Comparison of Acute Neurobehavioral and Cholinesterase Inhibitory Effects of N-Methylcarbamates in Rat

Katherine L. McDaniel,* Stephanie Padilla,* Renée S. Marshall,* Pamela M. Phillips,* Lynda Podhorniak,† Yaorong Qian,† and Virginia C. Moser*1

*Neurotoxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, US Environmental Protection Agency, Research Triangle Park, North Carolina 27711; and †Analytical Chemistry Branch, Office of Pesticide Programs, US Environmental Protection Agency, Fort Meade, Maryland 20755

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While the cholinesterase-inhibiting N-methyl carbamate pesticides have been widely used, there are few studies evaluating direct functional and biochemical consequences of exposure. In the present study of the acute toxicity of seven N-methyl carbamate pesticides, we evaluated the dose-response profiles of cholinesterase (ChE) inhibition in brain and erythrocytes (RBCs) as well as motor activity (both horizontally and vertically directed) and clinical signs of overt toxicity. The chemicals tested were carbaryl, carbofuran, formetanate, methiocarb, methomyl, oxamyl, and propoxur. All were administered orally, and rats were tested in 20-min activity sessions beginning 15 min after dosing; tissues were collected immediately after activity sessions. In general, motor activity was a sensitive measure of ChE inhibition for all these carbamate pesticides, and vertical activity showed the greatest magnitude of effect at the highest doses compared to either horizontal activity or ChE inhibition. Brain and RBC ChE activities were generally affected similarly. Pearson correlation coefficients of within-subject data showed good correlation between the behavioral and biochemical end points, with brain ChE inhibition and horizontal activity showing the highest correlation values. Determination of benchmark dose levels for 10% change in each end point also revealed that these two measures produced the lowest estimates. Thus, motor activity decreases are highly predictive of ChE inhibition for N-methyl carbamates, and vice versa.

Key Words: N-methyl carbamates; neurotoxicity; motor activity; cholinesterase; rats.

The acute neurobehavioral effects of N-methyl carbamate insecticides are primarily due to overstimulation of the cholinergic system as a result of central and peripheral cholinesterase (ChE) inhibition. These effects include lowered activity levels, fasciculations, salivation, lacrimation, with full-body tremors and dyspnea at high doses. There are currently 11 N-methyl carbamates registered for use in the United States (USEPA, 2006).

The temporal aspects of inhibition are limited by carbamylation and decarbamylation of the esterase enzyme (Aldridge and Reiner, 1975; Casida, 1963; O’Brien et al., 1966). Generally, decarbamylation of ChE is more rapid than dephosphorylation, and thus N-methyl carbamate pesticides are relatively short acting in terms of both ChE inhibition and the resultant acute neurotoxicity as compared to organophosphorus pesticides. The majority of the methylcarbamates are metabolized within 12 h after oral dosing, and there is a rapid excretion of metabolites in the urine (Dorough 1970). This rapid reversibility is one reason for their continued use as insecticides in a variety of applications.

While these pesticides have been widely used for decades, much of the available toxicity literature has focused on just a few of them. Of these studies, even fewer have examined functional outcomes as a direct consequence (e.g., in the same subject) of ChE inhibition, especially at sublethal doses. In addition, we could find almost no studies of a systematic comparison across this group of chemicals, with the exception of earlier studies from our laboratory (Padilla et al., 1996, 2007). Such comparisons are necessary for understanding acute neurotoxicity of these chemicals as a function of biochemical changes, i.e., ChE inhibition.

Most studies in which ChE inhibition is measured report the presence (or lack) of overt signs of toxicity (e.g., tremors, salivation, lacrimation, etc.). In some studies, toxic signs were reported at doses producing greater than about 35% inhibition of brain and/or blood ChE, although this level varied with the different chemicals assessed. Such observations are available for oxamyl (Fayez and Kilgore, 1992; Kennedy, 1986), carbofuran (Ferguson et al., 1984), and carbaryl (Orzel and Weiss, 1966). Other investigators have sought to objectively or quantitatively measure these behavioral changes with which to compare ChE inhibition. There are, however, fewer of such studies involving only propoxur (Kobayashi et al., 1988; Ruppert et al., 1983; Thiesen et al., 1999), and carbaryl...
to provide relative sensitivity of the activity measure. In addition, a scoring of overt toxicity signs was included to provide relative sensitivity of the activity measure.

