THE NEMATODE, *CAENORHABDITIS ELEGANS*, AS A MODEL FOR MAMMALIAN CHOLINERGIC NEUROTOXICITY

by

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(Under the Direction of Phillip L. Williams)

ABSTRACT

Assessing the toxicity of compounds acting on the nervous system has proved to be difficult, as measurable endpoints are often subjective and fraught with confounding variables. This dissertation examines the potential of *Caenorhabditis elegans* to serve as a toxicological model for mammalian neurotoxicity. The intent is to focus on one component of *C. elegans*' nervous system, the cholinergic nervous system, and determine how its responses to well-characterized agonists and antagonists compare to the responses of mammals. The literature pertaining to the acetylcholinesterase enzymes and the acetylcholinergic receptors of *C. elegans* is reviewed, as well as the literature pertaining to toxicological studies using *C. elegans*. In the first experiment, *C. elegans* was exposed to the reversible AChE-inhibitor carbamate class of pesticides in order to determine whether the endpoint, movement, could be used with reversible compounds by predicting their relative potencies in mammals. Given the functional redundancy of *C. elegans*' AChE enzymes, movement was measured to assess whether there was a difference in the contributions of either of the two main classes of AChE enzymes, and whether this difference influences the sensitivity of measuring movement in the second experiment. Next, agonists and antagonists that are well-characterized in ACh-activated receptors of vertebrates

and some nematode species were exposed to *C. elegans*, and measuring movement was used to determine whether their actions would be the same in *C. elegans*. Finally, some preliminary work on a new method to recover *C. elegans* from soil for toxicity testing is discussed.

INDEX WORDS: *Caenorhabditis elegans*; carbamate; pesticide; screen; toxicity; acetylcholinesterase

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# DEDICATION

This work is dedicated to my family – whether by birth or by marriage, for teaching me the lessons of life that truly matter.

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# CHAPTER 1

# INTRODUCTION

Since research with *Caenorhabditis elegans* began in earnest in the 1970s, researchers have examined *C. elegans*' potential as an organism for toxicological studies. Numerous studies have used *C. elegans* as a toxicological model, and these studies share a common aspiration: to take advantage of the astonishing degree to which *C. elegans* has been characterized and the ease of use of this simple organism to assess the toxicity of compounds in a rapid, sensitive, cost-effective and humane way.

Assessing the toxicity of compounds acting on the nervous system has proved to be particularly difficult, as measurable endpoints are often subjective and fraught with confounding variables. Even the simplest mammalian species is capable of relatively complex behavior. Sydney Brenner, the scientist responsible for identifying *C. elegans*' potential as a model organism, recommended that the study of behavior address two questions: "the genetic specification of the nervous system" and "the way nervous systems work to produce behavior" (Brenner 1974.). To do so, he chose an organism whose simple nervous system was amenable to study. *C. elegans*' nervous system is particularly advantageous to toxicity studies, as it produces only a few simple behaviors.

This dissertation examines the potential of *C. elegans* to serve as a toxicological model for mammalian neurotoxicity. The intent is to focus on one component of *C. elegans*' nervous system and determine how its responses to well-characterized agonists and antagonists compare to the responses of mammals. We have chosen to examine the cholinergic nervous system principally for the following reasons. First, the cholinergic nervous system is of ecological

relevance. Many environmental pollutants target either the acetylcholinesterase (AChE) enzymes or the acetylcholine(ACh)-activated receptors. Second, it is of interest for several important disease pathways, such as Alzheimer's and Parkinson's diseases. Finally, given the involvement of the cholinergic system in the locomotion of *C. elegans*, movement of populations of *C. elegans* may be measured as an endpoint. Movement has proven to be a quantitative, reproducible and automated method of recording behavioral toxicity in *C. elegans*.

Chapter 2 contains a review of the literature pertaining to the topics discussed throughout this dissertation. The next four chapters summarize experiments performed on the topics outlined below.

