Neurotoxicological Interactions of a Five-Pesticide Mixture in Preweanling Rats

Virginia C. Moser,*† Jane Ellen Simmons,† and Chris Gennings‡

*Neurotoxicology Division and †Experimental Toxicology Division, National Health and Environmental Effects Research Laboratory/Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina; and ‡Department of Biostatistics, Virginia Commonwealth University, Richmond, Virginia

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The estimation of risk following exposure to mixtures is an important feature of pesticide risk assessment. Also of concern is the potential for increased sensitivity of the young to pesticide toxicity. We have conducted interaction studies using a mixture of five organophosphorus (OP) pesticides (chlorpyrifos, diazinon, dimethoate, acephate, and malathion) in both adult (published previously) and preweanling rats using a fixed-ratio ray design. In the present study, cholinesterase inhibition and behavioral changes (motor activity, gait, and tail-pinch response) were measured in 17-day-old Long-Evans male rats following acute exposure to the OPs. The ratio of pesticides in the mixture reflected the relative dietary exposure estimates projected by the U.S. Environmental Protection Agency Dietary Exposure Evaluation Model. Dose–response data were collected for each OP alone, which were used (alone or in conjunction with the mixture data) to build an additivity model to predict the effects of the pesticide mixture along a ray of increasing total doses, using the same fixed ratio of components. The mixture data (full ray) were similarly modeled and statistically compared to the additivity model along the ray. Since malathion has been shown to produce synergistic interactions with certain OPs, it was of interest to evaluate the influence of malathion in this study. A second pesticide mixture, without malathion (reduced ray), was tested using the same dose levels of the remaining four OPs. Analysis of the full ray revealed significant greater-than-additive responses for all endpoints. The magnitude of this shift ranged from two- to threefold for estimates of the ED$_{20}$ and ED$_{50}$. The deviation from additivity was also detected in the reduced ray for all but two endpoints (motor activity and tail-pinch response); however, for all endpoints, the reduced ray was significantly different from the full ray. Thus, greater-than-additive responses were detected in preweanling rats with this OP mixture, and this effect can only partially be attributed to the malathion in the mixture.

Key Words: mixtures; cumulative risk; neurotoxicity; age susceptibility; chlorpyrifos; acephate; malathion; dimethoate; diazinon.

Many pesticides are used together or in a pattern that results in exposure to multiple pesticides over time, and these exposures may result in unexpected adverse health consequences to the exposed population. Thus, regulations that are based on the toxicity of these pesticides alone may not adequately protect human health from the adverse effects of cumulative exposures. Knowledge of how these compounds may interact and produce effects is required for scientifically based risk assessment of chemicals for which such cumulative exposures are likely. The need for more data on these exposures was highlighted in the 1996 Food Quality and Protection Act (FQPA), which directed the U.S. Environmental Protection Agency (EPA) to consider cumulative (multiple chemicals) and aggregate (multiple routes) exposures in the risk assessment process. Furthermore, the FQPA required that extra caution be taken when setting regulatory levels for chemicals to which children are expected to be exposed. Incorporating these changes into the risk assessment process has presented challenges to the EPA due to the lack of data in these areas, and research is needed to decrease the uncertainties associated with these regulatory decisions.

Early studies of binary pesticide mixtures indicated deviations from the expected additivity response in about half of the pairs tested (e.g., Casida et al., 1963; Cohen, 1984; DuBois, 1961, 1969; McCollister et al., 1959). It is also well known that malathion toxicity is potentiated by organophosphorus (OP) pesticides which inhibit the carboxylesterase (CaE)-mediated hydrolysis of malaoxon (e.g., Cohen and Murphy, 1971a,b, 1974; Frawley et al., 1957; Murphy and DuBois, 1957; Murphy et al., 1959, 1976). More recently, we reported that a mixture of five OPs produced greater-than-additive responses on measures of cholinesterase (ChE) inhibition (brain and blood) and behavior in adult rats (decreased motor activity, and gait changes) (Moser et al., 2005). Tests of this same mixture but without malathion still showed greater-than-additive responses on most measures, although it was evident that malathion played a role in the magnitude of the interaction (Moser et al., 2005). We suggested that detoxification factors may influence this synergy, i.e., a kinetic interaction.

