Impact of Chemical Proportions on the Acute Neurotoxicity of a Mixture of Seven Carbamates in Preweanling and Adult Rats

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Statistical design and environmental relevance are important aspects of studies of chemical mixtures, such as pesticides. We used a dose-additivity model to test experimentally the default assumptions of dose additivity for two mixtures of seven N-methylcarbamates (carbaryl, carbofuran, formetanate, methomyl, methiocarb, oxamyl, and propoxur). The best-fitting models were selected for the single-chemical dose-response data and used to develop a combined prediction model, which was then compared with the experimental mixture data. We evaluated behavioral (motor activity) and cholinesterase (ChE)-inhibitory (brain, red blood cells) outcomes at the time of peak acute effects following oral gavage in adult and preweanling (17 days old) Long-Evans male rats. The mixtures varied only in their mixing ratios. In the relative potency mixture, proportions of each carbamate were set at equitoxic component doses. A California environmental mixture was based on the 2005 sales of each carbamate in California. In adult rats, the relative potency mixture showed dose additivity for red blood cell ChE and motor activity, and brain ChE inhibition showed a modest greater-than additive (synergistic) response, but only at a middle dose. In rat pups, the relative potency mixture was either dose-additive (brain ChE inhibition, motor activity) or slightly less-than additive (red blood cell ChE inhibition). On the other hand, at both ages, the environmental mixture showed greater-than additive responses on all three endpoints, with significant deviations from predicted at most to all doses tested. Thus, we observed different interactive properties for different mixing ratios of these chemicals. These approaches for studying pesticide mixtures can improve evaluations of potential toxicity under varying experimental conditions that may mimic human exposures.

Key words: dose additivity; pesticide mixture; carbaryl; carbofuran; formetanate; methiocarb; methomyl; oxamyl; propoxur; cholinesterase; behavior; age; rat.

Environmental exposures generally involve multiple chemicals and pathways. These real-world exposures vary temporally and spatially in terms of specific chemicals and their relative proportions. There is no doubt that humans are exposed to a multitude of pesticides, given their widespread agricultural, industrial, and residential uses. There is, however, concern that the cumulative toxicity of these chemicals could be greater than that predicted by individual assessments. Research concerning mixture toxicity for risk assessment is a key ongoing environmental health issue.

Chemical mixture research has moved forward with a greater emphasis and understanding of experimental design. While many studies of mixtures have focused on binary combinations, there is a need to evaluate more complex and environmentally relevant mixtures. Statistical methodologies now exist to evaluate interactions for larger numbers of chemicals in defined mixtures. The ratios of components in a mixture can greatly influence the type of interaction; witness, for example, the ratio-specific synergistic effects of chloral hydrate and ethanol in a “Mickey Finn” (Gessner and Cabana, 1970). Furthermore, there is value in testing multiple ratios for describing the range of deviations from, or consistency with, dose additivity that may be observed (Jonker et al., 2005). Despite this, few studies of mixtures evaluate the effects of varying the composition.

N-methylcarbamate pesticides produce acute neurotoxicity by inhibition of acetylcholinesterase (ChE) at cholinergic nerve terminals, producing a profile of toxicity that includes nausea, sweating, miosis, gastrointestinal stimulation, lacrimation, salivation, twitches, tremors, and convulsions (Ecobichon, 2001). In laboratory animals, measurements of ChE inhibition and behavioral changes have been shown to be sensitive indicators of exposure, occurring at levels that do not produce overt toxicity. Because of their common mode of action, the default assumption for risk assessment is that carbamates exert toxicity in a dose-additive manner, that is,
the chemicals combine as if they were dilutions of the same chemical (U.S. EPA, 2000). This was the basis for the joint risk assessment conducted on 10 registered carbamates by the U.S. Environmental Protection Agency (U.S. EPA, 2007).