## Experiments

### 1. AChE-Reversibility

The experiment by Cole, et al., (2004) showed similar responses among *C. elegans* and rat and mice to the organophosphate (OP) class of pesticides that are irreversible AChE inhibitors in mammals. However, evidence in the literature suggests reversibility of both the OP and carbamate classes of pesticides in nematodes. Bunt (1975) found that herbivorous parasitic nematodes recovered following exposure to carbamates. Using *Meloidogyne javanica* and histochemical staining, Cuany, et al. (1984) measured substantial AChE recovery following exposure to aldicarb, a carbamate, and approximately 45% recovery after exposure to ethoprop, an OP. Mulder and Bakker (1988) determined that the dephosphorylation rate for ethoprop was similar to that observed for aldicarb. Opperman and Chang (1992) found nearly complete restoration of enzymatic activity 24 hours after aldicarb exposure, and ~10% activity for fenamiphos, an OP, using crude nematode AChE. They also noted that the partial recovery of enzymatic activity after fenamiphos exposure was sufficient to restore normal motility, but

allowed that more subtle effects on nematode behavior, although not observed, may have been present. We began a reversibility experiment of our own by testing methyl parathion, an organophosphate (OP). Surprisingly, worms paralyzed by this potent OP were found to revert to control movement levels after approximately 3 hours post-exposure recovery, even at the highest concentrations of the movement-concentration response curve determined by Cole, et al. (2004). The speed at which paralysis due to methyl parathion exposure reversed coupled with the short lifespan of *C. elegans* led us to question whether *C. elegans* would serve as an accurate model following exposure to non-lethal concentrations of AChE-inhibiting compounds that were reversible in mammals.

Therefore, we investigated whether *C. elegans*' could be used as a screen for vertebrate toxicity by assessing whether *C. elegans* displays similar toxicity as rats and mice to reversible acetylcholinesterase (AChE) inhibitors. We also sought to corroborate that the toxicity mechanism is the same. To determine relative potencies, movement-concentration curves were generated, $50^{th}$ percentiles for movement were located, ranked and compared statistically to rat and mouse oral acute LD50's. The ranking was significantly correlated to rat and mouse rankings ($\alpha = 0.05$). We measured a concentration-dependent decrease in AChE activity correlating to a decrease in movement for each carbamate, suggesting that the mechanism of toxicity is the same. Finally, as seen in mammals, inhibition of AChE activity occurred before a movement decrease. The response of *C. elegans* to carbamate exposure shows significant correlation to rat and mouse data.

2. AChE-Functional Redundancy

*Caenorhabditis elegans* is capable of relatively normal behavior with the loss of one of its two main genes for AChE, and it remains viable, but markedly less mobile, with the loss of both. Possible explanations for the functional redundancy between the *ace-1* and *ace-2* genes despite differing areas of expression include 1) the acetylcholine escapes from a synapse and migrates to a muscle cell or vice versa and is hydrolyzed, or 2) the AChE migrates between muscle cells and neurons (Johnson et al. 1988). Subtle differences in locomotion might also exist between the ACE-1$^-$ and ACE-2$^-$ strains. The sensitivity of the endpoint of movement would be negatively affected should one class of AChE affect movement much more than the other, or if one class of AChE was inhibited much more than the other.

Therefore, we explored the precision of measuring movement by measuring and comparing movements of wild-type strains to those lacking one of the 2 dominant genes for AChE. We then compared the sensitivities of the 3 strains to an AChE-inhibitor (propoxur) by generating movement-concentration curves, identifying concentrations that decreased movement by 50% (EC50s), and comparing them. The order of movement of unexposed samples of the strains is: N2 (wild-type) > ACE1$^-$ > ACE2$^-$; therefore, loss of the *ace-2* gene is more detrimental to movement. EC50s show an order of: N2 ≈ ACE2$^-$ < ACE1$^-$. Therefore, the enzymes encoded by *ace-1* were more susceptible to propoxur than those of *ace-2*. In conclusion, measuring movement was sufficiently precise to record differences following genetic manipulation and further chemical exposure.