**MATERIALS AND METHODS**

**Chemicals**

All chemicals were received from Chem Service (West Chester, PA) at ≥99% purity. The carbamates tested were carbachol (1-naphthylenol methylcarbamate), carbofuran (2,3-di-hydro-2,2-dimethyl-7-benzofuranol methylcarbamate), formetanate (N,N-dimethyl-N’-[3-[(methylamino)carbonyl]oxy]phenyl)methanimidamide), methiocarb (3,5-dimethyl-4-(methylthio)phenol methylcarbamate), methomyl (N-[[(methylamino)carbonyl]oxy]ethanimidothioic acid methyl ester), oxamyl (2-[(dimethylamino)carbonyl]oxy)-2-oxethanidothioic acid methyl ester), and propoxur (2-(1-methyl-ethoxy)phenol methylcarbamate). Corn oil was purchased from Sigma Chemical Co.

Solutions of methomyl, oxamyl, and formetanate were dissolved directly in deionized water on the morning of the study. Carbaryl, methiocarb, and propoxur were each weighed out the evening before the study, placed in corn oil, warmed in a 37°C water bath, vortexed vigorously every 10 min for 1 h, and then stirred overnight. Carbofuran was dissolved in a small volume of acetone (2.5% of the total volume), corn oil added, and vortexed every 10 min and the vial placed in the hood (uncovered) for 30 min to allow the acetone to evaporate. The vehicle for the carbofuran control animals was prepared in the same manner.

**Dosing Solution Analysis**

**Samples in water vehicle (formetanate, methomyl, and oxamyl).** All samples were stored at 4°C until analysis. After vigorous vortexing, 200 µl aliquots of each dosing solution were either diluted volumetrically with ChlorAC buffer (Pickering Laboratories, Mountain View, CA) or transferred directly to an autoinjector vial without further dilution, depending upon the labeled concentration. For the formetanate analyses (Niemann 1993), a high performance liquid chromatograph (HPLC) equipped with a DAD detector set at 250 nm and a 15 cm × 4.6 mm (i.d.) SCX column was used. The (1:1) isocratic mobile phase consisted of acetonitrile/water (premixed 50:50) and 0.4M ammonium phosphate buffer (pH 3.0) at a flow rate of 1.0 ml/min and a sample injection volume of 10.0 µl. For the oxamyl and methomyl analyses, an HPLC equipped with a 15 cm × 3.0 mm (i.d.) C18 column and a DAD detector at 220 nm was used since concentrations ranged from 0.1 to 2.5 mg/ml. The isocratic mobile phase consisted of acetonitrile/water (7:93) at a flow rate of 1.0 ml/min with a sample injection volume of 10.0 µl. Duplicate samples of the same solutions differed by an average of 0.9%.

**Samples in corn oil vehicle (carbaryl, carbofuran, methiocarb, and propoxur).** All samples were stored at room temperature until analysis. Although the corn oil samples were shaken vigorously and well mixed, the analystes remained in suspension in the samples containing high carbamate concentrations. For analysis, the corn oil samples were vigorously shaken for a few minutes and then 200 µl aliquots were transferred into 4 ml vials and diluted by adding an appropriate amount of dichloromethane. One milliliter of the diluted corn oil samples was injected onto a gel permeation chromatograph that was calibrated with corn oil and dioctyl phthalate standard to separate carbamates from the corn oil matrix. The fraction containing the carbamate was collected and concentrated to dryness under a stream of N2. The residue was redissolved in acetonitrile/water (1:1) for instrument analysis. A HPLC equipped with a 25 cm × 4.6 mm (i.d.) C18 column and a UV detector at 254 nm was used for propoxur, carbayl, methiocarb, and carbofuran analyses. Samples were analyzed isocratically using a mobile phase of acetonitrile/water (1:1 or 7:3) at a flow rate of 1.2 ml/min. Duplicate samples differed by an average of 11.1%.

