Appendix II.B.5

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Preliminary N-Methyl Carbamate Pesticides Cumulative Risk Assessment

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a. Introduction

The following text provides the information mentioned in the Hazard/RPF chapter of the Preliminary Cumulative Risk Assessment of the NMCs.

The following topics are covered in this Appendix:

- information provided in standard operating procedures for the measurement of tissue and blood ChE activity;
- investigation of reactivation in sampling handling and AChE analysis during EPA's time course and dose-response experiments;
- summary of motor activity experiments performed in EPA's dose-response experiments and;
- summary of protocol and preliminary results of seven chemical (equipotent) mixture experiment.

b. Comparison of Submitted Protocols used for the Measurement of Tissue and Blood ChE Activity in Rats

Table III.B.5-1 provides a summary of the ChE measurement protocols submitted for registration studies to EPA[1]. The protocols available at present time indicate that the experimental conditions among laboratories vary but that dilutions are generally limited to approximately 1:20 and that samples are frozen immediately. A reagent test kit from Boehringer Mannheim was indicated in three of the protocols. The Hitachi 717 was indicated in three protocols. Although information regarding the time of sample handling is more limited, the available information suggests that reasonable precautions were taken in these studies to reduce reactivation prior to analysis.

[1] SOPs for pirimicarb were provided to EPA on 7/25/2005. The evaluation of pirimicarb SOPs will be included in the revised risk assessment.

Table II.B.5-1. Comparison of the submitted protocols for the *N*-methyl carbamates used in measuring ChE activity in tissue and blood.

SOP Details	Methomyl	Oxamyl	Formetanate	Carbofuran
Mrids	44487501, 44472001	44254401, 44420301, 44472001	not indicated	45675701
Tissue	Rat RBC, tissue	Rat RBC, tissue	plasma, whole blood, brain	whole blood, plasma or serum, RBC, brain
Test kit	BMD ChE Reagent Set #124117	BMD ChE Reagent Set #124117	BMC ChE kit #124117	None, Reagent Recipe given
Instrument	Hitachi 717	Hitachi 717	Hitachi 717	Gilford Instruments System 2600
Anticoagulant	EDTA	EDTA	Heparin	EDTA
Centrifugation speed	speed, time, temp not indicated	speed, time, temp not indicated	plasma: 800 g 10 minutes	1600 g for 10 min @ 0-5°C
Centrifuge refrigerated?	not indicated	not indicated	Yes	0-5°C
Dilution	1:20 Triton	1:20 Triton	not specified Sorenson's buffer	1:20 Triton X (ice-cold)
Temp	tissue frozen -70C, RBC samples not indicated	tissue frozen -70C, RBC samples not indicated	not specified	stored at -72C until assay; assay at 30C
Sample on ice	tissue sample snap frozen if not analyzed immediately; RBC not indicated	tissue sample snap frozen if not analyzed immediately; RBC not indicated	yes, record time of sample receipt in lab	on ice until delivered to lab; processed samples frozen until analysis
Time limitation for sample life	not indicated	not indicated	2 hrs, time of sample and analysis	thawed RBC sample within 30 minutes.
Time of receipt of sample?	not indicated	not indicated	yes	record of time at each step in processing
# and Time of a sample batch for processing	not indicated	not indicated	30 max at a time, record of time of steps in process	24 samples in a batch, ~1 hr till frozen
Time from sample drawn to sample assayed	not indicated	not indicated	max time not indicated	RBC: 1 to 7 days

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c. Investigation of Reactivation in Sampling Handling and AChE Analysis

In response to questions raised by the SAP in February, 2005 concerning the *in vitro* reactivation of the cholinesterase activity in the samples from the *N*-methyl carbamate-treated animals in NHEERL experiments, the Agency conducted a series of experiments.

On March 27, 2004, we conducted the initial assessment of the level of cholinesterase activity in brains from animals treated with different dosages of oxamyl. We used only ½ of the brain (sectioned down the middle, sagitally) for this assessment. The other ½ of the brain was kept in the -80°C freezer frozen (without homogenization). About one year later on April 22, 2005, we assessed the activity in the other half of those brains under various conditions:

① Is reactivation caused by storage in a freezer for one year? The same conditions were used for this assay as were originally used for cholinesterase activity assessment to determine if storage for a year had caused reactivation of any activity. (Diluted 1:3 during homogenization and then immediately analyzed for cholinesterase activity using the radiometric assay).