There are few studies testing the hypothesis of dose additivity in carbamates (Padilla, 2006). An in vitro study of Chinook salmon olfactory ChE inhibition indicated that carbaryl and carbofuran combined in a dose-additive manner (Scholz et al., 2006), yet another study from the same laboratory reported greater-than-predicted ChE inhibition in juvenile Coho salmon exposed to combinations of high, but not low, doses of carbaryl and carbofuran (Laetz et al., 2009). Another article explored in vitro ChE properties of the binary mixtures of aldicarb and either carbofuran or carbaryl in a tissue-free system (Kok and Hasirci, 2004). Using a full-factorial design, less-than-additive inhibition was observed at the high, but not low, aldicarb concentrations. A combination of aldicarb, carbofuran, and oxamyl suggested potentiation of aldicarb and carbofuran effects in terms of rat plasma ChE inhibition (Iyanwura, 1989). Finally, acute exposure of adult rats to a mixture of carbaryl and propoxur resulted in dose additivity on an electrophysiological measure and small deviations from additivity for ChE inhibition (less-than additive for brain, greater-than additive for red blood cell [RBC]; Mwanza et al., 2012). There are more studies on mixtures of carbamate plus organophosphate pesticides, but given the differences in their binding characteristics with acetylcholinesterase, those findings may not be generalized to carbamate-carbamate interactions.

We have previously shown that a mixture of five organophosphate pesticides produced greater-than-additive interactions on ChE inhibition and behavioral effects, measured in both adult and preweanling rats (Moser et al., 2005, 2006). Here we use a modified and extended approach to study the mixtures of seven carbamates in differing proportions, utilizing the same endpoints. Specifically, we use a statistical approach that includes consideration of model fit to the individual chemical dose-response data, comparison of the parameters of the dose-additive model developed from single-chemical data to those of the model fit to the mixture data, and comparison of the predicted model to the actual experimental data (Hertzberg et al., unpublished). This study had several objectives, to test whether: (1) mixtures of these seven carbamates deviate significantly from dose additivity in their acute neurological effects, (2) different mixing ratios influence this outcome, and (3) the outcome differs, using the same carbamate mixtures, in young compared with adult rats.

**MATERIALS AND METHODS**

**Chemicals.** All carbamates were purchased from Chem Service (West Chester, PA) at ≥ 99% purity. The carbamates were carbaryl (1-naphthylalenol methylcarbamate), carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate), formetanate hydrochloride (N,N-dimethyl-N-[3-[[methylamino]carbonyl]oxy]phenyl)methanimidamide), methiocarb (3,5-dimethyl-4-(methylthio)phenylmethylcarbamate), methomyl (N-[[methylamino]carbonyl][oxy]ethanimidothioc acid methyl ester), oxamyl (2-(dimethylamino)-N-[[methylamino]carbonyl]oxy)-2-oxothioimidodithioc acid methyl ester), and propoxur (2-(1-methyl-ethoxy) phenylmethylcarbamate). For the ChE assay, [3H]acetylcholine iodide (76 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) and other reagents (Sigma Aldrich, St Louis, MO) were obtained at reagent-grade purity.

Because there were differences in the solubility of these chemicals, two solutions were prepared. The first solution included the water-soluble formetanate, oxamyl, and methomyl in deionized water. The second solution was made with the carbofuran first dissolved in a small amount of acetone (2.5% final volume, which was then allowed to evaporate), and the remaining chemicals were added and all were suspended in corn oil (Mazola).

**Rats.** Adult (~90 days old) male and timed-pregnant female Long-Evans hooded rats (Charles River Laboratories, Raleigh, NC) were housed on heat-treated pine shavings (Beta-Chip) in an AAALAC International-accredited animal facility with regulated temperature (72 ± 2°F) and humidity (50% ± 20%). Feed (Purina FormulaLab Diet no. 5008 for dams; Purina Rodent Chow #5001 for adults) and water (filtered tap) were freely available. Data for each carbamate mixture were collected in separate groups of rats. All studies were approved by the NHEERL Institutional Animal Care and Use Committee.