Standard carbamate solutions were analyzed prior to the analysis of actual samples to establish the calibration curve. Procedural blanks (corn oil or distilled water) and matrix spikes (corn oil or distilled water fortified with appropriate amounts of carbamate) were also processed and analyzed along with each batch of corn oil and water dosing solution samples as quality control samples. Water samples fortified with 0.505 mg/ml oxamyl and 0.462 mg/ml methomyl yielded 102 and 92.3% recoveries, respectively. The water sample fortified with formetanate at 0.338 mg/ml yielded 100% recovery. Two spiked samples of corn oil fortified with carbofuran at 0.5 mg/ml yielded recoveries of 112 and 109%. Propoxur and methiocarb fortified at 2 mg/ml yielded recoveries of 104 and 101%, respectively; carbaryl spiked at 1 mg/ml yielded a recovery of 102%.

**Rats**

Adult (~97 days old) male Long-Evans rats (Charles River Laboratories, Raleigh, NC) were used for all experiments. Rats were singly housed on heat-treated pine shavings in temperature- and humidity-controlled facilities which maintained current accreditation with Association for Assessment and Accreditation of Laboratory Animal Care International. Data for each carbamate pesticide were collected in separate groups of rats.

**Experimental Design**

The rats were weighed the day prior to dosing and assigned to a dose group using stratification of weights. On the morning of testing, each rat (n = 10/dose group) received the treatment in a single oral gavage dose (given at 1 ml/kg). Approximately 10–12 min later, each animal was visually examined and received a score, termed “Tox Score.” The Tox Score was a ranked, global description of degree of overt cholinergic signs, including, but not limited to, lacrimation, miosis, fasciculations, smacking, tremors, polyuria, and diarrhea (any of these alone or in combination). Rats were scored as 1, normal; 2, some effects that were not very obvious; and 3, severe and obvious effects. The examiners had no knowledge of the treatment of the animals.

Motor activity assessment began 15 min after dosing, and the session length was 20 min. Activity was monitored in a photocell-based chamber shaped like a figure eight (Reiter, 1983). A set of eight photocells spread throughout the chamber measured horizontal activity, and a bank of photocells placed 14 cm above the flooring measured vertical activity.

At the end of the activity session, half of the rats (n = 5/dose group) were removed and immediately decapitated under CO2 anesthesia for blood and brain collection. The other half of the rats were returned to their home cages and were not used further. Thus, tissues were collected within 35–40 min after dosing. Whole brain was collected and immediately placed on dry ice. Trunk blood was collected in a heparinized tube and centrifuged at 1000 × g for 10 min to separate plasma and RBC. The RBC fraction was diluted 1:3 (1 part RBC plus two parts 0.1M sodium phosphate buffer, pH 8.0 containing 1% Triton). Brain and diluted RBC were stored at ~80°C until assayed.

**ChE Assay**

The brain tissue was thawed on ice and prepared on the day of analysis. The brain was weighed and diluted (weight/volume) with 0.1M sodium phosphate buffer, pH 8.0 containing 1% Triton. The final dilution was 1:3. The brain tissue was homogenized on ice using a Polytron (model PTK100, probe 3012/2TM, 20,000 rpm, Brinkman Industries, Westbury, NY) for 20 s. Special care was taken to limit reactivation of the brain and RBC carbamylated ChE. Tissue dilution was kept to a minimum, and tissues were not further diluted until the
exact moment of adding the substrate at the beginning of the assay. Tissues were kept on ice until the exact moment of the assay, and the time between homogenization (brain) and assay was minimized (preliminary experiments indicated that brain homogenate diluted 1:3 kept on ice for 90 min did not show any significant reactivation).

The radiometric assay was essentially as described by Johnson and Russell (Johnson and Russell, 1975), with a total reaction volume of 100 µl with a final substrate concentration of 1.2mM acetylcholine iodide spiked with 0.1 µCi of [³H]acetylcholine iodide (76.0 mCi/mmol, Perkin Elmer Life Sciences, Boston, MA). The assay was conducted at 26°C using an incubation of 1 min for brain homogenate and 3 min for RBC. After the reaction was stopped and scintillant was added, activity was counted within 24 h of the assay in a Beckman scintillation counter (model LS6000LL, Fullerton, CA). Counting efficiency, as determined by an external quench standard, was approximately 62%. On the day of each assay, reference standards (serial dilutions of control rat brain homogenate kept frozen at −80°C) were analyzed immediately before the experimental tissues to ascertain that the assay was performing correctly. These reference values varied no more than 10% over the course of the experiments.