② Is reactivation promoted by dilution of the homogenate? The homogenate (1:3 dilution) was immediately diluted again to create a total dilution of 1:9 and the cholinesterase activity was assessed using the radiometric assay. This was to determine the effect of dilution on the reactivation of carbamate-inhibited cholinesterase activity.

③ Is reactivation promoted by allowing the tissues sit on ice for 1.5 hours? The homogenate 1:3 was allowed to sit on ice for 1.5 hours before assessment of activity. This was to determine the effect of time on the reactivation of carbamate-inhibited cholinesterase activity.

④ Is reactivation possible in these tissues? The homogenate (1:3) was subjected to a marked dilution (1:40) and increase in temperature (37°C) to measure the amount of reactivation that was possible in these samples over a period of 12 minutes.

The results of all four of these experiments are presented in Figure II.B.5-1 below. Storage of the tissue in the freezer for one year before analysis did not appear to differ from the original dose-response assessment. Allowing the homogenized tissue to sit for 1.5 hours before analysis produced slight (≤5%) reactivation in the 0.5 and 1.0 mg/kg dosage groups, but no reactivation in the highest (1.5 mg/kg) dosage group. Dilution of the tissue, however, had much more of an effect on the level of cholinesterase reactivation with a 7-10% increase in activity, especially at the dosage groups with more inhibition). Therefore it has to be assumed that this level of reactivation does occur during the initial 1:3 homogenization, but is unavoidable as the homogenization process is necessary for further handling of the tissue. Finally, if the carbamate-inhibited tissue is encouraged to reactivate by substantial dilution of the

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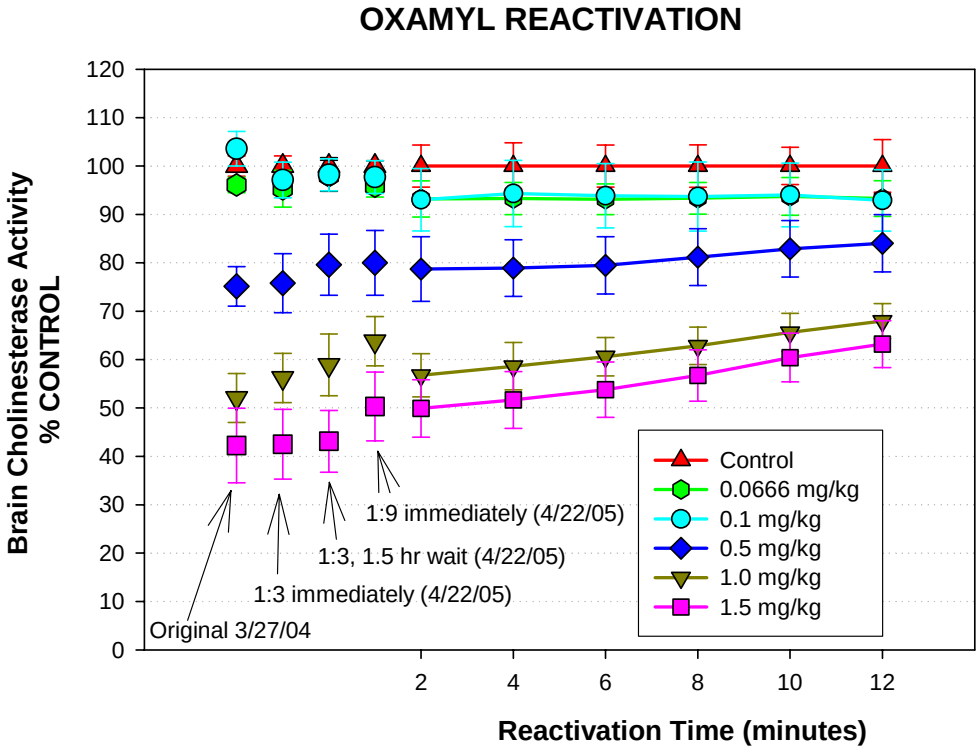
tissue and increases in temperature, one can see a progressive gaining of cholinesterase activity in all dosage groups that exhibited cholinesterase inhibition.