Over the past decade, considerable research has been directed toward defining age-related sensitivity differences to
a number of pesticides, including OPs (recently reviewed in Vidair, 2004). It has been suggested that for some OPs, kinetic factors, e.g., detoxification pathways, play a greater role in age-dependent susceptibility than do other factors, such as differences in the target enzyme (Benke and Murphy, 1975; Brodeur and DuBois, 1967; Chanda et al., 2002; Mortensen et al., 1996). Since the age-related differences in sensitivity may be greatly influenced by the development of certain detoxification systems, these differences between young and adult rats may also influence the interactions that occur following exposure to multiple OPs.

This current study expands our findings in adult rats by testing the OP mixture in preweaning rat pups. OP pesticides were chosen for study due to their widespread agricultural use, established common mode of action (inhibition of acetylcholinesterase; Mileson et al., 1998), and our experience with evaluating their effects in both young and adult rats (Moser 1999, 2000a). The class of registered OPs has been subject to cumulative risk assessment as mandated by FQPA for pesticides that share a common mechanism, which assumes additivity in response (USEPA, 2002). We expected that the greater-than-additive responses that we observed in adult rats would also occur when testing the mixture in young rats. Our hypothesis was that since responses we observed in adult rats would also occur when testing the mixture in young rats. Our hypothesis was that since young rats are more sensitive to at least some of the components in the mixture, probably due to kinetic differences, then the magnitude of the interaction would be greater as well.

**METHODS**

**Chemicals.** Chlorpyrifos (O,O-diethyl-O-[3,5,6-trichloro-2-pyridyl]phosphorothioate; purity, 99.2–99.9%), acephate (O,S-dimethyl acetylphosphorothioate; purity, 98.7%), diazinon (O,O-diethyl-O-[2-isopropyl-4-methyl-6-pyridimyl]phosphorothioate; purity, 99.2–99.4%), and malathion (S-[1,2-dicarboxyethyl]-O,O-dimethyl dithiophosphate; purity, 98.2–99.5%) were obtained from Chem Service (West Chester, PA) and dimethoate (O,O-dimethyl-S-[N-methylcarbomoylmethyl]phosphorodithioate; purity, 98.7%) was a gracious gift from Cheminova (Lemvig, Denmark). Acetate was dissolved in deionized water. The other pesticides were dissolved in a vehicle of corn oil which contained 5% ethanol as this was required to dissolve the mixture-dose levels. Actual mixture doses and pesticide proportions were obtained from DuPont/NEN Life Science Products (Boston, MA) and unlabeled acetylcholine iodide from Sigma (St Louis, MO). All other reagents were obtained from commercial sources and were of the highest available grade.

**Animals.** Timed-pregnant Long-Evans rats were obtained from Charles River Laboratories (Raleigh, NC) and individually housed on heat-treated pine shavings with feed (Purina Rodent Chow 5001) and water (filtered tap) freely available. Day of birth was considered postnatal day (PND) 0, and the pups were randomly culled across litters (pups combined and randomly redistributed across dams) to six males and two females on PND4. Only male pups were used in this study. All male pups within a litter were dose, with no more than one pup within a litter receiving the same dose. The animal facility was accredited fully by the Association for Assessment and Accreditation of Laboratory Animal Care International and maintained at 70 ± 2°F, 50 ± 10% humidity, with a 12-h light/dark cycle.

**Behavioral testing.** The rat pups were tested using the same neurobehavioral endpoints used in the adult OP mixture study (Moser et al., 2005). Upon removing the pup from the cage, the observer noted the presence of miosis, mouth smacking, salivation, or lacrimation. The rat was then placed in an open field for observation, and tremors, gait abnormality, and the response to a tail pinch were scored, using criteria previously described for preweaning rats (Moser, 2000b). Motor activity was then measured during a 30-min session, using an automated chamber shaped like a figure eight (Reiter, 1983). The same observer conducted all studies and was unaware of the dose level of each rat.

Immediately after the motor activity assessment, rats were decapitated quickly under CO₂-induced anesthesia. The brain was rapidly removed, and trunk blood was collected in heparinized tubes. All tissues were stored at −80°C until the time of assay.

**ChE assay.** A radiometric assay was used to determine brain and blood ChE activity (Johnson and Russell, 1975). Both tissues were diluted in two volumes of 0.1M sodium phosphate buffer (pH 8.0) with 1% Triton X-100, followed by homogenization (brain only) for 30 s (Polytron homogenizer, Kinematica Model PT3100, Littau, Switzerland). The final acetylcholine iodide concentration was 1.2mM.

