Pharmacology Biochemistry and Behavior, Vol. 58, No. 1, pp. 15–23, 1997

0091-3057/97 $17.00 + .00

ELSEVIER

PII S0091-3057(96)00458-3

The Relationship of Oral Chlorpyrifos Effects on Behavior, Cholinesterase Inhibition, and Muscarinic Receptor Density in Rat

AMY C. NOSTRANDT,*[1] STEPHANIE PADILLA† AND VIRGINIA C. MOSER†[2]

*Curriculum in Toxicology, University of North Carolina, Chapel Hill, NC 27599
†Neurotoxicology Division (MD-74B), National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711

Received May 1996; Accepted 19 August 1996

NOSTRANDT, A. C., S. PADILLA AND V. C. MOSER. *The relationship of oral chlorpyrifos effects on behavior, cholinesterase inhibition, and muscarinic receptor density in rat.* PHARMACOL BIOCHEM BEHAV **58**(1) 15–23, 1997.—Behavioral changes and tissue cholinesterase (ChE) inhibition were examined in animals treated with the commonly used insecticide chlorpyrifos. Adult male rats were dosed by gavage with 0, 10, 30, 60, or 100 mg/kg chlorpyrifos. Rats (n = 20/dose group) were evaluated using a functional observational battery (FOB) and an automated measure of motor activity. All rats were tested the day before dosing and at 3.5 h (the time of peak effect) after dosing; half of these (n = 10/dose) were sacrificed immediately after testing for tissue collection. The remaining rats were tested again at 24 h, followed by sacrifice. The following tissues were collected from each animal: half brain, individual brain areas from the other half of the brain (frontal cortex, hippocampus, striatum, hypothalamus, cerebellum, pons/medulla), retina, liver, heart, diaphragm, *quadriceps femoris* muscle, and blood (separated into whole blood, plasma, and erythrocytes). ChE activity was measured in all tissues, and muscarinic receptor density was assessed as quinuclidinyl benzilate (QNB) binding in all brain regions, heart, and retina. The lowest dose produced no behavioral effects but did produce significant ChE inhibition in most tissues at 3.5 h. Higher doses produced more ChE inhibition and cholinergic signs of toxicity. Partial recovery from behavioral effects was evident at 24 h, with little or no corresponding recovery of ChE activity. Apparent downregulation of muscarinic receptor density was noted only in striatum and pons/medulla of rats treated with the highest dose of chlorpyrifos. Correlations for behavioral and biochemical effects were generally poor because: a) the low-dose effects on ChE inhibition were not reflected in behavioral signs, and b) behavioral signs showed recovery at 24 h, whereas ChE activity did not. Examination of data for individual rats indicated that >60% of brain ChE inhibition was reached before neurobehavioral effects were evident. © 1997 Elsevier Science Inc.

Chlorpyrifos Cholinesterase inhibition Neurobehavior Functional observational battery Muscarinic receptors Rat

CHLORPYRIFOS is a broad-spectrum organophosphate insecticide that is widely used in agricultural, commercial, and domestic applications and thus has the potential for occupational exposure and environmental contamination [for a recent review, see (5)]. Although chlorpyrifos is known to be an inhibitor of cholinesterase (ChE) activity, and inhibition of acetylcholinesterase has been associated with clinical signs of acute toxicity of organophosphate pesticides (11,22), little work has been done to determine the precise relationship between various levels of sublethal ChE inhibition and alterations in behavior. In a recent review, D'Mello (9) concluded that correlations between central and/or peripheral ChE inhibition and behavioral effects are lacking.

The working hypothesis is that ChE inhibition increases cholinergic neurotransmission and thereby produces behavioral changes, although there is some disagreement as to whether a threshold exists, i.e., a critical level of inhibition that is required before functional changes are observed (2). Some investigators [e.g., (10,15)] report no simple correspondence of effects; rather, they associate different effects with different

[1]Present address: Food and Drug Administration, Center for Drug Evaluation and Research, 5600 Fisher's Lane, Rockville, MD 20857.
[2]To whom requests for reprints should be addressed. E-mail: moser.ginger@epamail.epa.gov

levels of inhibition. Furthermore, the slopes of dose–response curves of various ChE-inhibiting compounds are different, depending on the end point (24). The mechanisms by which behavioral effects are produced may include other actions in addition to ChE inhibition, such as receptor modulation. Although it is known that some ChE inhibitors interact directly with muscarinic receptors *in vitro* [e.g., (38,39)], the behavioral consequences of these actions have not been studied. On the other hand, many studies have noted that decreased muscarinic receptor number, presumably due to high concentrations of acetylcholine, appears to help the organism survive repeated exposure to anticholinesterases [reviewed in (6)].

Concurrent measurement of ChE inhibition, receptor binding, and behavioral effects is needed to understand the actions of ChE inhibitors, including their similarities and differences in terms of dose–response and time course. In the present study, using rats exposed to one of several doses of orally delivered chlorpyrifos, we: a) systematically evaluated a range of behaviors, using a battery of tests that assess neurobehavior, and b) in the same subjects, determined the degree of ChE inhibition in a number of peripheral tissues and areas of the central nervous system at a time closely approximating that of the behavioral observations.

MATERIALS AND METHODS

Animals

All animals were adult (approximately 70 days old) male Long–Evans hooded rats [CRL:(LE)BR] obtained from Charles River, Inc. (Raleigh, NC, USA). Average body weight at the beginning of the study was 350 ± 24 g (±SD). The animals were housed individually on hardwood chip bedding and allowed free access to food (Purina Rat Chow #5002) and deionized water. The animal facility was maintained at 22 ± 1°C and 55 ± 5% humidity, with a 12 L:12 D cycle (lights on at 0630 h). Observational testing took place within the animal rooms, and rats were transported to another room in the facility for motor activity assessment.

Chemical Treatment

Chlorpyrifos (99.5% pure, Chem Serve Co., West Chester, PA, USA) was dissolved in corn oil (Mazola®). Doses of 0, 10, 30, 60, and 100 mg/kg were administered by oral gavage in a volume of 1 ml/kg. Each treatment group consisted of 20 rats, and half of each dose group was sacrificed at each time point (n = 10/dose/time point).

Tissue Collection

All rats were evaluated behaviorally at 3.5 h postdosing [previously determined to be the time of peak effect, or TOPE; (24)] and half of each group was sacrificed by decapitation under CO_2-induced anesthesia. The remaining animals were tested again at 24 h after dosing and then sacrificed. Immediately following sacrifice, the following tissues were collected: brain, retina, liver, heart, diaphragm, and *quadriceps femoris* muscle. The brain was sectioned longitudinally (midsagittally) so that all anatomical regions were represented in one half-brain, and the remaining half-brain was further sectioned to give frontal cortex, hippocampus, striatum, hypothalamus, cerebellum, and pons/medulla. Dissections were conducted freehand with the guidance of a common atlas (30). Trunk blood was collected in heparinized tubes and, after removing an aliquot of whole blood, plasma and erythrocytes were separated by low-speed centrifugation (approximately 3500 rpm for 10 min). Thus, there were 15 tissues/fractions taken from each rat.