Figure II.B.5-1. Reactivation experiment with oxamyl



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d. Summary of Motor Activity Experiments Performed in EPA's Dose-Response Experiments

As part of EPA's dose-response studies, the acute neurobehavioral effects of *N*-methyl carbamate insecticides have been systematically compared. The results of neurobehavioral effects have been compared graphically to the results of AChE measurements. The use of an automated assessment of motor activity along with ChE activity measurements allows a functional outcome that can be directly related to the ChE inhibition in RBC and brain. The results presented below were presented at the March, 2005 meeting of the Society of Toxicology (Moser et al, 2005).

We evaluated all seven *N*-methyl carbamates as described in USEPA, 2005. Adult male Long-Evans rats (n=10/dose) were dosed by oral gavage with either carbaryl (3-50 mg/kg), propoxur (0.3-20 mg/kg), oxamyl (0.07-1.5 mg/kg), methomyl (0.1-2.5 mg/kg), methiocarb (0.5-25 mg/kg), formetanate (0.1-5 mg/kg), or carbofuran (0.1-1.5 mg/kg). Ten minutes after dosing, rats were scored for obvious signs of cholinergic toxicity ("tox score"). Horizontal and vertical activity were tabulated during subsequent 20-minute sessions in a figure-eight chamber. Brain and blood tissues were taken immediately thereafter to determine ChE activity as described above.

As shown in Figure II.B.5-2, exposure to each of the NMCs resulted in decreased motor activity. The vertical activity dose-response curves were significantly different from horizontal activity, with greater decreases in vertical activity at the higher doses. Nonetheless, the correlation between horizontal and vertical activity was good, indicating a high concordance of effect on both measures. Within-subject regressions indicated a better correlation between motor activity and brain ChE inhibition than with blood ChE. Brain ChE is equally or more sensitive at the low end of the dose-response.

The ranking of toxicity as measured by the "tox score" ranged from no effect (carbaryl, formetanate) to a dose-related increase in the incidence and severity (propoxur, carbofuran). Thus, some NMCs showed pronounced effects on motor activity at doses that produced little or no obvious toxicity as evaluated by this clinical observation. This illustrates that observable signs of cholinergic toxicity are not always predictive of the magnitude of behavioral neurotoxicity. In contrast, motor activity is a sensitive endpoint that reveals the biological relevance of ChE inhibition produced by these *N*-methyl carbamates.

For some NMCs, data are available for motor activity and clinical signs in studies submitted for pesticide registration. <u>The Agency has not yet extracted the motor activity data from registration studies for comparison with EPA's behavioral data or for formal comparison with ChE data</u>. However, evaluation of summary information provided in EPA's study reviews (ie, Data Evaluation Records, not provided here) indicates that ChE is equally or more sensitive than behavioral effects.