**Chemical treatments.** Single-chemical dose–response data were collected, followed by the mixture dose–response studies. Male pups were dosed on PND17. Following the same procedure as with the adult rats, neurobehavioral testing began 3.5 h after dosing, and rats were euthanized between 4.25 and 4.5 h after dosing. The acetate doses were administered by oral gavage at 1 ml/kg and the corn oil vehicle doses at 2 ml/kg. For the mixture study, the rats had to be gavaged twice. The water-dosing vehicle (acephate) was given first, followed immediately by the corn oil/ethanol-dosing vehicle (containing the other pesticides). The single-chemical dose–response curves included at least five dose levels plus control, with n = 10 per dose group. The mixture studies were conducted using six dose levels of each mixture plus control. In each mixture study, a single-dose group of each of the individual chemicals was included to assure that the chemical response was the same as that predicted from the earlier data (positive controls). Thus, the mixture study included six mixture-dose groups and control (n = 12 per dose group), and a single-dose level of each chemical (n = 8 per chemical).

Dietary exposure was estimated using the Dietary Exposure Evaluation Model (DEEM-FCID) software (US EPA, 2001). This is a probabilistic analysis conducted by combining representative data on concentrations of OP pesticides on foods, with distributions of anticipated consumption of these foods (US Department of Agriculture’s Continuing Survey of Food Intake by Individuals) by different segments of the U.S. population. The 95% exposure value of the general population was used for all pesticides. The proportions of the chemicals in the mixture were chlorpyrifos 0.031, acephate 0.040, diazinon 0.002, dimethoate 0.102, and malathion 0.085.

The mixture based on this composition was considered the “full ray.” The mixture-dose levels ranged from 10 to 165 mg/kg, and the pesticide proportions (mixing ratio) remained the same across dose levels. Actual mixture doses and their components are listed in Table 1. The second mixture (reduced ray) did not influence the interactions that occur following exposure to multiple OPs.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Doses (mg/kg) of Each Pesticide Comprising the OP Mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Full ray</td>
</tr>
<tr>
<td>10</td>
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<tr>
<td>20</td>
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<tr>
<td>40</td>
<td>7.0</td>
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<tr>
<td>60</td>
<td>10.5</td>
</tr>
<tr>
<td>100</td>
<td>17.5</td>
</tr>
<tr>
<td>165</td>
<td>28.9</td>
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</tbody>
</table>

*Malathion was not included in the reduced ray.
not contain malathion but did have the remaining four OPs at the same dose levels and therefore the same relative ratios as in the full ray. The reduced ray mixture-dose levels ranged from 1.8 to 28.9 mg/kg (Table 1).

Statistical methods. Data for eight behavioral endpoints were collected, but only three (motor activity, gait abnormality, and tail-pinch response) along with the ChE measurements, were analyzed using the additivity models. Gait abnormality and the tail-pinch response were ranked, but for these analyses, the data were converted to a binary response (i.e., presence or absence of gait abnormality and normal or decreased tail-pinch response). To constrain the probability of a response to be between 0 and 1, a logit link function was used. Total activity counts during the motor activity session as well as blood and brain ChE activity were continuous variables. Control values for blood ChE and motor activity did not differ statistically across studies, so they were combined for subsequent analyses. However, because of differences in control values across studies, brain ChE activity was normalized as a proportion of the respective study control value and analyzed as such.

The additivity model is based on either the single-chemical dose–response data or the single-chemical and mixture data combined and a definition of dose additivity (e.g., Berenbaum, 1985). If the observed response along the fixed-ratio mixture ray is more extreme than that predicted under additivity, then it is reasonable to claim a greater-than-additive response (synergism); if the response is less extreme than that predicted under additivity, a less-than-additive response (antagonism) can be claimed; otherwise, the curves are coincident and departure from additivity cannot be claimed for the mixture ray considered.

The development of the methods for these data is presented in several papers (Casey et al., 2004, 2005; Gennings et al., 2004b; Moser et al., 2005). When the data supported the hypothesis of a common maximum effect (plateau) for each of the pesticides in the mixture, the “single chemical required” (SCR) method of analysis (Casey et al., 2004) was conducted to test the hypotheses of additivity. When the data demonstrated different plateau levels, the “flexible SCR” method of analysis (Gennings et al., 2004a) was used. A threshold additivity model (Gennings et al., 1997) was used where possible with the SCR analysis, but if the data fit a threshold, or breakpoint, outside the experimental range, then the corresponding generalized linear model or nonlinear model was used.