Statistical Analyses

Two-way analyses of variance were conducted with a grouping factor of dose and either type of activity (horizontal and vertical) or tissue (brain and RBC) as within-subject factors. Significant interactions were followed by step-down analyses of individual dose-response data for each measure, with Dunnett’s t-test to determine which dose groups were significantly different from control. In addition, paired t-tests were used at each dose level to compare horizontal versus vertical activity and RBC versus brain ChE activity on a percent control basis; Tukey’s correction of the p-value was used for these multiple tests.

Pearson’s product moment correlation coefficients (r) of within-subject data were calculated for combinations of horizontal, vertical, brain, and RBC ChE data. A t-test was used to test for differences of dependent correlations from the same sample (Blalock, 1972). This t-test was used to compare the correlation of brain or RBC ChE activity versus horizontal activity and brain or RBC ChE activity versus vertical activity.

Benchmark dose modeling software (version 1.4.1; USEPA, 2007) was used to calculate doses estimated to produce a 10% decrease (BMD10; i.e., 90% of control) in each end point. In all cases, the Hill model was used, with a correction for nonequal variances when they were present.

RESULTS

All dosing solutions were analyzed to determine the actual concentration of the N-methyl carbamate present; Table 1 compares the nominal and actual concentration at each dose level. The average difference between the nominal and actual concentration for each dose for each carbamate was 9.2%. Each carbamate was administered on two consecutive days with new dosing solutions made up each day. The average concordance between the day 1 and day 2 dosing solutions was 4.5%.

No lethality occurred in any of these studies, and at no time did the rats show severe toxicity, e.g., convulsions or respiratory distress. RBC ChE data were not available for one rat treated with the highest dose of carbaryl (50 mg/kg) due to excessive clotting.

Even though these dose-response data were collected over a period of months, the control data for motor activity and brain ChE activity were quite consistent as seen in Table 2. The most variable measure, RBC ChE activity, varied as much as twofold across studies. In order to compare dose response across end points, all data were converted to percent of the concurrent control value; however, statistical analyses were conducted on the raw data. Dose-response curves for each carbamate are presented in Figures 1–7. Individual brain ChE and horizontal activity data are plotted in Figure 8 for all carbamates combined. For a more accurate depiction of the relationships between the various dependent measures, Table 3 presents the Pearson correlation coefficients between them for each chemical.

ANOVA’s were conducted for each carbamate to determine whether the within-subject dose-response trend was the same or different for ChE inhibition in brain and RBC and for horizontal and vertical motor activity. For ChE inhibition by all chemicals, there were significant main effects of dose (all p values < 0.0001) as well as dose-by-tissue interactions (all p values < 0.0001). Similarly on the activity measures, there were significant effects of dose and dose-by-activity type interactions (all p values < 0.0001). Thus, step-down analyses were conducted for each dose-response dataset for each end point separately.

Horizontal activity correlated significantly and strongly with vertical activity, with Pearson correlation coefficients ranging from 0.702 (methomyl) to 0.958 (methiocarb); coefficients for

<table>
<thead>
<tr>
<th>Carbamate</th>
<th>Concentration (mg/ml; nominal/actual)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbaryl</td>
<td>0.0/0.2 3.0/4.6 7.5/8.5 15.0/15.9 30.0/29.3 50.0/53.4</td>
</tr>
<tr>
<td>Carbofuran</td>
<td>0/0 0.1/0.1 0.3/0.3 0.5/0.5 0.75/0.74 1.5/1.4</td>
</tr>
<tr>
<td>Formetanate</td>
<td>0/0 0.1/0.1 0.3/0.3 0.6/0.55 1.5/1.5 5.0/4.8</td>
</tr>
<tr>
<td>Methiocarb</td>
<td>0/0 0.5/0.6 2.0/2.0 5.0/4.5 12.0/11.8 25.0/25.4</td>
</tr>
<tr>
<td>Methomyl</td>
<td>0/0 0.1/0.1 0.25/0.23 0.6/0.6 1.25/1.18 2.5/2.3</td>
</tr>
<tr>
<td>Oxamyl</td>
<td>0/0 0.067/0.05 0.1/0.1 0.5/0.45 1.0/1.0 1.5/1.4</td>
</tr>
<tr>
<td>Propoxur</td>
<td>0/0 0.3/0.5 1.0/1.1 3.0/3.4 10.0/9.8 20.0/21.4</td>
</tr>
</tbody>
</table>
the other chemicals were between 0.8 and 0.9. Correlations between brain and RBC ChE activity were also significant, with coefficients from 0.767 (formetanate) to 0.877 (carbaryl).