3. Receptor Agonists and Antagonists vs. the Cholinergic System of *C. elegans*.

In this study, we focused on compounds affecting the acetylcholine (ACh)-activated receptors of *C. elegans*. To determine relative potencies, concentration-movement curves were generated using: the nicotinic agonists, nicotine and levamisole; the nicotinic antagonist, hexamethonium; and the muscarinic antagonist, atropine. We then co-exposed each compound to an acetylcholinesterase inhibitor, aldoxycarb (aldicarb-sulfone), and generated concentration-movement curves. Each compound produced a decrease in movement with increasing concentration. Using the concentration-movement relationship, we were able to record that both antagonists lessened the toxicity of aldoxycarb exposure at lower concentrations, while both agonists acted synergistically with aldoxycarb to increase the toxicity at higher concentrations. A comparison of the toxicities of nicotine and levamisole shows the latter to be approximately 4 orders of magnitude more potent at the 50$^{th}$ percentile for movement. Following exposure to these compounds, the response of *C. elegans,* as measured by movement, was similar to results seen in testing other nematodes and vertebrates measuring more traditional endpoints.

4. Adaptation of Movement Assay to Soil-Based Exposure.

*Caenorhabditis elegans* is currently used as a toxicity model for soil-based exposures using lethality. Although movement has been successfully used as an endpoint for behavioral toxicity in an aqueous medium for a number of chemical classes, its use has not been adapted to a soil medium. This chapter describes a preliminary attempt to adapt the method of measuring movement to a soil-based exposure using a newly developed approach to recover the worms. The new method of recovery was accomplished by a low-pressure, dilution of soil samples, i.e. flooding the worms out using ludox – a solvent that increases the buoyancy of worms and causes

them to float out of the soil. We have managed to extract clean samples of worms from soil that are suitable for measurement of movement. Results obtained using unexposed worm samples show average movement rates between 1-1.25µm/s, which is below the average control rate of 3.52µm/s obtained following a 4 hour aqueous exposure in K-medium. The next step in determining whether behavioral toxicity testing can be performed in a soil-based exposure is to determine whether average control movement rates can be raised high enough to distinguish effects at higher concentrations of compounds tested. We will begin by assessing whether a lack of food is responsible for relatively low control movement rates. If successful, the final phase of this experiment will use organophosphate (OP) pesticides to establish movement-concentration curves following a soil-based exposure.

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# CHAPTER 2

# LITERATURE REVIEW

This chapter provides an overview of the literature reviewed on three main topics: the current characterization of the AChE enzymes of *Caenorhabditis elegans*, that of the ACh-activated receptors of *C. elegans*, and finally, studies assessing toxicological endpoints of *C. elegans* following exposure to toxicants. Some of the material is presented in subsequent chapters where material was submitted for publication; however, the purpose of this section is to provide a more detailed reporting of these topics.

## Introduction

The presence of the neurotransmitter, ACh, in nematodes was first described in *Ascaris suum* (Mellanby 1955), where it was observed that ACh was found in Ascaris head and body walls, and that the head contained ten times more ACh than the body wall. Del Castillo et al. (1963) used electrophysiological techniques on *Ascaris lumbricoides* to establish that ACh is a neurotransmitter in nematodes. Early characterization of the cholinergic nervous system of nematodes was performed in the larger parasitic species, such as *A. suum*, whose size (up to 30cm - adult length) facilitated electrophysiological experiments. The development of sophisticated genetic and molecular biological techniques, and the emergence of *C. elegans* as a genetic tool, provided an alternative to electrophysiological experiments and allowed exploration of the nervous system of this smaller nematode.

The behavior of AChE mutants of *C. elegans* and of the wild-type treated with an AChE inhibitor provided evidence that ACh is a neurotransmitter in *C. elegans*, as well as triggering

greater exploration of the nematode cholinergic system (Culotti et al. 1981; Johnson et al. 1981). The characterization of the cholinergic system of *C. elegans* has since advanced greatly, in some areas surpassing the level of information gathered over decades of research on the larger, parasitic species.