The SCR methodology uses the single-chemical dose–response data to develop a model that predicts the response of the mixture ray under additivity. The flexible SCR method includes both the single-chemical and the mixture data, but only the single-chemical parameters in the model predict the response of the mixture under additivity. The experimental mixture data along the fixed-ratio ray were fit to a similarly parameterized model. Before testing for departure from additivity, a goodness-of-fit test was performed on the model to ensure that it adequately fit the mixture data. In the event of significant lack-of-fit, higher order terms were added until an appropriate model was found for the mixture data. In the SCR analysis, the test of additivity is a test of coincidence of these two models along the specified fixed-ratio mixture, and Wald-type tests were used to test this hypothesis of additivity. Essentially, an overall test of additivity was used to determine if the single-chemical parameters adequately described the mixture data, and if not (at p < 0.05), subset analyses were conducted to test for significant interactions on each ray alone.

Preliminary analysis of the single-chemical data for brain and blood ChE provided evidence that there was some difference in the plateau (asymptote) levels of the five pesticides. Thus, the flexible approach described in Gennings et al. (2004a) was used to estimate an additivity model and to predict along the specified fixed-ratio rays. A likelihood ratio test, as described in Gennings et al. (2004a), was used to test whether the mixture data are adequately described by only single-chemical parameters and a constraint of additivity (i.e., planar contours of constant response).

Another approach to test the hypothesis of additivity with the “flexible” model uses a likelihood ratio test to compare the additivity model with the sample means at each of the mixture-dose groups. This was accomplished one ray at a time. When the overall test of additivity on each ray was rejected, single degree of freedom tests were conducted at each dose group, i.e., a “means mixture model” (with p < 0.05 per comparison) (Crofton et al., 2005).

The full and reduced rays were compared using the method described by Casey et al. (2004), which required correcting the curve from the full ray so that both rays fall in the same dose range. That is, the effect of removing malathion from the mixture was tested by comparing the dose–response curves for the two rays while noting that under the hypothesis that removing malathion does not alter the shape of the dose–response curve, the slope parameter for the reduced ray is equal to the slope parameter for the full ray × the factor (1 – a5) for a5 = 0.825, the proportion of malathion in the full mixture (see Casey et al., 2004, for details). When a difference is found between the full and reduced rays in the appropriately corrected comparison, then evidence for the effect of malathion in the interaction of the five pesticides can be claimed.

The estimation of these nonlinear models was done in SAS version 8.2 using the maximum quasi-likelihood criterion in a Nelder–Mead direct-search algorithm embedded in the nonlinear programming procedure in SAS, the Gauss–Newton iterative algorithm in PROC NLIN, and a Fisher scoring algorithm in PROC GENMOD.

The magnitude of change is important for understanding the biological significance of nonadditivity. For purposes of illustration, point estimates were calculated from the equations using the predicted additivity model and the actual mixture model, and the ratio of these estimates can indicate the magnitude of difference. Doses were calculated that (1) produced a 20% (ED20) or 50% (ED50) change from control (ChE activity or activity counts) or (2) produced an abnormal response in 20% (ED20) or 50% (ED50) of the treatment groups (gait score and tail-pinch response). When the SCR method of analysis was used (motor activity, gait, and tail pinch), the predicted ED20 and ED50 values were obtained directly based on the estimated additivity model, whereas when the flexible approach was used (brain and blood ChE), the values were indirectly estimated from the additivity model using only single-chemical data and the constraint of additivity. Note that these values are meant only to show the relationship between the additivity and mixture data, and no confidence limits are provided.

RESULTS

No lethality occurred during any of the chemical testing. For the pesticides tested individually, only a few signs of cholinergic toxicity were observed; however, this was not the case with the mixture-treated rats (see below). Prediction intervals (data not shown) were estimated for each of the single-chemical dose–response curves which showed similarity to the positive control data (shown as filled triangles in Figs. 1 and 3) from the mixture studies. Thus, there was no significant evidence of a shift in the dose–response curves across studies or that the double-dosing procedure used in the mixture study influenced the outcomes.