Behavioral Testing

All rats were tested using a functional observational battery (FOB), which consists of home cage, handling, open field, and manipulative neurobehavioral evaluations. Procedural details and scoring criteria for the FOB protocol are provided in McDaniel and Moser (23). Evaluations were first made while the rat was in the home cage. The observer evaluated each animal's posture and palpebral closure, and the presence or absence of convulsions was noted. If convulsions were present, they were categorized further. Each rat was then removed from the home cage and briefly held in the hand. The presence or absence of spontaneous vocalization, piloerection, and other fur and skin abnormalities were then noted, as well as the ease of removal and handling. Lacrimation, salivation, and ptosis were also noted and scored. Other signs such as exophthalmus, crustiness around the eyes, bite marks on the tail or paws, missing toe nails, or emaciation (shallow stomach, protruding spinal vertebrae) were recorded.

The rat was then placed on the top of a laboratory cart (60 × 90 cm) with a perimeter barrier (6.5 cm high) covered with a clean absorbent pad. The rat was observed moving about on the cart undisturbed for 3 min, and during that time the frequency of rearing responses was recorded. At the same time, gait characteristics were noted and ranked, and arousal, tremor, convulsions, and abnormal postures were evaluated. At the end of the 3 min, the number of fecal boluses and urine pools on the absorbent pad were recorded. Reflex testing then consisted of recording each rat's responses to the approach of the blunt end of a pen, a touch of the pen to the posterior flank, and an auditory click stimulus using a metal clicker. Responsiveness to a pinch on the tail (using forceps) and the ability of the pupil to constrict to light were then assessed. These measures were followed by a test for the aerial righting reaction, then by measures of forelimb and hindlimb grip strength, body weight, rectal temperature, and finally hindlimb landing foot splay. The entire battery of tests required approximately 6–8 min per rat.

Motor activity data were collected shortly after FOB testing, using a maze composed of a series of interconnected alleys shaped like a figure-eight with two blind alleys projecting from the central arena (33). Six pairs of phototransmitter diodes were equally spaced around the figure-eight portion of the maze, and one pair was located in each of the blind alleys (total of eight detectors). Activity was recorded by a microprocessor as photocell interruptions during a 1-h session.

Baseline evaluations were conducted the day before dosing, and the next day all rats were tested between 3.25 and 3.75 h postdosing. Immediately thereafter, the rats were placed in the motor activity chambers for 1 h. Upon removal from the activity devices, half of the rats were sacrificed for collection of tissues; the remaining animals were tested again at 24 h, followed by sacrifice. Thus, tissue collection actually took place approximately 4.5 or 25 h after administration of chlorpyrifos. Dosing and testing of both sets of rats were combined into one study; although this was necessarily spread out over several days (1.5 weeks), the dose and fate of the rats were counterbalanced across day and time of day. Dosing took place between 0800 and 1200 h, and testing began at approximately 1115 h each day. The observer was blind with respect to the treatment condition of the rats.

Previous studies have revealed 10 measures that are consistently altered following acute exposure to several different ChE inhibitors (24). In the present study, only those 10 measures were evaluated for correlations with the biochemical data.

Quantification of Muscarinic Receptor Density

Muscarinic receptor density was determined by binding of [^{3}H]quinuclidinyl benzilate (QNB; New England Nuclear, DuPont, Boston, MA, USA), as described by Yamamura and Snyder (41), in all brain regions as well as in heart and retina. Brain tissues and heart were homogenized at approximately 50 mg/ml in 0.05 M sodium phosphate buffer (pH 7.4). Retinae were homogenized in pairs in approximately 2 ml of the same buffer. Tissues were centrifuged and homogenized two times, then incubated at 37°C with at least 0.07 μCi of [^{3}H]QNB (specific activity, 32.9 Ci/mmol), with and without atropine (concentration, 5 μM), until equilibrium was reached (1 h). Binding was corrected for protein content as determined by the method of Lowry et al. (20).

Cholinesterase Assay

Cholinesterase assays were performed either by using a microtiter plate assay [detailed in (27)] based on the spectrophotometric method of Ellman and coworkers (13) using acetylthiocholine iodide, or by using the radiometric method of Johnson and Russell (17) with [^{3}H]acetylcholine iodide (New England Nuclear, DuPont; specific activity, 74.8 mCi/mmol). Brain tissues, muscle tissues, retina, and plasma were analyzed by the spectrophotometric method, and whole blood, erythrocytes, and liver were analyzed by the radiometric method. For brain tissues, heart, and retina, 1–10 μl of remaining tissue preparations from the muscarinic binding assay were used in the microassay procedure. Plasma samples of 2 μl were used. Diaphragm and *quadriceps* tissues were homogenized 1:20 in 0.1 M sodium phosphate buffer (pH 8.0) with 1% Triton X-100 added and were centrifuged to remove connective tissue; 10–15 μl was used in the assay. Whole blood and erythrocytes were assayed for ChE by the radiometric method (17) due to interference of hemoglobin with the Ellman assay in those tissues. Each was diluted 1:2.5 in 0.1 M sodium phosphate buffer with 1% Triton X-100 added (pH 8.0). Assay volumes were 15 μl for whole blood and 20 μl for erythrocytes. The radiometric assay was also used to analyze liver ChE, because background absorbance in the Ellman assay is unacceptably high due to the high glutathione content of that tissue. An 80-μl volume of a 1:10 homogenate in 0.1 M sodium phosphate buffer (pH 8.0) was used. Activity was corrected for protein content (20).

TABLE 1

ACUTE EFFECTS OF CHLORPYRIFOS ON THE ENDPOINTS OF THE NEUROBEHAVIORAL TEST BATTERY

Test Measures	3.5 h	24 h
Autonomic		
*Lacrimation	100	—
*Salivation	100	—
*Pupil response (miosis)	100	—
Defecation	30, 60, 100 ↓	—
Activity		
*Motor activity	30, 60, 100 ↓	60, 100 ↓
Rearing	30, 60, 100 ↓	60, 100 ↓
Home cage posture (flattened)	100	100
Convulsive		
*Tremors	60, 100	—
*Smacking	30, 60, 100	—
Neuromuscular		
*Gait score (ataxic, uncoordinated)	30, 60, 100	60, 100
*Landing foot splay	60, 100 ↑	—
Righting reaction	100	—
General reactivity		
Arousal	60 ↓	60 ↓
Removal reactivity	60, 100 ↓	—
Sensorimotor		
*Tail pinch response	60, 100 ↓	—
Click response	30, 60, 100 ↓	—
Approach response	—	60 ↑
Physiological		
*Body temperature	30, 60, 100 ↓	100 ↓
Body weight	60, 100 ↓	30, 60, 100 ↓

Chlorpyrifos had no effect on urination, forelimb or hindlimb grip strength, handling reactivity, touch response, or piloerection. The doses (mg/kg) that produced effects significantly different from the control group are listed, along with the direction of change, where applicable. n = 10/dose/time point. Asterisks mark the 10 measures that are most reliably altered by treatment with ChE inhibitors (24).