**Acetylcholinesterases of *C. elegans***

There are three functioning classes of AChE in *C. elegans:* AChE A, B, and C. The two main functioning classes are AChE A, which is comprised of a 13s (IV), an 11.4s (III) and a 5.4s (IA) separated form all encoded by the gene *ace-1* on the X chromosome (Johnson et al. 1981), and AChE B, which is comprised of a 7.3s (II) and a 5.1s (IB) separated form encoded by the *ace-2* gene on chromosome I (Culotti et al. 1981). A third functional class, AChE C, is encoded by the gene *ace-3* on chromosome II (Johnson et al. 1988). A fourth gene also exists, *ace-4*, which is thought to encode a non-functional protein (Combes et al. 2003). The three functional classes were originally grouped by their catalytic activity, and indeed, *in vitro* data shows similar within-class $K_m$s and significantly different between-class $K_m$s (Johnson et al. 1988; Johnson and Russell 1983). However, *in vitro* catalytic activity alone will not predict the relative contributions of each class towards movement of *C. elegans*. Another important factor is differences in the locations each class is expressed. Early histochemical staining showed a wide overlap of AChE A, B and C in *C. elegans* tissues (Culotti et al. 1981; Johnson et al. 1988). Later studies using Green Fluorescing Protein (GFP) expression found *ace-1* in all body-wall and vulval muscle cells (Culetto et al. 1999), *ace-2* almost exclusively in neurons, and *ace-3* in several muscle cells of the pharynx and in the two Canal Associated Neurons (CAN cells) (Combes et al. 2003). Despite the distinct areas of expression, however, loss of either the *ace-1*

or *ace-2* gene produces a worm with no observable movement defect, whereas loss of both *ace-1* and *ace-2* produces a mutant whose movement is clearly impaired, yet still viable. Additionally, AChE activity in the *ace-1* deficient strain (VC505 or ACE1$^-$) and the *ace-2* deficient strain (GG202 or ACE2$^-$) are roughly equivalent, while that of the double mutant ACE-1$^-$2$^-$ (strain GG201) is <5% of the total AChE activity measured (but not required for 'normal' functioning) in the N2 strain (Combes et al. 2000).

**Cholinergic Receptors of *C. elegans***

ACh-activated GPCRs

Although pharmacological experiments suggested the presence of inhibitory muscarinic ACh-activated G protein-coupled receptors (GPCRs) (Lewis et al. 1980), mAChRs were first demonstrated in *C. elegans* using binding studies by Culotti and Klein (1983). It was the first characterization of binding sites for any neurotransmitter in nematodes. The completion of sequencing *C. elegans*' genome revealed the sequences of at least 3 genes encoding ACh-activated GPCRs (Bargmann 1998).

The first characterization of a mAChR in *C. elegans* began with Lee, et al. (1999), who isolated a cDNA clone, co-expressed it with a G protein-gated inwardly rectifying K+ channel (GIRK1) in *Xenopus* oocyte, and found that ACh was able to elicit a current. These authors found significant differences in the pharmacological responses of this receptor, whose gene was then named *gar-1* and the receptor denoted as GAR-1, to the responses of mAChRs of pig cardiac muscle. Additionally, amino acid sequencing found <35% homology between *gar-1* to the 5 human mAChR subtypes. This methodology was then used to characterize the two remaining genes, subsequently named *gar-2* and *gar-3* (Hwang et al. 1999; Lee et al. 2000).

There are appreciable sequence and pharmacological differences among the receptors encoded by the three genes: *gar-1*, *gar-2* and *gar-3*. The 'receptor encoded by *gar-2*' (GAR-2) was found to be similar in sequence to GAR-1, and showed even greater pharmacological differences to vertebrate mAChRs than the GAR-1 receptor (Lee et al. 2000). The 'receptor encoded by *gar-3*' (GAR-3), however, possesses significant sequence similarity to vertebrate mAChRs and displays virtually identical ligand-binding specificity indicating that GAR-3 is a member of the conventional mAChR family (Hwang et al. 1999). To date, homologues of *gar-1* and *gar-2* have not been reported in other organisms, yet it is not known whether *gar-1* and *gar-2* are found only in *C. elegans*, as these receptors may have been overlooked in other species because mAChR-specific compounds used in vertebrates are not very effective on these receptors.

Expression studies using GFP-reporter gene fusion show non-overlapping expression patterns for the three genes. The gene, *gar-2*, is expressed in a subset of *C. elegans*' neurons that are distinct from those expressing *gar-1*. These neurons include: some head neurons with ciliated endings, which are putative sensory neurons; in many cells of the ventral cord, which are considered to be motor neurons; and in the hermaphrodite-specific neuron (HSN) motor neurons, which innervate vulval muscles in the hermaphrodite (Lee et al. 2000). The gene *gar-1* was expressed in a subset of neuronal cells different from those expressing *gar-2*, which include some head neurons with ciliated endings, as well as the nucleus periventricularis magnocellularis (PVM) neuron (Lee et al. 2000). GFP expression of *gar-3* is shown in the pharyngeal muscle, in pharyngeal neuron I3, and in neurons of the extra-pharyngeal nervous system (Steger and Avery 2004).