There were 10 rats/dose for both mixtures tested at both ages. Adults were acclimated to the facility for at least one week before dosing. Dams were allowed to deliver naturally, with day of birth noted as postnatal day (PND) 0. On PND3, rat pups were cross-fostered across all dams, assuring that littersmates were spread across litters. All litters were culled to eight pups, with six males in each. Testing occurred on PND17. The male pups were dosed in a split-litter design, such that each pup in a litter received a different dose; thus, 10 litters were used to supply 10 pups at each of the six dose levels.

**Experimental design.** The pesticides were administered at two different mixing ratios, listed in Table 1. The relative potency ratios were based on the oral potencies of the chemical components, specifically the doses causing 10% inhibition (ED10) of brain ChE activity (Woody Setzer, U.S. EPA, personal communication), using dose-response data presented in McDaniel et al. (2007). This theoretically

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Proportion of Individual Carbamates Within Each Mixture, Total Doses Administered, and Potency-Weighted Score*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixture proportion</td>
<td>Potency-weighted score</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>0.42</td>
</tr>
<tr>
<td>Propoxur</td>
<td>0.29</td>
</tr>
<tr>
<td>Methiocarb</td>
<td>0.20</td>
</tr>
<tr>
<td>Methomyl</td>
<td>0.05</td>
</tr>
<tr>
<td>Formetanate</td>
<td>0.02</td>
</tr>
<tr>
<td>Oxamyl</td>
<td>0.01</td>
</tr>
<tr>
<td>Carbofuran</td>
<td>0.01</td>
</tr>
<tr>
<td>Total doses</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>0.3, 1.3, 3.7, 8.6, 15.9 mg/kg</td>
</tr>
<tr>
<td>Environmental mixture</td>
<td>0.2, 0.9, 2.6, 6.0, 11.1 mg/kg</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>0.41</td>
</tr>
<tr>
<td>Carbofuran</td>
<td>0.39</td>
</tr>
<tr>
<td>Oxamyl</td>
<td>0.13</td>
</tr>
<tr>
<td>Methomyl</td>
<td>0.04</td>
</tr>
<tr>
<td>Formetanate</td>
<td>0.03</td>
</tr>
<tr>
<td>Methiocarb</td>
<td>0.003</td>
</tr>
<tr>
<td>Propoxur</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*Mixtue proportion divided by ED10 for brain ChE inhibition in adult rats.
combines the seven chemicals in proportions that reflect similar potencies or hazards. The chemical proportions in the California environmental (CE) mixture were derived from the relative amounts of each chemical sold (a surrogate for relative exposure through agricultural uses) in the state of California in 2005 (prediction.ca.gov/docs/prr/prrmain). Total mixture doses for PND17 pups were adjusted downward slightly (70% of adult) as previous data from our laboratory have shown that pups are somewhat more sensitive to acute doses of some of these carbamates (Moser et al., 2010); however, the mixing ratios remained the same (Table 1).

Rats were allocated to treatment groups by weight stratification for adults, and random selection from male pups within each litter. The mixtures were administered in two doses by oral gavage, with the water dosing solution immediately followed by the corn oil dosing solution. Control rats received two doses, first of water and second of corn oil with 2.5% acetone. The dosing solutions were administered at essentially the same time because these carbamates have similar time-courses, and to remain consistent with the time at which the single-chemical dose-response data were collected. Adults were dosed at 1 ml/kg of each solution, whereas the young rats received doses at 2 ml/kg solution to provide greater accuracy in the dosing volume. All dosing solutions were prepared on the day of dosing.