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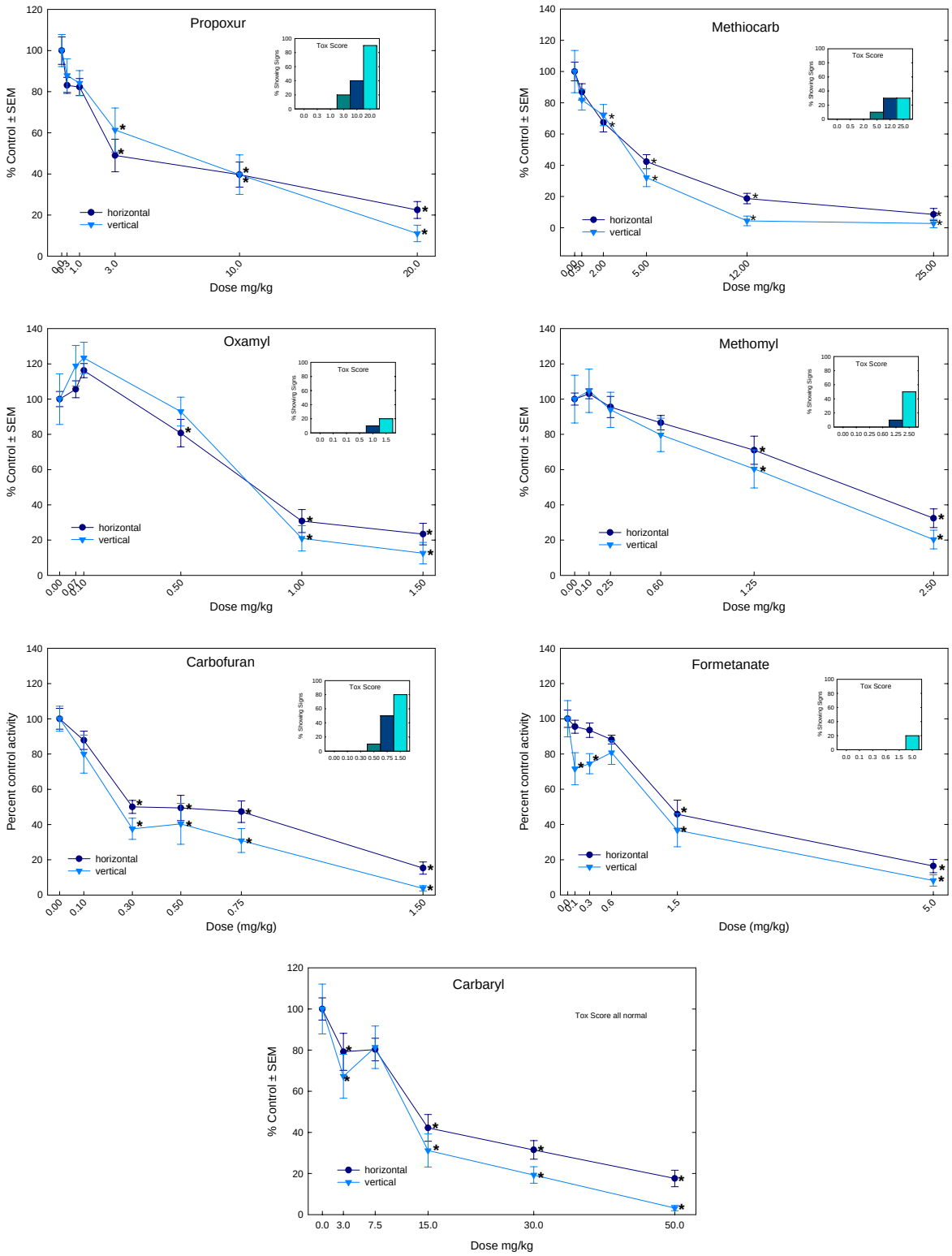

Figure II.B.5-2. Plots of motor activity data from NHEERL experiments for seven *N*-methyl carbamates

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e. Summary of Protocol and Preliminary Results of Seven Chemical (Equipotent) Mixture Experiment

A mixture study was conducted using 7 *N*-methyl carbamates (carbaryl, carbofuran, formetanate, methiocarb, methomyl, oxamyl, and propoxur). A dose-additive experimental design was used and the proportion of the carbamates in the mixture was based on their potency: they were combined in an equipotent manner using the individual-chemical benchmark values as the point of comparison. The composition of the mixture is shown in Table II.B.5-2.

Table II.B.5-2. Composition of a seven chemical mixture with the NMCs

***N*-Methyl Carbamate Pesticide**	**Percentage of Mixture**
Carbaryl	41.60%
Carbofuran	1.46%
Formetanate	1.63%
Methiocarb	19.60%
Methomyl	5.05%
Oxamyl	1.49%
Propoxur	29.10%

The mixture study consisted of 13 dosage groups with 10 male, Long-Evans rats (90 days old) in each group. The *N*-methyl carbamates were given in two vehicles: either corn oil (carbaryl, carbofuran, methiocarb and propoxur) or water (formetanate, oxamyl, and methomyl). Every animal received both vehicles, one dose immediately after the other. Five different dosage levels of the mixture were given, predicted to produce <5%, 10%, 25%, 45% or 60% brain cholinesterase inhibition, and also each *N*-methyl carbamate was given alone at a previously tested dosage to confirm the original dose-response data (7 single-chemical experimental groups). The control group received the vehicles only. The study was staggered over four consecutive days with all dosage levels being tested each day. All dosing solutions were made fresh each day. The animals were dosed, clinical observation of the degree of cholinergic toxicity was recorded 10 minutes after dosing, and then motor activity was assessed for the next 20 minutes. Immediately after removal from the activity chambers, the animals were briefly anesthesized with CO_2 and killed by 40 minutes after dosing. Brains were taken and frozen at -80°C until analysis; RBCs were prepared from trunk blood samples. Cholinesterase activity was determined using the radiometric assay as previously described.