Brain ChE

Figure 1 provides dose–response data for brain ChE inhibition produced by each of the five pesticides. The plots for two pesticides provide an indication of a threshold within the experimental region: these were chlorpyrifos (threshold estimate, 1.5 mg/kg; 95% confidence limits, 1.1–1.8) and malathion (83.5 mg/kg; 95% confidence limits, 70.0–96.9). The dose–response curves for the other three pesticides drop even at the lowest dose group tested.

The data indicated different plateaus (i.e., maximal inhibition) for the different pesticides (Fig. 1), as well as the mixtures (Fig. 2). The full ray mixture had the lowest plateau
(estimated at 7.4% of control), while diazinon and dimethoate had the highest plateau (32.1% of control). The reduced ray had a plateau similar to that of acephate, chlorpyrifos, and malathion, which were not significantly different (22–26% of control).

As seen in the mixture dose–response data in Figure 2, there was a significant departure from additivity on both the full and reduced rays ($p < 0.001$ for each). The model fit to the actual mixture data falls below the model estimated under an assumption of additivity for both fixed ratios, indicating evidence of a nonadditive interaction (synergy). The hypothesis that malathion had no effect in the mixture was also rejected ($p < 0.001$), indicating that the presence of malathion had an impact on the dose responsiveness of the other four pesticides in the mixture. The full and reduced rays were different from each other, and the deviation from predicted appeared to be greater with the full ray.

**FIG. 1.** Individual dose–response data for brain ChE, presented as a percentage of control activity. Filled circles indicate data from the dose–response studies, and filled triangles indicate data from the positive control groups during the mixture studies. Line shows the fitted curve.
Using the means model for the mixture data, the overall test of additivity was again rejected ($p < 0.001$). The change in the parameter estimates for the single-chemical dose–response curves was large enough that all mixture-dose means were significantly lower (all $p$ values $< 0.001$) than the predicted model. In other words, at all dose levels, the mixture-dose means were lower than predicted, indicating that the deviation from additivity was significant even at the lowest dose tested (10 mg/kg).

The $ED_{20}$ and $ED_{50}$ values predicted for the full and reduced rays are presented in Table 2. The shift in effective doses for inhibiting brain ChE was about twofold for the full ray and somewhat less for the reduced ray.

### TABLE 2

<table>
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<tr>
<th>Endpoint</th>
<th>Predicted</th>
<th>Actual</th>
<th>Ratio</th>
<th>Predicted</th>
<th>Actual</th>
<th>Ratio</th>
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<td>83.2</td>
<td>3.5</td>
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</table>

$^a$No significant difference between predicted and actual rays.

### Blood ChE

The individual dose–response data for blood ChE are presented in Figure 3. As with brain ChE, the data provided indication of a threshold for malathion (27.1 mg/kg, 95% confidence limits, 8.9–45.3).

The full ray mixture had the lowest plateau (0.2% of control), while diazinon and dimethoate had the highest (14.9% of control). The reduced ray had a plateau similar to that of acephate, chlorpyrifos, and malathion (3–9% of control) (see Figs. 3 and 4).

The additivity models predicted by the individual dose–response curves are shown in Figure 4, along with the models fit to the experimental mixture data. For both the full and reduced rays, the model fit to the mixture data falls below the model estimated under an assumption of additivity, and the likelihood ratio tests of additivity were rejected ($p < 0.001$). Comparison of the two mixture rays showed that they were significantly different from each other ($p < 0.001$). Ratios of the $ED_{20}$ and $ED_{50}$ values indicated that the full ray showed slightly less than a twofold shift, while the reduced ray had ratios slightly more than 2. As with brain ChE, the means mixture model indicated significant differences between the mixtures and predicted means at all dose levels (all $p$ values $< 0.001$).

### Motor Activity

Overall, the group data for motor activity were quite variable, but the fitted dose–response curves showed negative slopes for all except diazinon. Thus, the diazinon slope was
removed from the model. None of the single-chemical data provided evidence of a threshold dose significantly different from zero.

While the overall test of additivity was rejected ($p < 0.001$), the test of additivity was rejected on the full ray ($p < 0.001$) but not on the reduced ray ($p = 0.168$). These models are shown in Figure 5. There is evidence that malathion interacts with the remaining chemicals, and the full ray was significantly different from the reduced ray ($p < 0.001$). As indicated in Table 2, the magnitude of difference for the full ray was greater at the ED$_{50}$ (ratio of 2.6) than at the ED$_{20}$ (1.3 ratio) value.