Testing occurred at the peak time for maximal effects. Previous time-course studies have shown this to be around 40 min for each individual chemical (Moser et al., 2010; Padilla et al., 2007). Fifteen minutes after dosing, rats were placed in motor activity chambers shaped like a figure eight (Reiter, 1983). Photobeam spaces around the chamber detected movement, i.e., photobeam breaks, over 20-min sessions. At that time any obvious signs of cholinergic toxicity (e.g., tremors, lacrimation, salivation) were noted. Immediately after the activity session, rats were euthanized for tissue collection (35–40 min after dosing). Whole brain was collected and immediately placed on dry ice. Trunk blood was collected in heparinized tubes and centrifuged at 1000 × g for 10 min to separate plasma and RBC. The RBC fraction was diluted 1:3 (1 part RBC plus 2 parts 0.1M sodium phosphate buffer, pH 8.0 containing 1% Triton). Brain and diluted RBC were stored at −80°C until assayed.

Cholinesterase assay. On the day of assay, brain tissue was thawed on ice, weighed, and diluted 1:3 with chilled buffer (0.1M sodium phosphate buffer, pH 8.0, 1% Triton), then homogenized on ice for 20 s. The radiometric assay of Johnson and Russell (1975) was used to minimize tissue dilution as well as incubation time and temperature. Tissue was incubated with [3H]acetylcholine iodide (final substrate concentration 1.2 mM) in a total reaction volume of 100 μl at 26°C for 30 s (brain homogenate) or 2 min (RBC). The reaction product, [3H]acetate, was counted within 4 h of the assay in a Beckman scintillation counter (model LS6000LL, Fullerton, CA). All samples were run in duplicate, and all duplicates were within 20%. Negative values after blank subtraction were set to zero.

Statistical analyses. The null hypothesis for risk assessment of chemicals with a common mode of action is dose addition (U.S. EPA, 2000). To evaluate this for each endpoint, the data were analyzed in several steps (Hertzberg et al., unpublished) using SAS NL MIXED (v9.1, SAS Institute Inc., 2004). First, five regression models (e.g., exponential, logistic) were selected as candidate dose-response functions representing a range of curve shapes. All were fit via nonlinear regression to each individual’s dose-response data, taken from published data for adult (McDaniel et al., 2007) and PND17 (Moser et al., 2010) rats, and preferred models were identified. Model preference was based on adequate lack of fit statistics (preferre nce is p > 0.10, marginal fit if 0.05 < p < 0.1) and the magnitude of the corrected Akaike Information Criterion (AICc), which balances goodness of fit against model complexity (Burnham and Anderson, 2004). The preferred dose-response model (which best fits the majority of the seven single chemical data sets) was used to develop the combined prediction model as the dose-additive version and evaluated as to how well the prediction model fit the composite of all the component data sets. The two models that were used for fitting the data reported here were exponential functions with three parameters (exp 3, equation 1, where x is the exposure level and plat is the high-dose plateau response) and exponential with two parameters (exp 2, equation 2). Their corresponding dose-additive prediction models (Berenbaum, 1985) are shown in equations 3 and 4.

\[
y = plat + \exp(\alpha + \beta x) \quad (1)
\]

\[
y = \exp(\alpha + \beta x) \quad (2)
\]

\[
y = plat + \exp(\alpha + \sum_{i=1}^{7} \beta_i x_i) \quad (3)
\]

\[
y = \exp(\alpha + \sum_{i=1}^{7} \beta_i x_i) \quad (4)
\]

The experimental mixture data were fit and evaluated using the same functional form as the preferred component model. Because of the ray design (fixed component proportions), the mixture model used total mixture dose for the exposure level. This mixture (experimental) model was first evaluated for how well it described the mixture data. The prediction model (based on component data) was evaluated for how well it predicted the mixture response, testing for model consistency using a Wald test (Wald, 1943). Lastly, differences in model (mixture vs. prediction) parameters and in predicted responses at each mixture dose were compared, using both Hochberg and step-down Bonferroni corrections for multiple comparisons; resultant p values < 0.05 were considered statistically significant. The models were then used to determine doses estimated to produce 20% (ED20) and 50% (ED50) effect in order to derive the magnitude of differences between the predicted and actual data.