### Carbaryl

At the lowest dose (3 mg/kg), motor activity (horizontal and vertical) was significantly depressed but not at the next higher dose (7.5 mg/kg), which did lower brain ChE (Fig. 1). Doses of 15 mg/kg and higher significantly lowered all parameters. Paired analyses revealed that the inhibition of RBC and brain were similar across all doses. Motor activity was decreased to a greater degree, in terms of percent control, than ChE activity, and vertical activity was more affected than horizontal at all doses except 7.5 mg/kg. Tox scores were all “1”s (normal). Within-subject correlations between ChE and motor activity are presented in Table 3. While RBC ChE correlations appeared higher, these differences were not significant. Vertical activity was a better predictor of both brain and RBC ChE inhibition than horizontal activity. BMD10 values were similar for brain ChE activity and both forms of motor activity (0.9–2.3 mg/kg), but the BMD10 value for RBC ChE was much higher (13.1 mg/kg).

### Carbofuran

The lowest dose of carbofuran (Fig. 2) significantly decreased brain ChE activity but not RBC ChE or motor activity. At each dose level, brain and RBC ChE inhibition were similar. The higher doses produced relatively greater decreases in motor activity, with vertical activity significantly more affected than horizontal at the two highest doses. There was a graded increase in the number of rats showing toxicity as assessed by the Tox Score, with average scores of 1.1, 1.5, and

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<table>
<thead>
<tr>
<th>Carbamate</th>
<th>Horizontal</th>
<th>Vertical</th>
<th>Brain</th>
<th>RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbaryl</td>
<td>158.0 ± 8.5</td>
<td>45.6 ± 5.5</td>
<td>7004.9 ± 156.3</td>
<td>311.8 ± 24.4</td>
</tr>
<tr>
<td>Carbofuran</td>
<td>160.1 ± 9.4</td>
<td>49.5 ± 3.5</td>
<td>7158.4 ± 201.4</td>
<td>428.3 ± 19.4</td>
</tr>
<tr>
<td>Formetanate</td>
<td>178.9 ± 8.7</td>
<td>49.0 ± 5.0</td>
<td>7363.4 ± 248.4</td>
<td>323.1 ± 46.3</td>
</tr>
<tr>
<td>Methiocarb</td>
<td>177.2 ± 10.6</td>
<td>45.2 ± 6.1</td>
<td>6551.6 ± 214.1</td>
<td>187.9 ± 20.3</td>
</tr>
<tr>
<td>Methomyl</td>
<td>168.5 ± 5.8</td>
<td>50.8 ± 6.9</td>
<td>6516.3 ± 125.6</td>
<td>331.2 ± 16.8</td>
</tr>
<tr>
<td>Oxamyl</td>
<td>158.3 ± 6.8</td>
<td>38.1 ± 5.5</td>
<td>6728.2 ± 125.3</td>
<td>294.0 ± 45.2</td>
</tr>
<tr>
<td>Propoxur</td>
<td>171.5 ± 11.5</td>
<td>48.9 ± 3.8</td>
<td>6159.3 ± 292.7</td>
<td>344.2 ± 12.5</td>
</tr>
</tbody>
</table>

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**FIG. 1.** Effects of carbaryl on motor activity (horizontal and vertical) and ChE activity (brain and RBC) expressed as percent control. * indicates doses significantly different from control; n = 5/dose for ChE measurements, n = 10/dose for motor activity.

**FIG. 2.** Effects of carbofuran on motor activity (horizontal and vertical) and ChE activity (brain and RBC), expressed as percent control. * indicates doses significantly different from control; n = 5/dose for ChE measurements, n = 10/dose for motor activity.
2.4 (on a 1-to-3 scale) at 0.5, 0.75, and 1.5 mg/kg, respectively. The higher correlations (Table 3) for brain ChE and motor activity compared to RBC ChE were not significantly different. BMD analyses produced similar BMD10 values for all end points (0.04–0.09 mg/kg).