Another interesting discovery resulting from the sequencing of *C. elegans*' genome was the discovery that, unlike mammalian mAChR genes, all three *gar* genes of *C. elegans* were found to contain introns, which suggested the possibility of alternative splicing. In fact, it was subsequently shown that *gar-1* and *gar-2* exist in three alternatively spliced forms each (*gar-1a*, *gar-1b*, *gar-1c* and *gar-2a*, *gar-2b*, *gar-2c*, respectively) while alternative splicing of the *gar-3* gene generates two functional GAR-3 isoforms, *gar-3a* and *gar-3b* (Park et al. 2003; Park et al. 2000; Suh et al. 2001). Any pharmacological or physiological significance of alternative splicing has not been established, as researchers have yet to find differences in coupling activity, drug specificity or spatial expression patterns of the isoforms to date, although differences in isoform abundance have been recorded.

The characterization of mAChRs in *C. elegans* has been greatly aided by the sequencing of the *C. elegans*' genome; however, progress has been slower on elucidating their function. Unlike AChEs or nAChRs, there are no GAR mutants to study. Never the less, we know that GAR-1 and GAR-2, when expressed in *Xenopus* oocytes, activate the GIRK1 channel, and this appears to be mediated by a $G_i$ protein but not by a $G_o$ protein in *Xenopus* oocytes, because none of the GAR-1 or GAR-2 isoforms produced the transient calcium activated chloride current, which is known to be stimulated by $G_o$ or $G_q$ proteins (Lee et al. 2000; Suh et al. 2001). In contrast, GAR-3 activates the endogenous $Cl^-$ channel in *Xenopus* oocytes. These results imply that the signal transduction pathway mediated by GAR-3 is distinct from those mediated by GAR-1 and GAR-2 (Lee et al. 2000).

The physiological role of GAR-3 is better understood than those of either GAR-1 or GAR-2. GAR-3 appears to couple to the activation of phospholipases C and D (Min et al. 2000). Using CHO cells stably expressing GAR-3b, Park, et al. (2006) observed that carbachol (an

AChE inhibitor) stimulated cAMP production in a dose- and time-dependent manner. The stimulating effect of carbachol was suspended by the muscarinic antagonist, atropine, indicating that the cAMP production is specifically mediated by GAR-3b. When the cells were treated with BAPTA-AM and EGTA, which reduce the cytosolic calcium level, carbachol-stimulated cAMP accumulation was inhibited by approximately 56%. Inhibition of PKC by chronic treatment with phorbol 12-myristate 13-acetate (PMA) decreased carbachol-stimulated cAMP production by as much as 68%, therefore, it appears that $Ca^{2+}$ and PKC are critically involved in GAR-3b-mediated cAMP formation. They further observed that carbachol-stimulated cAMP production was further enhanced by treatment with pertussis toxin (PTX), which is known to inactivate $G_i$ and $G_o$ proteins. This observation indicated that GAR-3b couples to a PTX-sensitive G protein, presumably $G_i$, to attenuate the cAMP accumulation. Given that GAR-3b stimulates cAMP production in CHO cells, this suggests that GAR-3b couples to both stimulatory and inhibitory pathways to modulate the intracellular cAMP level.

Perhaps the clearest explanation of a physiological role for any mAChR in *C. elegans* comes from an experiment performed by Steger and Avery (2004); these researchers found that arecoline acts through GAR-3 to promote pharyngeal muscle contraction, and that GAR-3 activity in the pharyngeal muscle has at least two distinct components, one affecting the relationship between excitation and contraction in the pharyngeal muscle (EC coupling) and the other affecting membrane potential. GAR-3 regulates intracellular $Ca^{2+}$ levels or manages the activity of $Ca^{2+}$-dependent processes to optimize pharyngeal pumping. In this way, the GAR-3 signaling pathway differs substantially from muscarinic cascades in vertebrate smooth muscle. Rather than simply facilitating muscle contraction, GAR-3 signaling may subtly adjust the kinetics of pharyngeal muscle function.