RESULTS

The mixture doses used here included levels of the individual components that were less than their respective ED10s for brain ChE inhibition. For adults, this included the lowest and next lowest doses of both mixtures, but for pups this included only the lowest dose as all higher doses contained “active” levels of carbofuran (a very potent carbamate in pups). On the other end of the dose response, the highest doses were not severely toxic; tremors, salivation, or lacrimation were not observed in either age group at any time. There were, however, clear effects on all three endpoints, approaching maximal effect in some cases (e.g., RBC ChE inhibition). Thus, the doses effectively bracketed appropriate dose ranges of the individual components.

In adult rats, there were acceptable models for the individual dose-response data for each endpoint. Overall, a three-parameter exponential model had adequate fits and lowest AICc values for most of the chemicals, even if it was not the most preferred for all. Thus, the exp 3 (equation 1) model was chosen to develop the prediction model and to fit the experimental data for both mixtures. For brain ChE, RBC ChE, and motor activity, the worst fits were obtained with carbofuran, carbaryl, and oxamyl, respectively. When relating the mixture proportion to the ED10, oxamyl and methomyl had the highest importance in terms of potency in the environmental mixture; the importance was equal across chemicals in the relative potency mixture (Table 1). Thus, the less than optimal fit for oxamyl may have impacted the motor activity prediction model for the environmental mixture.
In the PND17 rats, the exp 3 model was acceptable for almost all chemicals for the measurements of brain and RBC ChE inhibition. For brain ChE, only oxamyl showed poor fit with this model. On the other hand, motor activity data for several of the chemicals did not show clear, monotonic decreases (Moser et al., 2010); thus, model fits were less consistent and more variable. The exp 2 (equation 2) model was acceptable for five of the chemicals, but the data for formetanate and methomyl did not have any acceptable fits: these were the chemicals that showed inverted U-shaped or flat dose response. The exp 2 model was chosen to fit the predicted dose response for motor activity.

For brain ChE inhibition following exposure of adult rats to both mixtures (Fig. 1), the Wald test of consistency between the prediction model and the modeled experimental data rejected the null hypothesis of dose additivity (relative potency \( p = 0.0311 \), environmental \( p < 0.0001 \)). The experimental data were shifted to the left, indicating a greater than expected response, i.e., synergism. For the relative potency mixture, separate comparisons showed that while all the model parameters were significantly different, only the middle dose group (3.74 mg/kg) was different from predicted. For the environmental mixture, the deviation from dose additivity was due to differences in slope, and doses of 0.6 mg/kg and above were significantly different from predicted.

In rat pups (Fig. 1), the Wald test for the brain ChE inhibition data was not significant for the relative potency mixture, whereas the environmental mixture was different from predicted (\( p < 0.0001 \)) and showed greater than additive response. The deviation from dose additivity in the environmental mixture was due to significant differences in all three parameters, and responses at all mixture doses were significantly lower from predicted.

In the analysis of adult RBC ChE inhibition (Fig. 2), the Wald test for consistency between the predicted and experimental models did not reject the null hypothesis of dose additivity for the relative potency mixture, but it did for the environmental mixture (\( p = 0.0010 \)). Analyses of the environmental mixture did not reveal significantly different parameters at the \( p < 0.05 \) level, although the difference in slopes was suggestive (\( p = 0.0584 \)). The middle dose groups (0.6–2.8 mg/kg) were significantly different from predicted, and the response was greater than predicted. Although the high dose group was not different from predicted, this may reflect maximal inhibition in both the predicted and experimental models.

In contrast to the adults, the Wald test for the RBC ChE inhibition in PND17 rats (Fig. 2) rejected the null hypothesis for both mixtures (relative potency mixture \( p = 0.0242 \), environmental mixture \( p < 0.0001 \)). With the relative potency mixture for RBC ChE inhibition, comparisons of the models did not reveal any significant parameter differences, so while dose additivity is rejected, there is no obvious cause. Only the middle dose groups (0.9, 2.6 mg/kg) were significantly less effective than predicted. Analyses of the environmental mixture revealed that all model parameters were significantly different, and the middle dose groups (0.4–2 mg/kg) showed greater than predicted response. The high dose groups in both predicted and experimental models produced close to maximal inhibition and were not different.