Formetanate

Motor and ChE activity were decreased in a similar manner across most doses of formetanate (Fig. 3). The two lowest doses, however, significantly decreased vertical activity, whereas the middle dose group did not reach statistical significance. Paired comparisons indicated differences between horizontal and vertical activity at 0.1, 0.3, and 5 mg/kg but at not the intermediate doses. Only two rats in the highest dose group showed overt toxicity as assessed by the Tox Score. Brain and RBC ChE correlated with both measures of activity (Table 3). The lower vertical activity correlation coefficients with ChE inhibition were probably due to the nonmonotonic decrease in vertical activity at the two lowest doses. The BMD10 values were similar across end points (0.13–0.53 mg/kg).
Methiocarb

Methiocarb (Fig. 4) showed a differentiation between RBC and brain ChE activity at the lowest dose due to an increase in RBC ChE activity. The differentiation between ChE activity and motor activity effects were most clear at the higher three doses. For example, vertical activity was about 5% of control levels while brain ChE activity was about 55% (i.e., 45% inhibition). While a few rats in the three higher dose groups had Tox Scores greater than “1”, the incidence did not exceed 30%. Correlations between ChE activity and motor activity were generally high (~0.9). Brain ChE activity was significantly more predictive than RBC only for horizontal activity. As with carbaryl, the BMD10 value for RBC ChE was highest (5.9 mg/kg), but the other end points had similar values (0.4–1.1 mg/kg).

Methomyl

In contrast to methiocarb, methomyl (Fig. 5) showed little differences in the dose-response profiles for the various end points, except at the highest dose, where vertical activity was decreased more than the other end points. Paired analyses indicated that brain and RBC ChE inhibition were not different at any dose, and vertical activity was significantly different from horizontal only at 2.5 mg/kg. Horizontal activity was more highly correlated with brain and RBC ChE activity, but neither form of ChE measure was significantly better at predicting either form of activity. At the highest dose, half of the rats showed overt toxicity (scores of “2” in the Tox Score). There were essentially no differences among the BMD10 values for the different end points (0.38–0.76 mg/kg).

Oxamyl

There were no significant changes in any end point observed at the two lower doses of oxamyl (Fig. 6). At the higher doses, RBC ChE was inhibited to a greater degree than brain, and in fact, the magnitude of change was more similar to the motor activity decreases. Only one and two rats in the 1 and 1.5 mg/kg dose groups, respectively, showed overt toxicity (Tox Scores of “2”). Correlation coefficients were high, with brain ChE being significantly more predictive than RBC ChE for horizontal but not vertical activity. BMD10 values were very similar (0.25–0.51 mg/kg).
**Propoxur**

At the effective dose range (3–20 mg/kg), propoxur produced greater effects on motor activity than ChE activity. While an intermediate dose (10 mg/kg) indicated that RBC ChE activity was significantly decreased more than brain, this was the only dose that showed such separation of effect. Likewise, vertical activity was significantly lower than horizontal at the highest dose only. A graded increase in incidence of overt toxicity was observed with the Tox Score (average scores of 1.2, 1.4, and 2.1 at 3, 10, and 20 mg/kg, respectively). Brain ChE activity correlated significantly better than did RBC ChE with both horizontal and vertical motor activity. The BMD10 value for horizontal activity was the lowest (0.27 mg/kg), with the ChE values showing the highest BMD10s (2.7 and 3.5 mg/kg for brain and RBC, respectively).

When all these carbamate data were combined into a single correlation analysis (Table 3), the correlations between brain ChE and both forms of activity were significantly higher than the correlations with RBC ChE activity.

**DISCUSSION**

For each of the seven chemicals tested, motor activity depression was generally greater than the ChE inhibition, and vertical activity was depressed to a greater extent than horizontal activity at the high end of the dose-response curves. This greater sensitivity of vertical activity could be due to other influences on the behavior (e.g., vestibular effects) or the configuration of the chambers (rearing is only measured in the central arena). When focusing on the low end of the dose response, however, significant motor activity decreases were not observed in the absence of significant ChE inhibition. BMD analyses showed lower BMD10 estimates for horizontal (but not vertical) activity compared to ChE inhibition only for two pesticides (methiocarb and propoxur). Most of these carbamates showed, in general, similar ChE inhibition in brain and RBC. On the other hand, oxamyl produced significantly more inhibition in RBCs than in brain across most doses—this same pattern of inhibition was also noted in the time-course assessment (Padilla et al. 2007). Despite this, BMD10 values for brain ChE inhibition were numerically lower than RBC for carbaryl, formetanate, methiocarb, and methomyl; this difference could reflect the lower variability of the brain ChE data.