As can be seen from Figure II.B.5-3 below, increasing dosages of the mixture produced increasing decrements in brain cholinesterase activity.

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Moreover, the dose-additive model predicted the degree of cholinesterase inhibition within the 95% confidence limits of each predicted value.

It is important to note that the single dose repeat of each *N*-methyl carbamate (open symbols, Figure II.B.5-4) overlapped the original dose-response curve values for each methyl carbamate, except for methomyl and propoxur which showed 5-10% less activity.

Figure II.B.5-3. Brain cholinesterase results from seven chemical equipotent mixture

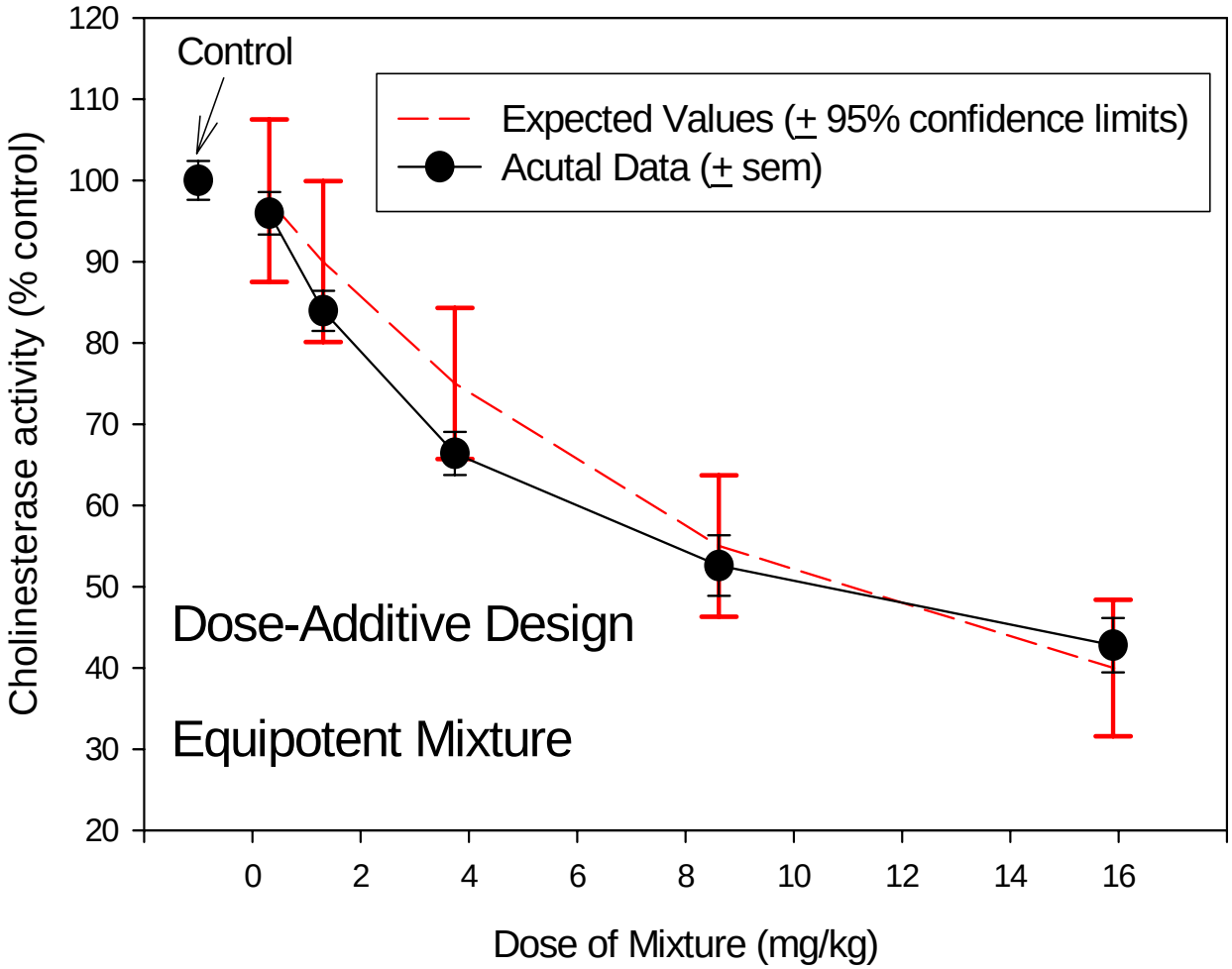

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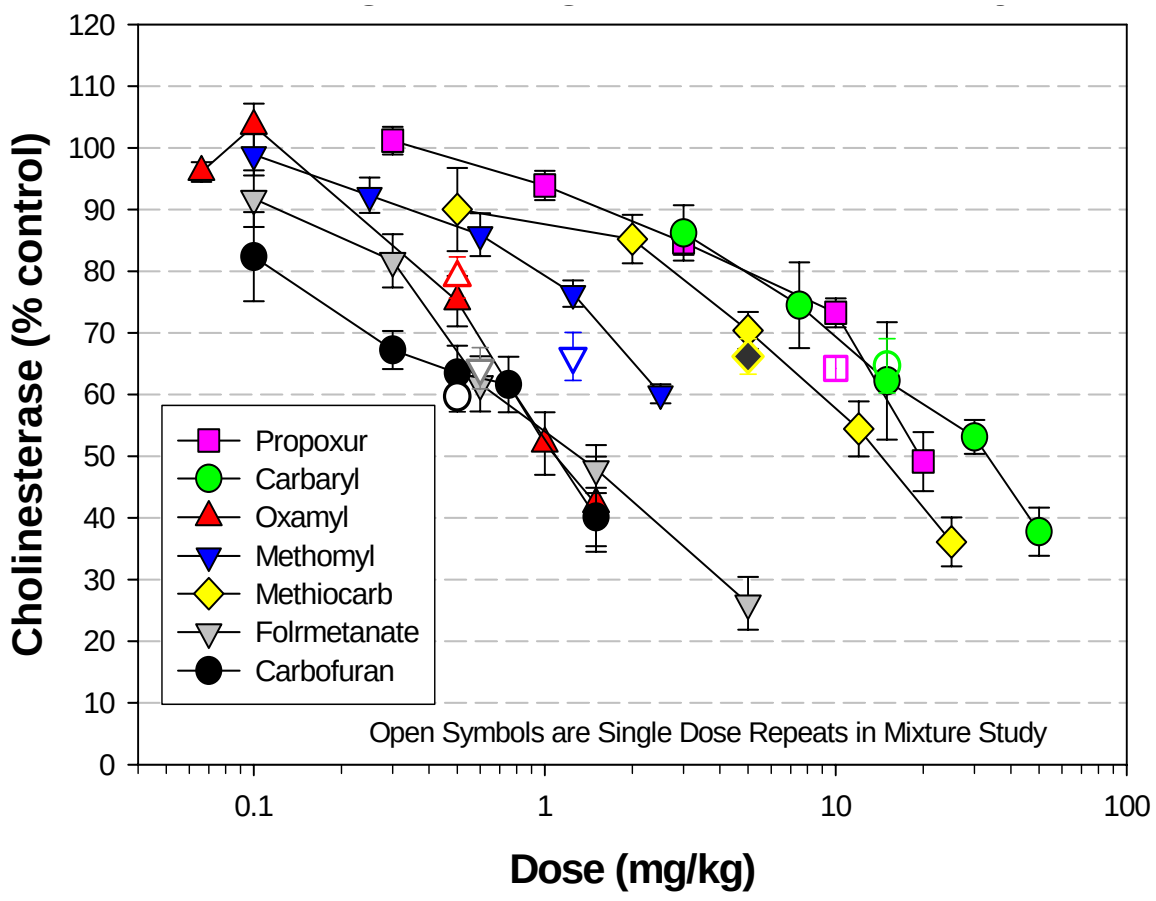
Figure II.B.5-4. Comparison of brain cholinesterase from dose-response and single dosing in the mixture study



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f. References

Moser, V.C., Phillips, P.M., McDaniel, K.L., Marshall, R.S., Padilla, S., Lowit, A. 2005. Comparison of acute neurobehavioral effects of *N*-methyl carbamate insecticides. Presented at the 44th Annual Meeting of the Society of Toxicology; New Orleans, Louisiana.