<u>ACh-activated ionic channels</u>

The nicotinic acetylcholinergic receptors (nAChRs) of *C. elegans* show surprising complexity and diversity with regards to subunit composition and expression patterns. Nicotinic acetylcholinergic receptors are members of the dicysteine loop ligand-gated ion channel superfamily. They are pentamers, consisting of a transmembrane domain and an extracellular, ACh-binding domain. The monomers, or subtypes, of the receptor dictate the ligand-binding properties, as well as determining channel-selectivity for cations or anions. As such, the diversity of the receptor subtypes predicts the diversity of the receptors' function and pharmacology. In *C. elegans*, over 50 Open Reading Frames have been identified that encode proteins with amino acid sequences showing substantial similarity to those of previously characterized nAChR subtypes of various organisms (Jones and Sattelle 2003). So far, 29 nAChR subtypes have been identified in *C. elegans* (Brown et al. 2006). While work continues in this area, this is the largest known family of nAChR subtypes identified in any species. By sequence homology, five groups of subtypes named after the first subtype characterized have been identified: deg-3-like; acr-16-like; unc-38-like; acr-8-like and unc-29-like (Jones and Sattelle 2003). A table summarizing the properties of these five subtype groups is listed at the end of this chapter (Table 2.1).

Subtypes with two adjacent cysteine residues in loop C are essential for ACh-binding, and are referred to as α subtypes. Subtypes lacking the cysteine doublet are classified as non-α subtypes. Alphas are required for binding; so their diversity, as well as the presence of non-α subtypes, adds to the variety of the physiological and pharmacological properties of the receptor.

So far, there are over 20 α subtypes identified in *C. elegans*, a number twice that identified in vertebrates (Mongan et al. 1998; Robertson et al. 1994).

Expression studies in *C. elegans* utilizing GFP-labeling show a diverse expression pattern of the receptor subtypes. Some nAChR subtypes are specific to neurons (acr-5, deg-3, des-2), while others are present in both body wall muscle and neurons (lev-1, unc-29, unc-38, unc-63), and lev-8 (formerly acr-13) is expressed in a variety of regions including muscle cells, neurons and epithelial-derived socket cells (Jones and Sattelle 2003; Sattelle et al. 2002). Even though expression is known for only a small proportion of the *C. elegans*' nAChR family, current data suggests that expression patterns cross tissue boundaries (Jones and Sattelle 2003). This contrasts to the concept of 'muscle' and 'neuronal' nAChRs of vertebrates.

While advancements in the genetic analysis of *C. elegans* has led to a considerable amount of information pertaining to the subunit composition and the expression of nAChRs in *C. elegans*, experiments to describe the pharmacology of these receptors in nematodes have been performed almost exclusively in larger, parasitic species. From these studies, nematode nAChRs have been pharmacologically divided into three categories: nicotine-sensitive (N-type), levamisole-sensitive (L-type), and bephenium-sensitive (B-type) (Martin et al. 2004). It was further shown using a contraction assay with *A. suum* that nicotine preferentially activates small-conductance channels and levamisole preferentially activates large-conductance channels (Levandoski et al. 2005). While it may be appropriate to apply general conclusions made in parasitic nematodes such as dividing the receptors based on agonist specificity or channel conductance to *C. elegans*, there remains a dearth of information on the effects of agonists and antagonists on *C. elegans*' nAChRs, especially involving tissue or cut-worm preparations. To date, most studies detailing the pharmacology of *C. elegans*' nAChRs are from subtype cDNAs

expressed in *Xenopus* oocytes, whose composition (homomers or combinations of subtypes) may or may not exist in wild-type *C. elegans*.