Statistics

Behavioral test measures are either continuous (which provides data on an interval scale), scalar (ranked based on a defined scale), or descriptive. Most of the measures can be grouped to assess broad domains of neurological function, such as autonomic, sensorimotor, neuromuscular, convulsive, reactivity, and activity. Analysis of each measure was carried out as previously described [see (7,25)]. Two-way ANOVAs

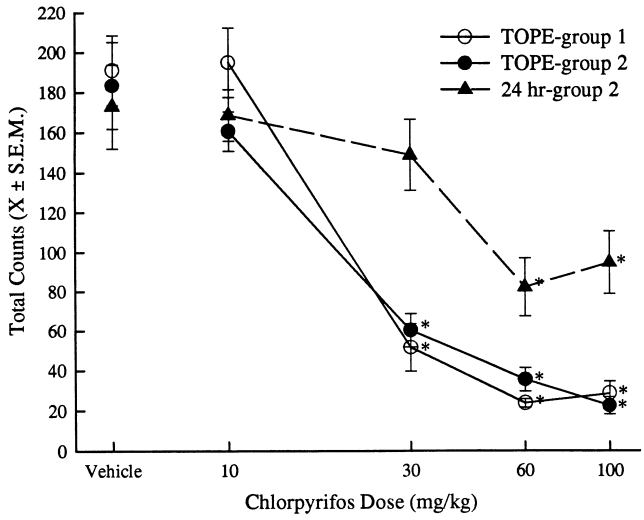

FIG. 1. Effects of chlorpyrifos on total counts (mean ± SEM) during the 1-h motor activity session. Data are shown for rats that were tested and then sacrificed at the time of peak effect (TOPE-group 1; n = 10/dose) and for rats that were tested at both the time of peak effect (TOPE-group 2) and at 24 h (24 hr-group 2; n = 10/dose). Asterisks indicate data significantly different from the corresponding control.

were conducted with a grouping factor of dose and time as repeated (within-subject) factors. When significant overall effects were obtained, analyses at each time point were conducted to determine which time points were significant and, at those times, which dose groups differed from the control, using Dunnett's *t*-test (for continuous data) or *t*-test contrasts (for rank-order data). Continuous data were analyzed by a general linear model [GLM; (37)], following adjustment for each rat's baseline value. Rank-order data were analyzed using a categorical modeling procedure [CATMOD; (37)], which fits linear models to functions of response frequencies, and then by weighted regression. In all cases, resulting probability values <0.05 were considered significant.

Analysis of neurochemical data (ChE activity, muscarinic receptor binding) employed a one-way ANOVA with dose as the only factor. All analyses were conducted on the raw data, although the data are presented here as percentage of corresponding control for each.

To determine correlations between ChE inhibition in various brain regions and behavioral measures, a matrix was constructed containing Pearson's correlation coefficients and chi-square tests (37).

RESULTS

In general, the selected dose range of chlorpyrifos was well tolerated. One rat dosed with the high dose (100 mg/kg) died before the 24-h sacrifice. At 3.5 h, rats treated with the two higher doses had lost 4–5% of predosing body weight. On the day after dosing, weight loss occurred in the three higher dose groups (≥30 mg/kg); the greatest loss (9%) was in the high-dose rats.

