

Why AChE activity should persist at similarly low levels across the brain is unknown at present. The virtual absence of AChE activity in brain cubes exposed to chlorpyrifos oxon argues strongly against the idea of protected cellular compartments whose AChE is not accessible to inhibitor. Another potential explanation is that the residual activity reflects a minor, widely distributed AChE isozyme that is relatively resistant to chlorpyrifos oxon. Organophosphate-resistant AChE has been observed after chronic treatment with paraoxon [26]. However, an oxon-resistant AChE isozyme would be at variance with our *in vitro* data showing that chlorpyrifos oxon inhibited AChE from chlorpyrifos-treated rats as readily as AChE from controls (Fig. 1).

We suggest an alternative idea, that residual AChE activity represents newly synthesized enzyme that has not yet encountered organophosphate. Although this idea is speculative at present, its toxicokinetic background is worth analyzing. Newly synthesized AChE could be responsible for residual enzyme activity during repeated administration of chlorpyrifos, provided that AChE synthesis were able to keep pace with AChE inactivation. This condition would be met if enzyme biosynthesis were rapid or toxicant activation were slow. A new equilibrium would arise in which the level of active AChE was determined by the relative rates of these two phenomena. If "residual" AChE activity did reflect new enzyme, its abundance at equilibrium would be proportional to the rate of enzyme synthesis. As stated earlier, we have not proved that AChE synthesis is accelerated after exposure to chlorpyrifos. Nevertheless, it remains possible that chlorpyrifos and related organophosphorus compounds promote residual AChE activity by enhancing enzyme biosynthesis. The relative uniformity of this activity, however, would predict that most parts of the brain produce AChE at similar rates, even though their normal enzyme levels are quite different.

Unfortunately, it is difficult to evaluate this prediction because we know so little about the regional distribution of AChE synthesis. There is no doubt that AChE turnover differs from one region of the brain to another when measured by the return of enzyme activity after irreversible inhibition [27–31]. After dosing rats with paraoxon, Chambers and Chambers [32] found that the recovery of AChE activity, as a *percentage* of control, was faster in the medulla oblongata than in the corpus striatum. Since the striatum contains 10 times more AChE than the medulla, this observation is consistent with similar absolute rates of enzyme production in the two areas. It is worth noting recent findings that AChE mRNA levels are actually lower in the striatum than in the medulla [33, 34]. This leads us to speculate that the normal region-to-region variations in brain AChE activity primarily reflect different enzyme lifetimes.

In conclusion, the long-lasting inhibition of AChE by chlorpyrifos is probably not associated with significant damage to AChE-expressing neurons in the brain, since message and protein levels were sustained. In fact, chlorpyrifos actually causes a modest increase in the amount of immunoreactive AChE protein, which would be consistent with some type of feedback regulation of enzyme production. Explanations for the slow recovery of enzyme activity must therefore focus on the propensity of this agent for depot storage and gradual bioactivation. The pattern of residual AChE activity in brain is consistent with the idea that new enzyme biosynthesis is sufficiently rapid, relative to chlorpyrifos activation, to stabilize brain AChE activity at a new equilibrium between 10 and 20% of control levels. The nature and source of this residual activity remain interesting topics for investigation.

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