Being an apical measure of nervous system function, motor activity is sensitive to perturbations of the motor, sensory, and/or integrative systems. This behavior may therefore reflect subtle effects of carbamates and serve as an early indicator of toxicity. With Pearson correlation coefficients generally greater than 0.8, these experiments show a clear correspondence between motor activity and ChE inhibition for all of these carbamate pesticides. Correlation analyses indicated that brain ChE activity was significantly better than RBC ChE when correlated with horizontal activity for propoxur, oxamyl, and methiocarb and for vertical activity only for propoxur. Comparing all carbamates (Fig. 8) reveals considerable overlap in the data. For almost all carbamates, a linear relationship between decreasing activity and ChE activity, i.e., no threshold was evident. Only the pattern for formetanate suggested a threshold, in that motor activity was not altered until brain ChE was around 50% of control levels. While correlation does not imply a causal mechanism, such data point to the high predictability of activity data (especially horizontal) for indications of underlying ChE inhibition (especially brain) and vice versa.

The Tox Score showed very different patterns across the studies. In a few cases (carbaryl, formetanate, oxamyl), only a few, or no, rats scored greater than “1”, even at doses producing considerable ChE inhibition and motor activity decreases. The lack of signs, for some, may have been a function of time of observation. Several animals scored a “1” at the time of scoring but either upon being put in or removed from the activity chambers, they showed clear signs of intoxication. Unfortunately, our protocol specified only one evaluation time, but the potential for end point-dependent time-course profiles has also been mentioned by others (e.g., Lammers and Kulig, 1997). Regardless, it is apparent from this comparison that the objective motor activity measurement was more sensitive and reliable for detecting subtle N-methyl carbamate neurotoxicity than the observation of clinical signs of toxicity.

In general, our data agree with the scant published literature of N-methyl carbamates in rats. The most neurotoxicity data are available for carbaryl. In a study using the same within-subject design as we did here, carbaryl (1.5–75 mg/kg ip) produced a linear correspondence between RBC ChE inhibition and motor activity decreases, and at the higher doses, activity was maximally depressed while RBC ChE was only about 60% inhibited (Padilla et al., 1996). Those results are very similar to the present findings. In other studies, Orzel and Weiss (1966) reported a correlation between the onset and duration of tremors and the degree of brain ChE inhibition (64%) produced by ip injections of carbaryl 25 mg/kg; in addition, blood glucose rose in a similar manner (Orzel and Weiss, 1966). On the other hand, in studying active avoidance behavior, Goldberg concluded that the behavioral disruption from carbaryl (5 and 10 mg/kg ip) was greater than could be accounted for by the degree of ChE inhibition (25–42% inhibition; Goldberg et al. 1965). Others have also reported behavioral alterations including decreased motor activity (8–28 mg/kg ip) with 16 mg/kg producing 58% brain ChE inhibition (Ruppert et al., 1983), hypothermia and decreased activity within 24 h after dosing at 25 and 75 mg/kg (Gordon and Mack 2001), and changes in measures of a functional observational battery (10 and 30 mg/kg ip; Moser et al., 1988). Comparatively speaking, the effective dose range in the present study (3–50 mg/kg po) is similar to these previous studies in terms of magnitude of behavioral change as well as ChE inhibition. Using much higher doses (50–200 mg/kg po), Ray and Poddar (1990) reported only 28–69% brain ChE
inhibition and a dose-dependent increase in the magnitude of tremors. By far the lowest effective doses were reported by Singh (1973), in which 0.56 and 2.24 mg/kg ip carbaryl decreased running wheel activity; unfortunately, ChE inhibition was not measured in that study.

In a previous study, propoxur showed the greatest inhibition of blood and brain ChE at 15–30 min after dosing with 2.1–70 mg/kg po; the time course of inhibition (20.9 mg/kg po producing about 42% blood ChE inhibition) correlated with the peak tissue levels as well as toxic signs (Krechen and Foss, 1982). Behavioral studies have reported decreased open-field activity and rearing at 6.2 mg/kg po (Agarwal et al., 1988), decreased open-field rearing and locomotor activity in figure-eight chambers at 2–8 mg/kg ip (2 mg/kg producing 47% brain inhibition; Ruppert et al., 1983), and decreased open-field rearing, ambulation, and active avoidance responding at 8.3 mg/kg po (producing 57% brain inhibition; Thiesen et al., 1999). Thus, the effective dose range for activity depression and enzyme inhibition in the present study (0.3–20 mg/kg) corresponds well with these earlier studies.