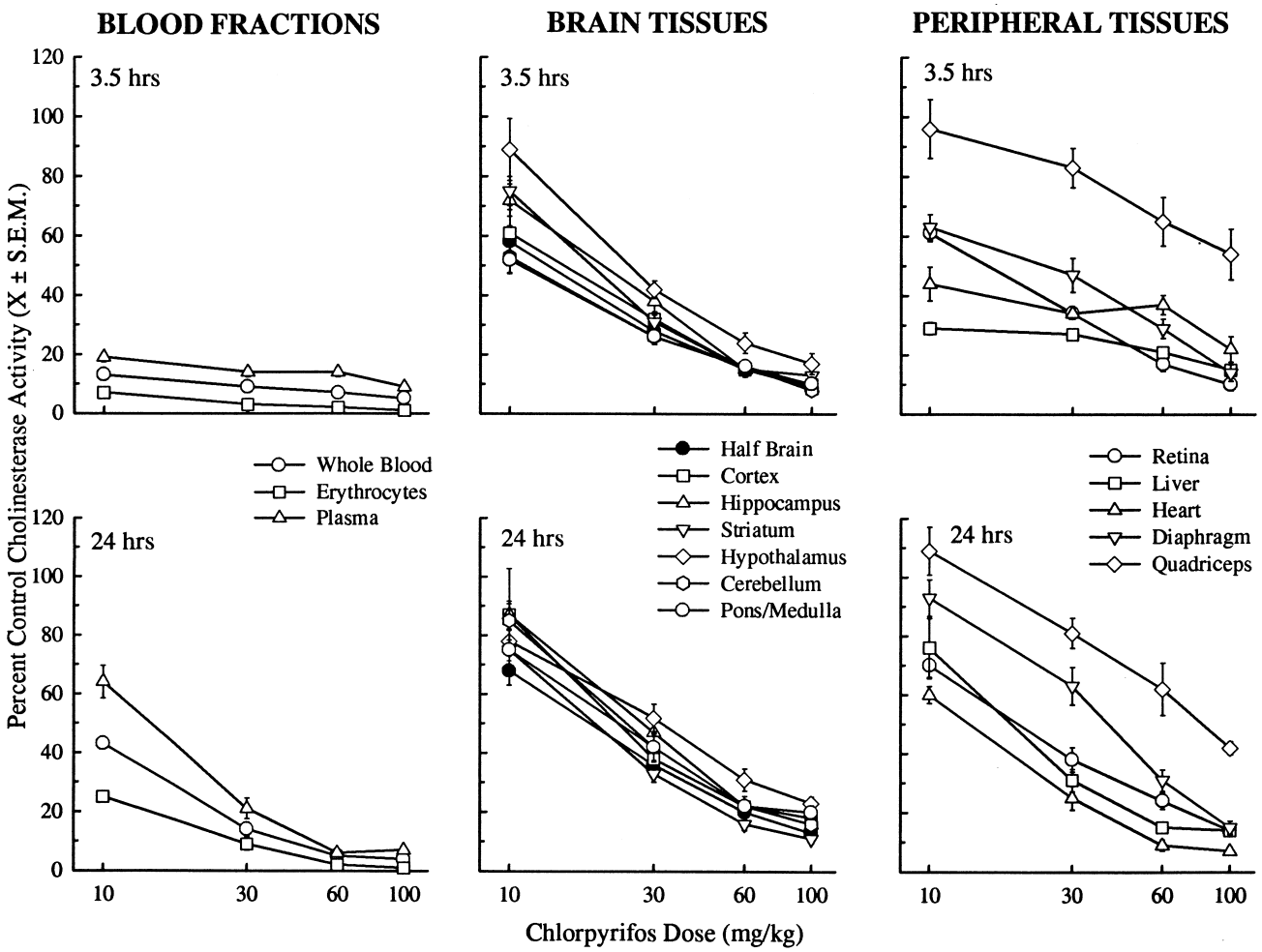

FIG. 2. Chlorpyrifos-induced ChE inhibition in blood fractions (left panels), brain tissues (middle panels), and peripheral tissues (right panels) taken after the 3.5-h (top) and 24-h (bottom) behavioral evaluations. Data (mean ± SEM, $n = 10$/dose) are expressed as percentage of appropriate vehicle control values. All instances where ChE activity was less than 80% of control were significantly different from the corresponding control ($p < 0.05$). Control ChE activities, expressed as nmol substrate hydrolyzed (see Materials and Methods)/min per mg protein (for tissues) or per ml (blood fractions), were as follows (mean ± SEM) for the 3.5-h and 24-h evaluations, respectively: whole blood, 732.5 ± 70.9, 771.5 ± 32.7; plasma, 283.9 ± 10.8, 292.7 ± 13.3; erythrocytes, 259.7 ± 17.4, 245.8 ± 14; half-brain, 163.1 ± 4.9, 173.8 ± 7.3; striatum, 612.5 ± 40, 798.7 ± 86.4; cerebellum, 55.0 ± 4.2, 50.4 ± 2.8; cortex, 126.3 ± 10, 109.8 ± 10.7; hippocampus, 93.7 ± 7.3, 96.6 ± 4.9; pons/medulla, 142.5 ± 7.8, 123.9 ± 7.8; hypothalamus, 57.7 ± 5.4, 68.0 ± 5.7; retina, 219.2 ± 11.7, 263.9 ± 26; heart, 16.5 ± 1.4, 17.1 ± 1.6; liver, 0.15 ± 0.01, 0.17 ± 0.02; diaphragm, 15.2 ± 1.2, 15.8 ± 0.9; *quadriceps*, 7.8 ± 0.9, 8.9 ± 0.3.

Behavioral Changes

The effects of chlorpyrifos on the measures of the screening battery, and effective doses, are presented in Table 1. Significant changes were noted in the 10 endpoints that previous studies revealed to be consistently altered following acute exposure to ChE inhibitors (24), as well as in some other measures. No behavioral changes were seen in the 10-mg/kg treatment group. At 3.5 h, the lowest behaviorally effective dose (30 mg/kg) produced hypothermia, smacking, uncoordinated and ataxic gait, reduced rearing and figure-eight maze activity, depressed response to the click stimulus, and decreased defecation in the open field. At higher doses, more endpoints were altered in a dose-dependent manner. The day after dosing, considerably fewer behavioral effects were observed, and most effects were obtained only in the two higher dose groups (except for body weight).

Figure 1 presents the effect of treatment on motor activity, plotted as a function of dose, for the 1-h test sessions. Data for the rats killed after the 3.5-h test (TOPE-group 1 rats) were not different from the 3.5-h data for rats that were also tested at 24 h (TOPE-group 2). This indicates that the magnitude of the acute effect was similar in both groups of rats, i.e., there was no difference between these cohorts. Additionally, in this figure, considerable behavioral recovery is evident 24 h after dosing.

Biochemical Changes

ChE inhibition produced by chlorpyrifos at 3.5 and 24 h in the various tissues is shown in Fig. 2. Inhibition of ChE was greatest in the three blood fractions and in liver at 3.5 h (71–99% inhibition), with little difference in the degree of inhibition as a function of dose in those tissues. A clearer dose–response relationship was observed in the brain areas (11–92% inhibition). Generally, ChE inhibition in half-brain appeared to be representative of that in individual brain regions, and the pattern of retinal ChE inhibition was similar to that of the brain. ChE inhibition in the muscle tissues also exhibited a clear dose–response relationship, with inhibition in heart > diaphragm > *quadriceps*. All tissues had significantly reduced ChE activity at all doses, with the following exceptions: hypothalamus at the 10-mg/kg dose and *quadriceps* at the 10- and 30-mg/kg dose levels.

In the two lower dose groups, some recovery of ChE activity was evident in all tissues at 24 h. On the other hand, at 24 h, the ChE inhibition produced by the two higher doses was equal to or greater than that noted at 3.5 h in blood components, muscle tissue, striatum, and liver.

Apparent downregulation of muscarinic receptor density was noted in pons/medulla at 3.5 h, and in striatum at 24 h, but only at the highest dose of 100 mg/kg (see Fig. 3). Muscarinic receptor density was decreased by approximately 23% in pons/medulla and 27% in striatum.

Comparisons of Behavioral and Biochemical Changes

Figure 4 presents the dose–response relationships of two neurobehavioral effects (motor activity depression and gait abnormalities) for comparison with ChE inhibition (brain, blood, and/or cerebellum). The most evident disparity between the ChE inhibition data and functional changes is seen at the 10-mg/kg dose, which inhibited ChE in most tissues but produced no effects on the FOB motor activity.

Figure 5 depicts two examples of correlations between specific behavioral measures and ChE inhibition at 3.5 h. Motor activity correlated best with pons/medulla ChE, and gait score correlated best with striatal ChE. The range of correlation coefficients for the analysis of behavioral measures and ChE inhibition at 3.5 h are summarized in Table 2, along with the tissue that produced the "best fit" (a judgment based on the *r*-value). From Table 2, it appears that some behavioral measures (e.g., activity, smacking, and temperature) were more closely related to brain ChE inhibition, whereas others (e.g., lacrimation and tremors) were better related to peripheral ChE inhibition; however, in some cases the *r*-values were