**Gait Score**

Gait abnormalities were observed in a dose–responsive manner with all five pesticides, but the maximal incidence
did not reach 100% for diazinon (60% at the high dose) or dimethoate (40% at the high dose). The estimates for dose thresholds were outside of the experimental regions for all pesticides.

The mixture data are presented in Figure 6. The hypothesis of additivity along each fixed-ratio ray was rejected on both rays (both $p$ values $< 0.001$), and the mixtures were associated with a greater-than-additive interaction. The comparison of the full and reduced rays also showed significant differences ($p = 0.002$), indicating that malathion is associated with the interaction. The ratio of point estimates for the full ray was somewhat greater (threefold) than that for the reduced ray (2.2-fold) (Table 2).

**Tail-Pinch Response**

The tail-pinch response (incidence of abnormal lowered response) data for diazinon and dimethoate were not dose responsive (no effects throughout the dose range), and their slope parameters were removed from the model. The remaining three pesticides showed a positive slope, but the responses were not seen in all the pups, even at the highest doses (maximal incidence of 50% for acephate, 60% for chlorpyrifos, and 30% for malathion).

The mixture data are presented in Figure 7, and the predicted models for both rays did not reach the maximum effect. The hypothesis of additivity along the full mixture ray was rejected ($p < 0.001$) but not along the reduced ray ($p = 0.247$).
The difference in ED$_{20}$ and ED$_{50}$ values was about 3.5-fold (Table 2). The test for an interaction due to malathion was also rejected ($p < 0.001$), indicating that malathion is associated with the interaction.

**Other Endpoints**

Miosis, mouth smacking, salivation, lacrimation, and fine tremors, indicative of cholinergic overstimulation, were seen only in the pups treated with the mixtures. The incidence of these signs is listed in Table 3 for the full and reduced rays. In general, these signs were not observed with any of the individual pesticides, except at doses much higher than those used in the mixtures. Therefore, Table 3 indicates the incidence of cholinergic signs at the doses for individual chemicals corresponding to the doses used at each level of mixture.

Statistical analyses were not conducted for these endpoints, so it is not possible to say whether these findings represent significant deviations from additivity, however suggestive it may be. There was essentially no difference between the full and reduced rays in the dose–response data for miosis. The reduced ray produced a higher incidence of smacking at the high dose but a lower incidence of tremors, compared to the full ray. Finally, the reduced ray did not produce salivation or lacrimation, even though both were seen with the full ray.
DISCUSSION

There were significant interactions, with evidence of departure from additivity (greater-than-additive responses, i.e., synergy), among the five pesticides along the full ray for all endpoints in this study. With the reduced ray, only the gait score and brain and blood ChE data were associated with departures from additivity. For all endpoints, the full and reduced rays were significantly different from each other, suggesting an influence of malathion. In addition, cholinergic signs (especially miosis, mouth smacking, and tremors) were clearly evident in the mixture groups, even though no such signs were observed at the corresponding levels of the individual pesticides. Such cholinergic effects corroborate the increased ChE inhibition detected in brain and blood. These findings are reminiscent of the synergy reported with the same mixture in adult rats (Moser et al., 2005). In that study, the full ray showed greater-than-additive responses for all but the tail-pinch response, and the reduced ray also deviated from additivity for ChE inhibition and motor activity.

While the largest difference in point estimates for the adults was twofold, the present data showed that the dose–response was shifted by factors up to 3.5. This difference could be due to the increased sensitivity of the young to some of these pesticides in these dose ranges. There is considerable literature on age-related differences with chlorpyrifos, diazinon, and malathion (e.g., Benke and Murphy, 1975; Moser, 1999, 2000a; Moser and Padilla, 1998; Padilla et al., 2004), but less is known regarding dimethoate and acephate. However, the higher sensitivity of the young was accounted for in the present study by using lower doses of the specific pesticides, and indeed the range of biological responses measured was similar to that of the adults.