Carbofuran 0.05 mg/kg po produced a 37% decrease in RBC ChE within 15 min without any signs of toxicity (Ferguson et al., 1984), whereas a higher dose of 0.69 mg/kg po did produce overt toxicity at 45 min (Renzi and Krieger, 1986) even though the measured ChE inhibition was reportedly the same. Higher doses (1–2.5 mg/kg sc) produced toxicity of increasing severity which developed within minutes and peaked at about 1 h after dosing; 1.5 mg/kg produced 50–75% inhibition measured in various brain regions (Gupta and Kadel, 1989). In the present study, 0.75 mg/kg po produced about 40% ChE inhibition, but motor activity was depressed by about 60%.

Oxamyl doses of 1–3.5 mg/kg po produced a range of toxic signs with a maximum of 54% brain ChE inhibition (Fayez and Kilgore, 1992), whereas oral doses of 4.86 mg/kg and higher produced lethality, yet no greater inhibition measured in blood (Kennedy, 1986). In the present study, doses of 1 and 1.5 mg/kg produced 80–90% blood inhibition and almost complete suppression of motor activity.

Formetanate decreased responding in an operant task at 0.03–0.75 mg/kg ip, but ChE inhibition was not measured (Moser and MacPhail, 1986, 1987). In the current study, this dose range produced up to about 50% suppression of both motor and ChE activities. We could find no other studies comparing ChE inhibition and toxic effects with formetanate, methiocarb, or methomyl.

The importance of the conditions of the assay used to measure carbamate-induced ChE inhibition is well known. Precautions must be taken to minimize reactivation of the enzyme to estimate more accurately the level of inhibition present in vivo. Temperature, dilution, and incubation time are factors that can significantly influence the data (Johnson and Russell, 1975; Nostrandt et al., 1993; Winteringham and Fowler, 1966). For example, Pickering and Pickering (1971) demonstrated that carbamate-induced inhibition was lessened by more than 50% with time after sample preparation and with the amount of tissue dilution. It is conceivable that many previous studies did not account for this factor in their interpretation of data, which could make comparisons of effective doses across studies more difficult. Moreover, this could explain reports (e.g., Goldberg et al., 1965; Renzi and Krieger 1986) wherein behavioral changes were measured at dose levels without apparent ChE inhibition; however, it is difficult to discern information about the assays from published papers.

We used the radiometric method in the present study to determine the underlying degree of ChE inhibition because this method allows analysis of very concentrated samples. This was very important to lessen the degree of decarbamylation. By keeping tissues cold or frozen at all times and including short incubation times and minimal dilutions, we have tried to limit reactivation to the extent possible. In the present study, a small degree of reactivation could influence the statistical outcomes, especially at the lower doses. This could lead to data wherein motor activity appears somewhat more affected than ChE (i.e., propoxur) or why brain ChE may appear more inhibited than RBC (e.g., carbofuran). The exact degree to which this could occur is impossible to determine. It must be noted that a recent publication (Lassiter and Brimijoin, 2007) reports a sensitive assay for ChE activity in carbamate-treated animals, especially in very low dose situations. This may be superior to the use of the radiometric assay especially for determination of the BMDs.

The use of the BMD modeling allowed prediction of doses producing low-level effects and the 95% lower limits on those estimates. The current analyses indicated that carbofuran was the most potent N-methyl carbamate, and carbaryl was the least potent; the other chemicals were intermediate. The precise BMDs depended on the end point assessed, but overall, the BMDs for brain ChE and horizontal activity were the lowest. These results stress the importance of evaluating both function and enzyme inhibition for the overall assessment of these pesticides.

In summary, these studies provide quantitative within-subject dose-response data for motor activity and ChE inhibition for seven N-methyl carbamates. Correlations were generally high between the functional and biochemical changes but better overall for brain compared to RBC ChE. Motor activity decreases were highly predictive of ChE inhibition for N-methyl carbamates and vice versa. Furthermore, with the possible exception of oxamyl, these current data support the use of brain ChE activity over RBC when evaluating neurotoxicity for these chemicals.

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REFERENCES