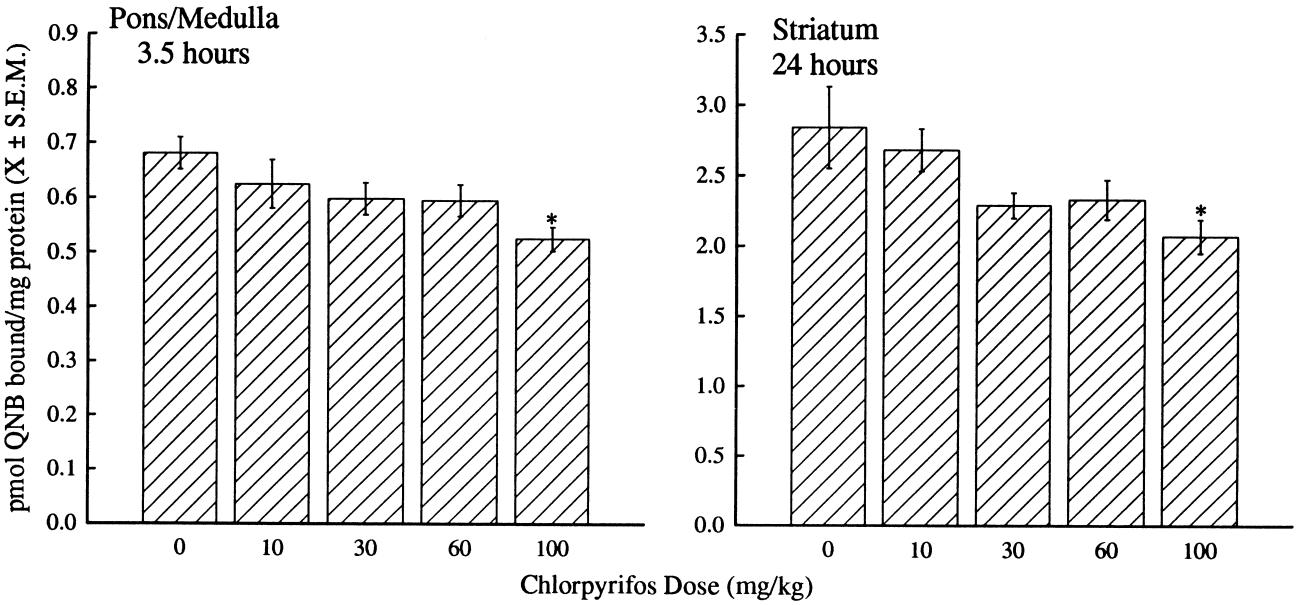

FIG. 3. Effects of chlorpyrifos on muscarinic receptor binding in the pons/medulla tissues (3.5-h evaluation) and the striatum (24-h evaluation). Asterisks indicate data significantly different from the corresponding control.

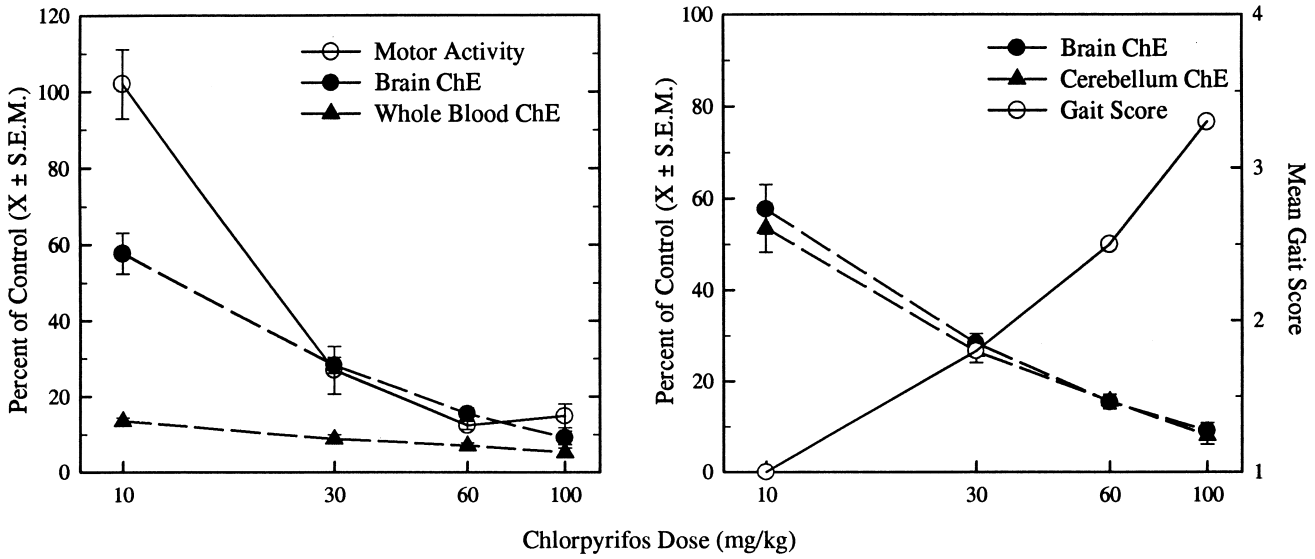

FIG. 4. Comparison of chlorpyrifos-induced behavioral changes and ChE inhibition, expressed as percentage of appropriate control values (mean ± SEM; n = 10/dose), at the 3.5-h evaluation. Data presented are effects on motor activity, compared with ChE activity in whole blood and brain (left panel), and mean ranking of gait abnormalities (1 = no abnormality, 4 = severe abnormality), compared with ChE activity in brain and cerebellum (right panel). Control motor activity counts were 191 ± 17.5 (mean ± SEM); see Fig. 2 for control ChE activity values.

comparable. Among the three blood components, whole blood generally produced higher r-values than did plasma or erythrocytes; likewise, diaphragm showed somewhat higher r-values than did most other peripheral tissues. Examination of individual brain areas, however, did not reveal any section that routinely correlated best with these measures. At 24 h, recovery of behavioral alterations and ongoing ChE inhibition resulted in fewer significant correlations (only gait score, temperature, and motor activity), and these were rather low (all r-values were ≤ 0.6, most ≥ 0.45; data not shown).

Another way to examine relationships between ChE inhibition and behavioral signs is to stratify the data in terms of