The adult motor activity data (Fig. 3) for the relative potency mixture did not show evidence of deviation from dose additivity, whereas the Wald test for the environmental mixture was significant (\( p < 0.0001 \)) and suggested a greater than additive response. There were marginal differences in the plateau parameter (\( p = 0.08 \)), but all doses of 0.6 mg/kg and greater were significantly different from predicted. In pups, the Wald test rejected additivity for the environmental mixture.
For the environmental mixture, the slope parameters were significantly different, and the highest dose groups (1 mg/kg and greater) were different from predicted. There were generally modest differences between the predicted and actual ED20 values, although the differences were greater in the PND17 rats than in adults (Table 2). For pups and adults, the ratios were at least 1.5-fold or greater for the environmental mixture (indicating synergy), whereas for the relative potency mixture, the mixture responses were similar to or even greater than predicted. In adult rats, the largest degree of synergy was observed with RBC ChE inhibition by the environmental mixture, which showed an ED20 value that was 2.4-fold smaller than predicted. The differences in point estimates for the environmental mixture were somewhat greater in the PND17 rats, with point estimates shifted about two- to almost threefold. The comparisons between predicted and actual ED50 values gave the same profile (data not shown).

### DISCUSSION

This study provides an example of experimental design and statistical analyses appropriate for addressing the hypothesis of dose additivity in a mixture of seven carbamates. This goes beyond early studies of simple binary mixtures. From a statistical standpoint, this approach could be applied to a mixture of any number of components. A limiting factor, however, is that well-defined individual-chemical dose-response data are needed for all the component chemicals. Defined multipesticide mixtures have been evaluated in only a few other studies, including four or five organophosphates (Moser et al., 2005, 2006), 11 pyrethroids (Wolansky et al., 2009), and 15 mixed-class pesticides (Dolara et al., 1994).

The relative potency mixture appeared dose additive for most endpoints at both ages and only showed marginal deviations from additivity for adult brain ChE inhibition (greater than additive, one dose) and pup RBC ChE inhibition (less than additive, middle doses). In contrast, for the environmental mixture, more obvious greater than additive responses were observed in both age groups for all endpoints. This synergism was generally evident at all but the lowest dose tested in adults and pups; however, with PND17

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### TABLE 2

<table>
<thead>
<tr>
<th>Predicted Mixture Ratio</th>
<th>Adult</th>
<th>PND17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative potency mixture</td>
<td>2.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Brain ChE*</td>
<td>3.3</td>
<td>0.50</td>
</tr>
<tr>
<td>Motor activity</td>
<td>1.3</td>
<td>0.44</td>
</tr>
<tr>
<td>Environmental mixture</td>
<td>0.86</td>
<td>0.50</td>
</tr>
<tr>
<td>Brain ChE*</td>
<td>0.80</td>
<td>0.55</td>
</tr>
<tr>
<td>Motor activity*</td>
<td>0.58</td>
<td>0.33</td>
</tr>
<tr>
<td>PND17</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Relative potency mixture</td>
<td>0.50</td>
<td>0.77</td>
</tr>
<tr>
<td>Brain ChE</td>
<td>2.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Motor activity</td>
<td>0.44</td>
<td>0.19</td>
</tr>
<tr>
<td>Environmental mixture</td>
<td>0.19</td>
<td>0.12</td>
</tr>
<tr>
<td>Brain ChE</td>
<td>1.4</td>
<td>0.54</td>
</tr>
</tbody>
</table>

*Indicates that the overall hypothesis of dose additivity was rejected.
brain ChE inhibition, all dose groups were different from predicted. The few instances where the highest dose did not differ from predicted were probably due to the responses converging at the maximal effects. Thus, nonadditive responses were not limited to high doses.