Many steps along the metabolic/detoxification pathways of these OPs can be different in the young. The pesticides in this mixture are metabolized via liver hydrolysis to active (and more potent) metabolites (Hussain et al., 1985; Pond et al., 1995; Pope, 1999). Detoxification involves the B-esterases (e.g., CaEs), A-esterases, and microsomal enzymes which bind to and/or hydrolyze these pesticides (Aldridge, 1953; Chambers et al., 1994; Jokanović et al., 1996; Maxwell, 1992). All these pathways are less well developed in the young, and maturation of these systems tracks the decreasing sensitivity to acute exposure to chlorpyrifos and other OPs (e.g., Atterberry et al., 1997; Benke and Murphy, 1975; Brodeur and DuBois, 1967; Chanda et al., 1997, 2002; Moser et al., 1998; Sterri et al., 1985). We have suggested that the interactions in adult rats are due, at least in part, to kinetic factors (Moser et al., 2005). Given the importance of these metabolic and detoxification routes and considering the lesser activity of such in the young, we expected that the magnitude of synergy would be greater. Our findings somewhat confirm that hypothesis, lending credence to the kinetic theory; follow-up studies of tissue levels and analysis of detoxification pathways would be needed to further test this theory. However, the magnitudes of the differences were not striking, and given the lack of confidence limits on the point estimates, they may not be significantly different.

It is important to note that these ratios represent estimates, without confidence limits, and could be modified by the different analyses used (SCR vs. flexible SCR). Gennings et al. (in press) illustrated the impact that inclusion of the mixture data, as is done in the flexible SCR model, can have on the predicted response under additivity. The SCR method of analysis uses only single-chemical data in the development of the additivity model; mixture data are not included. In contrast, the flexible SCR model used both single-chemical and mixture data in the development of the additivity model, with only the single-chemical parameters being used to describe the predicted fit under additivity.

There were subtle differences in the biological responses between the young and adults (Moser et al., 2005). In the

<table>
<thead>
<tr>
<th>TABLE 3</th>
</tr>
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<tbody>
<tr>
<td>Incidence (% of subjects per group) of Cholinergic Signs in Rats Treated with the Full and Reduced (red.) Mixtures, as well as Individual (indiv.) Components</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Miosis</th>
<th>Smacking</th>
<th>Tremors</th>
<th>Salivation</th>
<th>Lacrimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>8</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
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<td>8</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>42</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>100</td>
<td>17*</td>
<td>33</td>
</tr>
</tbody>
</table>

* Effect of 5 mg/kg chlorpyrifos.

Note. Doses are indicated by 0 (control) and 1–6 representing increasing dose.
present study, ChE inhibition clearly reached a plateau (maximal inhibition) which varied for the different pesticides. This finding forced us to use the flexible SCR method of analysis, which allows for such differing plateaus (Gennings et al., 2004a). The pattern was consistent between both brain and blood ChE, in that the full ray showed the lowest plateau, dimethoate and diazinon had the highest plateau, and the reduced ray and other pesticides were intermediate. On the other hand, the brain ChE activity in adults dropped to a minimum of about 20–25% of control in all the adult dose–response data, and blood ChE was decreased to about 5% of control. Since the plateau reflects inhibition as well as resynthesis of the target enzyme, this difference may be due to the faster enzyme turnover in the young as well as differences in the rate of reactivation (e.g., Moser and Padilla, 1998). In addition, Mortensen et al. (1996) showed that the inherent sensitivity of the enzyme did not differ for several anticholinesterases, including two (chlorpyrifos oxon and malaoxon) that were used in this mixture. Tissue levels of the pesticides would be useful in resolving this question.

An advantage of the fixed-ratio ray design is that environmentally relevant combinations can be evaluated in an efficient manner. In the present study, we used the ratio predicted by the DEEM analysis for the general population (95th percentile). This was the same ratio used in our adult rat study (Moser et al., 2005) in order to allow direct comparisons. While these doses may be higher than environmental exposure, the relative proportions are more relevant to potential human exposure. Since these outcomes are specific for the ratio tested, it would be of interest to conduct a similar study using proportions specific for the younger population (e.g., infants and children).

In summary, this study showed greater-than-additive responses for blood and brain ChE, motor activity, gait abnormalities, and depression of a tail-pinch response following acute exposure to a mixture of five OPs. In all cases, the magnitude of interaction was only about two- to threefold. Tests of the same mixture but without malathion showed additivity for motor activity and the tail-pinch response but not the other endpoints. Thus, the nature of the interaction was similar across endpoints, with malathion playing a role but not being the critical factor in the response.

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REFERENCES