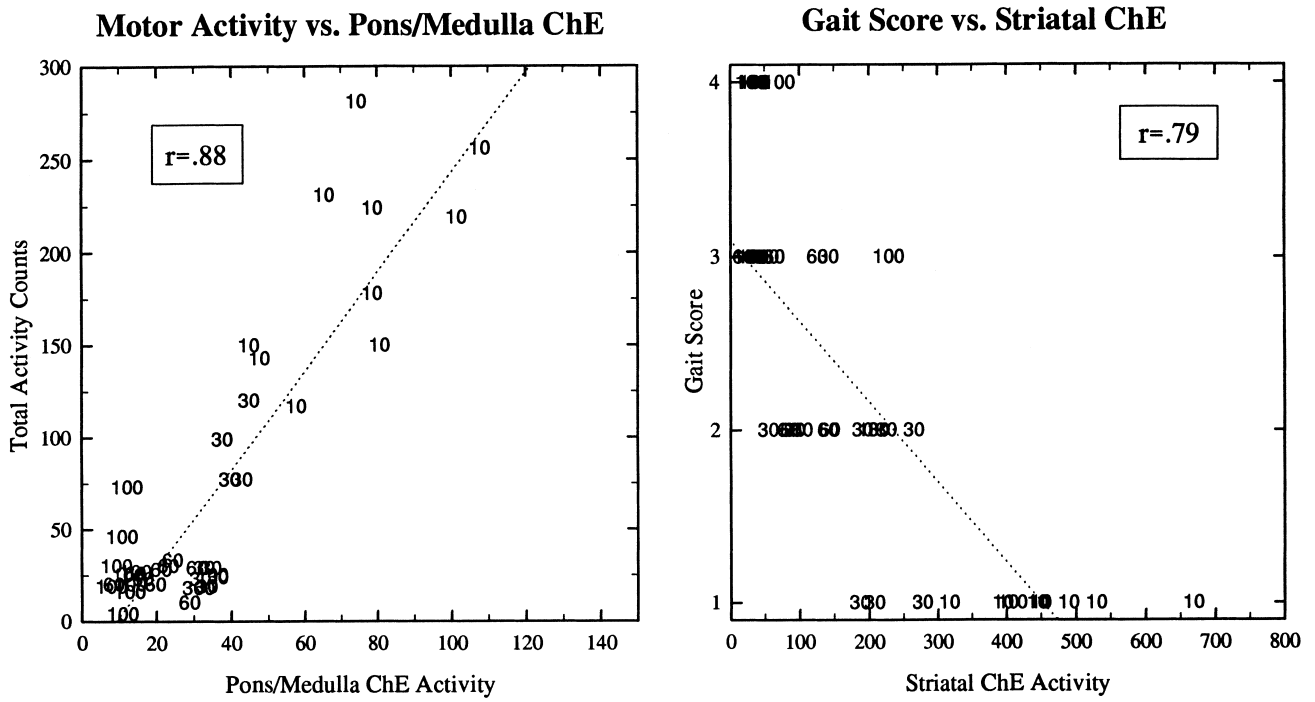

FIG. 5. Correlations between motor activity (total activity counts) and ChE activity (nmol substrate hydrolyzed/min/mg protein) in pons/medulla (left panel), and gait score (see Fig. 4 legend) and ChE activity in striatum (right panel), at the 3.5-h evaluation. Numbers indicate dose level (mg/kg) for each individual rat. See Fig. 4 for control motor activity values, see Fig. 2 for control ChE activity values.

TABLE 2

CORRELATION COEFFICIENTS FOR BEHAVIORAL MEASURES AND ChE INHIBITION AT 3.5 h

	Brain Portions		Blood Components		Peripheral Tissues	
Test Measure	Correlations	Best Fit	Correlations	Best Fit	Correlations	Best Fit
Lacrimation	0.43–0.48	Hippocampus	0.41–0.49	Whole blood	0.48–0.61	Liver
Salivation	—		0.33–0.36	Whole blood	0.34–0.36	Liver
Smacking	0.59–0.67	Hippocampus	0.46–0.51	Whole blood	0.46–0.52	Diaphragm
Tremors	0.52–0.59	Cerebellum	0.52–0.66	Plasma	0.55–0.70	Diaphragm
Gait score	0.73–0.79	Striatum	0.57–0.70	Whole blood	0.54–0.83	Diaphragm
Tail pinch response	0.67–0.81	Hypothalamus	0.42–0.69	Whole blood	0.43–0.67	Diaphragm
Pupil response	0.38–0.45	Hippocampus	0.43–0.51	Whole blood	0.45–0.50	Diaphragm
Landing foot splay	0.32–0.36	Hypothalamus	0.34	Whole blood*	—	
Body temperature	0.60–0.69	Pons/medulla	0.51–0.52	Whole blood	0.32–0.56	Diaphragm
Motor activity	0.69–0.88	Pons/medulla	0.45–0.67	Whole blood	0.39–0.72	Diaphragm

The table lists only ranges of significant ($p < 0.05$) correlation coefficients (Pearson's *r*-values) for behavioral measures and ChE inhibition at 3.5 h. "Best Fit" refers to the tissue that had the highest *r*-value. Dashes indicate that there were no significant correlations.

*The only significant correlation.

ChE inhibition and to enumerate the effects seen at each range of values (see Table 3). The individual rat data for brain ChE (expressed as inhibition) were sorted into bins representing >90% inhibition, 80–90% inhibition, etc., instead of by dose level. The numbers of rats that fit into each of these categories were similar ($n = 7$–10) and mostly, but not completely, represented a dose response. For example, of the 10 rats with >90% brain ChE inhibition, eight had received the highest dose (100 mg/kg) and two had received the next highest dose (60 mg/kg). The incidence of each sign of toxicity was then tabulated for each of the groups (represented as bins). The incidence increased in the groups with more inhibition, i.e., the greater the inhibition, the more rats showed each sign of toxicity.

By examining all 10 "cardinal" cholinergic signs in this manner, we determined breakpoints, or thresholds, for each of the 10 signs. Table 3 shows these results only for whole blood and brain ChE inhibition, because these data are more likely to be collected by researchers than are brain regions or specific muscle tissues. Changes in gait, body temperature, and motor activity were observed in rats with brain ChE inhibition of 60–70%, but not in rats with <60% inhibition. Note that these were also the three most sensitive measures; each was significantly altered in the 30-mg/kg dose group. As brain ChE inhibition increased, the number of the signs of toxicity also increased. Although these data may be intuitive based on the statistical results from the end points (i.e., effective dose levels), this approach provides the clearest indication of the relationship between behavioral changes and ChE inhibition.

A similar approach using whole blood ChE inhibition shows a steeper curve, with 80% inhibition appearing to be a threshold before behavioral signs are observed. Note that, due to the marked depression of blood ChE at even the lowest dose, 25 of the 40 treated rats comprise the lowest ChE activity group (<90% inhibition). Thus, these data are skewed somewhat.

DISCUSSION

The data from this study show that, following a single oral dose, behavioral effects of chlorpyrifos are evident at doses ⩾30 mg/kg. Lower doses produced no observable changes, even

TABLE 3

INCIDENCE (%) OF EACH CARDINAL SIGN FOR EACH GROUP REPRESENTING BINS OF BRAIN ChE INHIBITION RATHER THAN DOSE GROUP

	Brain ChE Inhibition (number of rats in each bin)					Blood ChE Inhibition (number of rats in each bin)		
EndPoint	90–100% (10)	80–90% (8)	70–80% (7)	60–70% (7)	<60% (18)	90–100% (25)	80–90% (14)	<80% (11)
Temperature	100%	100%	86%	57%	0%	92%	36%	0%
Gait	100	100	57	71	0	84	43	0
Motor activity	90	88	86	57	0	84	36	0
Smacking	90	63	57	0	0	64	14	0
Foot splay	40	38	29	0	0	32	14	0
Tail pinch	70	38	0	0	0	40	0	0
Tremors	90	25	0	0	0	44	0	0
Lacrimation	80	13	0	0	0	32	7	0
Pupil response	60	0	0	0	0	24	0	0
Salivation	30	0	0	0	0	12	0	0

Listed for each individual endpoint is the percentage of rats either showing abnormalities in the measure or having individual data outside the range defined by the control group mean ± 2 SD.

though there was substantial ChE inhibition in most tissues. Furthermore, recovery of behavioral effects was evident the day after dosing, at a time of minimal recovery of ChE activity.

Due to the lack of direct correlation between behavior and ChE inhibition at low doses, linear correlational analyses between these parameters were not particularly effective. Instead, the data suggest the existence of a threshold level of activity, below which neurobehavioral changes occur. In this study, brain ChE levels representing ⩾60–70% inhibition were correlated with the occurrence of behavioral changes. In chlorpyrifos-treated rats, gait changes, depressed motor activity, and hypothermia were the most sensitive measures and occurred at this level of inhibition. On the other hand, the measures that are "classical" signs of a cholinergic crisis (e.g., lacrimation, salivation, miosis), were observed only in rats with >90% inhibition of brain ChE.