There were age-related differences in terms of the magnitude of synergy, but not in terms of dose-additivity conclusions. Preweanling rats are often more sensitive to ChE inhibitors than adults, which caused us to adjust the total dose levels down to prevent unwanted toxicity; however, the ratios were maintained to allow closer comparisons between the ages. Interestingly, the relative potency mixture showed dose additivity at both ages, even though the relative potency ratios were based on adult, not pup, ED10 values. Comparison of the doses used here and the ED10 values derived from PND17 data (Moser et al., 2010) showed that both mixtures, but especially the environmental, had considerably more carbofuran on a potency-weighted basis (data not shown).

The determination of dose-additivity rests to some extent on the model or approach used in the analysis. A previous analysis of the same relative potency data for adult brain ChE inhibition reported that the mixture effects at each individual dose were within the 95% confidence limits of the predicted response (U.S. EPA, 2007). That approach, however, did not model the mixture data and so could not compare the shapes of the predicted and mixture models. The model choice used may also impact the outcomes, which becomes particularly important where the individual chemicals suggest different dose-response shapes. In these analyses, the candidate models were selected not because of any biological representation but only based on their ability to conform to different variants of descending curves. The use of the preferred model based on goodness of fit and general shape, while statistically justified, may introduce uncertainty in determining the appropriate form for the prediction model. Less than optimal fits may impact the precision of the parameters used in the prediction and/or experimental models. On the other hand, an exponential function is founded in pharmacology and receptor theory and could be used regardless of individual fits; indeed, in this study the exponential was the preferred model for most of the dose-response data. Relatively poor fits may also reflect small sample sizes and number of doses, less than optimal dose selection without a full range of responses, and/or variability in the endpoint. Thus, well-defined dose-response curves are important for the approach described in this article.

Our results with seven carbamates should be compared with caution to in vivo studies using binary carbamate combinations. In previous studies (Laetz et al., 2009; Mwanza et al., 2012), the concentrations of the two carbamates were similar, but in this study the two mixtures differed in the proportions of key components. Specifically, methomyl and carbaryl were present in higher concentrations in the environmental mixture, while methiocarb and propoxur were much less prominent in the environmental mixture compared with the relative potency mixture. This observation may suggest these specific chemicals to be important influences in the deviations from additivity noted with the environmental mixture.

Chemical interactions at metabolic and/or target sites have often been proposed as explanations for deviations from additivity. Previous studies suggest either competition at the enzyme leading to less-than predicted inhibition (Kok and Hasirci, 2004) or competition for other binding or metabolic sites leading to less detoxification and greater-than predicted response (Iyaniwura, 1989; Laetz et al., 2009). Carbamates are metabolized via microsomal CYP enzymes, other esterases, and nonspecific proteins (Tang et al., 2006). One potential site of interaction is saturable binding to carboxylesterases. Studies have shown in vitro as well as in vivo inhibition of
carboxylesterases produced by carbaryl, carbofuran, metho-
myl, oxamyl, and propoxur (e.g., Barata et al., 2004; Costa and
Murphy, 1983; Ehrich et al., 1992; Fossi et al., 1992; Gupta
and Dettbarn, 1993; Gupta and Kadel, 1989, 1990; Parker
and Goldstein, 2000). This binding is biologically significant in
that coadministration of carboxylesterase inhibitors poten-
tiates the toxicity of carbofuran, oxamyl, and propoxur (Costa
and Murphy, 1983; Gupta and Dettbarn, 1993; Gupta and Kadel,
1989, 1990; Parker and Goldstein, 2000). Thus, a relatively
greater influence on carboxylesterases may have resulted from
higher concentrations of oxamyl and methomyl in the environ-
mental mixture, leading to increased toxicity. Unfortunately,
carboxylesterase detoxification for these seven carbamates has
not been systematically compared. Another potential kinetic
factor is differential absorption of the dosing solutions (water,
then oil vehicles) that were administered sequentially, which
could impact the mixture outcomes; however, it was important
that the vehicles matched those used for the single-chemical
dose-response data. The use of physiologically based phar-
macokinetic models holds promise for delineating these possi-
bilities as well as other potential kinetic interactions (Yang
et al., 2004).

The use of a ray design, in which the components remained
fixed across increasing doses, is more efficient than large-scale
full-factorial designs (e.g., Brunden and Vidmar, 1989; Casey
et al., 2004; Gennings, 1995). In particular, the ray mixture
can be adapted to address specific hypotheses or exposures.
Strategies to select specific exposure scenarios include using
ratios that reflect modeled or measured co-occurrence of expo-
sure or monitoring in humans. In our previous study of organo-
phosphates, the ratio of five pesticides was based on dietary
intake estimates (Moser et al., 2005, 2006). Similar approaches
for mimicking environmental exposures have been used by
others. For example, a mixture of four disinfection byproducts
was studied for hepatotoxicity (Gennings et al., 1997), using a
ratio that was based on the average seasonal proportion from 35
water-treatment facilities. In other studies, the thyroid effects
of 18 polychlorinated aromatic hydrocarbons were measured
using a ratio based on average concentrations of the chemicals
found in human breast milk (Crofton et al., 2005), and in vitro
cytogenicity of 15 pesticides were tested using a ratio propor-
tional to their average concentrations in foods in Italy (Dolara
et al., 1994). Considerations such as these maximize the rel-
relevance of the results while limiting the required number of
experimental studies.

In this study, the environmental mixture was considered to be
more environmentally relevant as it estimated actual use, albeit
in one state. There are, however, caveats to this assumption. For
example, California records are based only on agricultural use
and exclude home and garden, and most industrial and insti-
tutional uses. Coexposures to rapidly reversible carboxamides
would need to be almost simultaneous (e.g., within the same
meal). In addition, pesticide sales may not directly correlate
with exposure patterns due to differences in applications and/or
environmental persistence. Furthermore, California data may
not reflect national sales, and as California has large agricul-
tural areas, the pesticide use patterns may be very different in
more urban states. Finally, the environmental mixture reflects a
snapshot of one year, although comparison of sales data across
several years suggests that while exact proportions may differ,
overall the relative numbers have been fairly stable. Thus,
while it may be more difficult to simulate exposures on an exact
geographic and temporal scale, studies such as these can move
the science toward better understanding of influences of these
variables on the overall response.

For most occurrences where the data deviated from dose
additivity, the effects were greater than expected, indicating
synergism. In order to estimate the magnitude of differences
between the predicted and actual responses, the models were
used to derive doses estimated to produce 20% (ED20) or 50%
(ED50) effect to estimate the magnitude of the differences
(Table 2). This approach is consistent with using the ratio of
isoeffective doses used by U.S. EPA and others for estimating
the interaction magnitude (Boobis et al., 2010; Hertzberg et al.,
1999; Moser et al., 2005, 2006). In adult rats, there were gener-
ally less than twofold differences in these values; only the RBC
ChE inhibition estimates for the environmental mixture showed
slightly greater differences. The differences were greater in the
PND17 environmental mixture data, yet these were still less
than threefold. This general degree of synergy, reflecting rel-
atively modest increases in toxicity, was also reported in our
study on organophosphates and is consistent with the magni-
tude reported in other studies (reviewed in Boobis et al., 2011).

In summary, these data illustrate differences in outcomes
based only on the relative ratios of the mixture components.
The data were mostly dose additive with mixture ratios based
on relative potency factors (i.e., hazard-based mixture),
whereas greater-than additive, or synergistic, effects were noted
with mixture ratios based on amounts sold in California (i.e.,
exposure based). These results were similar across endpoints in
both adults and PND17 pups. The magnitude of the synergy was
up to twofold in adults, and up to threefold in pups. Additional
consideration of component proportions would contribute to a
better understanding of cumulative risk.

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REFERENCES