While this pattern of toxicity appears to agree with early assertions regarding thresholds [e.g., (2,36)], examination of data using another ChE inhibitor can lead to different conclusions. In a previous study from our laboratory (29), both neurobehavioral endpoints and cholinesterase activity in blood and brain were examined in animals exposed to paraoxon. In that study, good correlation was seen between brain ChE activity and a number of neurobehavioral measures; no threshold was evident. The FOB and motor activity tests were sensitive indicators of relatively low (i.e., 26%) brain ChE inhibition. It was concluded that the relationship between ChE inhibition and behavior may be a continuum, in which a low dose of paraoxon produces low levels of ChE inhibition and may produce subtle neurobehavioral changes that become more evident at higher doses and increased ChE inhibition. This was also the case for dichlorvos-induced conditioned taste aversions (34) and in another study reporting clinical signs of paraoxon (40). Clearly, in the present study, chlorpyrifos displayed a pattern different from the above-mentioned compounds. Thus, the relationship between neurobehavioral alterations and ChE inhibition may depend on the compound, test measure, tissue, dose, and time of assessment.

It is well known that repeated exposure to organophosphates often results in downregulation of muscarinic receptors, which is evident in altered binding of [^{3}H]QNB. This downregulation is offered as an explanation for the development of "tolerance" [i.e., decreasing clinical signs in the presence of substantial ChE inhibition; for a review, see (6)]. Specifically, marked downregulation of brain muscarinic receptors has been demonstrated after a single subcutaneous (s.c.) injection of chlorpyrifos (4,31). In the present study, significant receptor downregulation also occurred after a single oral dose of chlorpyrifos. The extent of downregulation, however, was much less than what was noted after a s.c. dose, and this change was noted in only two brain regions and only at the highest dose of chlorpyrifos (see Fig. 3). The s.c. route of administration probably provides a depot of chlorpyrifos that produces longer-lasting ChE inhibition (31), which in turn may be an initiating event in receptor downregulation.

D'Mello (8) noted that no single test has been shown to be representative of behavior and that anticholinesterases are not a homogeneous group of chemicals. Using the same end points as in the present study, the same conclusion was stressed in Moser (24). There is, therefore, a need for a more systematic evaluation of the effects of ChE-inhibiting agents on behavior and ChE inhibition. In addition, these anticholinesterases need to be evaluated individually, as differences may be revealed in the dose–response for specific endpoints [e.g., (16,21,24,34)]. The behaviors being studied may also greatly influence the results of comparisons across inhibitors [e.g., (26)].

Few studies have been published in which dose–response characteristics for both behavioral and neurochemical effects of pesticides have been examined, and even fewer have presented the results of both types of effects in the same animals (1,8,9,28). Hart (15) stated that no studies have "examined the shape of the relationship between acetylcholinesterase activity and . . . behavior." We have attempted to establish a systematic method for examination of both types of effects in the same subjects, at approximately the same point in time, that can be used in the study of multiple cholinesterase-inhibiting pesticides.

Previous studies that have addressed these issues [e.g., (3,12,14,18,19,31,32,35)] have noted dose-related behavioral effects and ChE inhibition, but often not in the same animals, or else only at one dose level. The results have been inconsistent, probably due to the various ChE inhibitors, test species, and behavioral endpoints employed. For example, Kurtz (19) reported a within-subject comparison of performance in an avoidance task and malathion-induced ChE inhibition, in which behavioral decrements occurred at doses that did not produce ChE inhibition, yet time-course data showed that ChE was inhibited when behavior was at control levels. This study contrasted with another from the same laboratory, in which the behavioral and biochemical effects of mobam correlated quite well (18). The one generality that emerges from these various studies is that behavioral alterations most often recover more quickly than does ChE activity.

In the present study, we have demonstrated neurochemical effects temporally coinciding with observed behavioral effects. Dose-related ChE inhibition was seen in a number of tissues, and when a threshold was exceeded, the inhibition corresponded to certain behavioral effects. In addition, muscarinic receptor downregulation was observed after an acute dose. It is unrealistic to expect that all anticholinesterase pesticides are alike in these respects, and thus the findings of this study may be specific to chlorpyrifos-induced toxicity. While this study describes a systematic evaluation of chlorpyrifos, caution should be taken in extrapolating the conclusions to other pesticides, other behavioral endpoints, or other species.

ACKNOWLEDGEMENTS

We acknowledge the excellent technical assistance of Sue Willig, Kathy McDaniel, Pam Phillips, and Renée Marshall for long hours of dosing and testing rats, dissecting tissues, and conducting neurochemical assays. A.N. was funded by the EPA/UNC Toxicology Research Program, training grant T901915, with the Curriculum in Toxicology, University of North Carolina at Chapel Hill. This manuscript has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use. Portions of this research were presented at the annual Society of Toxicology meeting, March 1995 (*The Toxicologist* 15:205; 1995).

REFERENCES

1. Annau, Z. A.: Effects of organophosphorus compounds. In: Chambers, J. E.; Levi, P. E., eds. Organophosphates: chemistry fate and effects. San Diego, CA: Academic Press; 1992:419–432.

2. Bignami, G.; Rosic, N.; Michalek, H.; Milosevic, M.; Gatti, G. L.: Behavioral toxicity of anticholinesterase agents: Methodological, neurochemical, and neuropsychological aspects. In: Weiss, B.;

Laties, V. G., eds. Behavioral toxicology. New York: Plenum Press; 1975:155–216.
3. Carr, R. L.; Chambers, J. E.: Acute effects of the organophosphate paraoxon on schedule-controlled behavior and esterase activity in rats: Dose–response relationships. Pharmacol. Biochem. Behav. 40:929–936; 1991.
4. Chaudhuri, J.; Chakraborti, T. K.; Chanda, S.; Pope, C. N.: Differential modulation of organophosphate-sensitive muscarinic receptors in rat brain by parathion and chlorpyrifos. J. Biochem. Toxicol. 8:207–216; 1993.
5. Cochran, R. C.; Kishiyama, J.; Aldous, C.; Carr, W. C., Jr.; Pfeifer, K. F.: Chlorpyrifos: Hazard assessment based on a review of the effects of short-term and long-term exposure in animals and humans. Food Chem. Toxicol. 33:165–172; 1995.
6. Costa, L. G.: Role of second-messenger systems in response to organophosphorus compounds. In: Chambers, J. E.; Levi, P. E., eds. Organophosphates: chemistry, fate and effects. San Diego, CA: Academic Press; 1992:271–432.
7. Creason, J. P.: Data evaluation and statistical analysis of functional observational battery data using a linear models approach. J. Am. Coll. Toxicol. 8:157–169; 1989.
8. D'Mello, G. D.: Neurobehavioral toxicology of anticholinesterases. In: Ballantyne, B.; Marrs, T. C., eds. Clinical and experimental toxicology of organophosphates and carbamates. Oxford, UK: Butterworth-Heinemann Ltd.; 1992:61–74.
9. D'Mello, G. D.: Behavioral toxicity of anticholinesterases in humans and animals—A review. Hum. Exp. Toxicol. 12:3–7; 1993.
10. Dutta, H.; Marcelino, J.; Richmonds, C.: Brain acetylcholinesterase activity and optomotor behavior in bluegills, *Lepomis macrochirus* exposed to different concentrations of diazinon. Arch. Int. Physiol. Biochim. Biophys. 100:331–334; 1992.
11. Ecobichon, D. J.: Pesticides. In: Amdur, M. O.; Doull, J.; Klaasen, C. D., eds. Casarett and Doull's toxicology: the basic science of poisons, 4th ed. New York: Pergamon Press; 1991:580–592.
12. Ehrich, M.; Shell, L.; Rozum, M.; Jortner, B. S.: Short-term clinical and neuropathologic effects of cholinesterase inhibitors in rats. J. Am. Coll. Toxicol. 12:55–68; 1993.
13. Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Featherstone, R. M.: A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88–95; 1961.
14. Goldberg, M. E.; Johnson, H. E.; Knaak, J. B.: Inhibition of discrete avoidance behavior by three anticholinesterase agents. Psychopharmacologia 7:72–76; 1965.
15. Hart, A. D. M.: Relationships between behavior and the inhibition of acetylcholinesterase in birds exposed to organophosphorus pesticides. Environ. Toxicol. Chem. 12:321–336; 1993.
16. Hoskins, B.; Fernando, J. C. R.; Dulaney, M. D.; Lim, D. K.; Liu, D. D.; Watanabe, H. K.; Ho, I. K.: Relationship between the neurotoxicities of soman, sarin and tabun, and acetylcholinesterase inhibition. Toxicol. Lett. 30:121–129; 1986.
17. Johnson, C. D.; Russell, R. L.: A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. Anal. Biochem. 64:229–238; 1975.
18. Kurtz, P. J.: Behavioral and biochemical effects of the carbamate insecticide, mobam. Pharmacol. Biochem. Behav. 6:303–310; 1977.
19. Kurtz, P. J.: Dissociated behavioral and cholinesterase decrements following malathion exposure. Toxicol. Appl. Pharmacol. 42:589–594; 1977.
20. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.
21. Lynch, M. R.; Rice, M. A.; Robinson, S. E.: Dissociation of locomotor depression and ChE activity after DFP, soman, and sarin. Pharmacol. Biochem. Behav. 24:941–947; 1986.
22. Marrs, T. C.: Organophosphate poisoning. Pharmacol. Ther. 5: 51–66; 1993.
23. McDaniel, K. L.; Moser, V. C.: Utility of a neurobehavioral screening battery for differentiating the effects of two pyrethroids, permethrin and cypermethrin. Neurotoxicol. Teratol. 15: 71–83; 1993.
24. Moser, V. C.: Comparisons of the acute effects of cholinesterase inhibitors using a neurobehavioral screening battery in rats. Neurotoxicol. Teratol. 17:617–625; 1995.
25. Moser, V. C.; McCormick, J. P.; Creason, J. P.; MacPhail, R. C.: Comparison of chlordimeform and carbaryl using a functional observational battery. Fundam. Appl. Toxicol. 11:189–206; 1988.
26. Moss, D. E.; Rodriguez, L. A.; McMaster, S. B.: Comparative behavioral effects of CNS cholinesterase inhibitors. Pharmacol. Biochem. Behav. 22:479–482; 1985.
27. Nostrandt, A. C.; Duncan, J. A.; Padilla, S.: A modified spectrophotometric method appropriate for measuring cholinesterase activity in tissue from carbaryl-treated animals. Fundam. Appl. Toxicol. 21:196–203; 1993.
28. Padilla, S.: Regulatory and research issues related to cholinesterase inhibition. Toxicology 102:215–220; 1995.
29. Padilla, S.; Moser, V. C.; Pope, C. N.; Brimijoin, W. S.: Paraoxon toxicity is not potentiated by prior reduction in blood acetylcholinesterase. Toxicol. Appl. Pharmacol. 117:110–115; 1992.
30. Paxinos, G.; Watson, C.: The rat brain in stereotaxic coordinates, 2nd ed. San Diego, CA: Academic Press; 1986.
31. Pope, C. N.; Chakraborti, T. K.; Chapman, M. L.; Farrar, J. D.: Long-term neurochemical and behavioral effects induced by acute chlorpyrifos treatment. Pharmacol. Biochem. Behav. 42: 251–256; 1992.
32. Reiter, L. W.: Acute and subacute parathion treatment: Effects on cholinesterase activities and learning in mice. Toxicol. Appl. Pharmacol. 25:582–588; 1973.
33. Reiter, L. W.: Chemical exposures and animal activity: Utility of the figure-eight maze. In: Hayes, A. W.; Schnell, R. C.; Miya, T. S., eds. Developments in the science and practice of toxicology. New York: Elsevier; 1983:73–84.
34. Roney, P. L., Jr.; Costa, L. G.; Murphy, S. D.: Conditioned taste aversion induced by organophosphate compounds in rats. Pharmacol. Biochem. Behav. 24:737–742; 1986.
35. Ruppert, P. H.; Cook, L. L.; Dean, K. F.; Reiter, L. W.: Acute behavioral toxicity of carbaryl and propoxur in adult rats. Pharmacol. Biochem. Behav. 18:579–584; 1983.
36. Russell, R. W.: Neurophysiological and biochemical correlates of effects of drugs on behaviour: The acetylcholine system. In: Steinberg, H.; deReuck, A. V. S.; Knight, J., eds. Animal Behavior and Drug Action. Churchill: London; 1964:144–159.
37. SAS Institute Inc.: SAS/STAT user's guide, version 6. Cary, NC: SAS Institute Inc.; 1990.
38. Volpe, L. S.; Biagnioni, T. M.; Marquis, J. K.: In vitro modulation of bovine caudate muscarinic receptor number by organophosphates and carbamates. Toxicol. Appl. Pharmacol. 78:226–234; 1985.
39. Ward, T. R.; Ferris, D. J.; Tilson, H. A.; Mundy, W. R.: Correlation of the anticholinesterase activity of a series of organophosphates with their ability to compete with agonist binding to muscarinic receptors. Toxicol. Appl. Pharmacol. 122:300–307; 1993.
40. Wecker, L.; Mobley, P. L.; Dettbarn, W.-D.: Central cholinergic mechanisms underlying adaptation to reduced cholinesterase activity. Biochem. Pharmacol. 26:633–637; 1977.
41. Yamamura, H. I.; Snyder, S. H.: Muscarinic cholinergic binding in rat brain. Proc. Natl. Acad. Sci. USA 71:1725–1729; 1974.