Since the realization that *C. elegans* possesses a large number of nAChR subunits, there has been much investigation as to what role these nAChR subtypes play in the functioning of *C. elegans*. Why would an organism as simple as *C. elegans* have such diversity? Generally, ionic channel receptor activation produces rapid changes in membrane potential that subsequently alter voltage-gated receptors and stimulate myriad cellular processes. In *C. elegans*, like vertebrates, nAChRs are involved in fast action responses, such as muscle activation and neuronal signaling. However, unlike vertebrates, *C. elegans* also possesses ACh-activated $Cl^-$ channels, which are thought to have an inhibitory function in *C. elegans* (Putrenko et al. 2005). In addition, the genes deg-3 and des-2, which are formed by splicing of a single primary transcript, have been found to be much more readily activated by choline than by ACh (Yassin et al. 2001). Because choline is a strong chemo-attractant to nematodes, it was postulated that the DEG3/DES2 nAChR was a chemoreceptor for choline. Supporting this hypothesis were the findings that the DEG3 receptor protein was localized to the sensory dendrites of putative chemosensory neurons and that deg-3 and des-2 deletion mutants exhibit strong deficits in chemotaxis to choline (Yassin et al. 2001). Finally, recent work has shown evidence that a GAR3 Gαq pathway promotes protractor muscle contraction by up-regulating nAChR signaling before mating, indicating that cross-communication takes place between the ACh-activated GPCRs and the nAChRs of *C. elegans* (Liu et al. 2007).

**Toxicological Studies Using *C. elegans***

This section reviews the toxicity studies conducted with *C. elegans*. Many studies have used chemicals recognized as human toxicants or environmental pollutants as research tools. For example, many AChE-inhibiting pesticides are routinely used in studies aimed at describing the physiological functions of molecular components of the cholinergic nervous system. However, the scope of this review is limited to those studies that either used *C. elegans* to characterize a toxicant, or used toxicants to assess *C. elegans*' potential as a toxicological model, thereby producing toxicity data of the compounds. This review omits studies that used endpoints not commonly used in toxicological studies, such as ecological endpoints (effects on community, diversity, etc.). Also, although many studies that exposed *C. elegans* to common toxicants in order to establish a mechanism of action are relevant to developing *C. elegans* as a toxicological model, they were not included in this review, as their emphasis was on using sufficiently high exposure concentrations to produce observable pathology, not necessarily on determining a concentration-effect relationship.

The toxicological studies conducted using *C. elegans* can be divided into three general categories based upon the endpoint measured: those using lethality, those using sublethal endpoints, and those using reporter genes. Fifteen studies were found to use lethality as an endpoint. Twelve studies were found to use sublethal endpoints, and 17 studies were found to use reporter genes. In addition, five studies used a combination of endpoints from more than one of these categories. The results for studies using the endpoints of lethality, sublethal endpoints and a combination are summarized in table 2.2, table 2.3 and table 2.4, respectively. In each table, the studies are listed in chronological order. Toxicological studies using reporter genes in *C. elegans* use either a tagged stress-response gene, such as HSP-16 (a heat-shock protein) or a

tagged gene of interest to toxicological processes, such as the metallothionein genes. When the tagged gene is up-regulated following exposure to a compound of interest, the resulting marker (usually fluorescence) is measured, and a concentration-effect relationship is established that is sensitive and potentially, ultra-specific to a cellular process of choice. Although an important means of utilizing *C. elegans* as a model toxicological organism, studies using reporter genes are not within the scope of this dissertation and will not be discussed further.

Together, the studies displayed in tables 2.2, 2.3 and 2.4 show a number of exposure mediums, exposure durations and chemicals tested. Interestingly, almost all studies cite a common desire to develop *C. elegans* as a toxicological model, because it is inherently practical as a laboratory organism and well-characterized. Potential uses cited for developing *C. elegans* into a toxicological model include: an ecological indicator of exposure; a rapid screening organism to predict toxicity towards vertebrates and as a means to investigate the mechanism of toxicants.

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Table 2.1 – *nAChR subtype groups in Caenorhabditis elegans.*

| **Group** | **Number of genes in group** | **α / β** | **Amino acid sequence similar to:** |
|---|---|---|---|
| unc-38 like | 3 | α | Insect α |
| unc-29 like | 4 | β | Drosophila ARD |
| acr-16 like | 9 | α | Vertebrate α 7 |
| deg-3 like | 8 | ? | Unique to nematodes? |
| acr-8 like | 3 | ? | Unique to nematodes? |

Table 2.2 – *Summary of C. elegans Toxicity Studies Utilizing Sublethal Endpoints*.

| REFERENCE | EXPOSURE MEDIUM | ENDPOINT MEASURED | CHEMICALS TESTED | EXPOSURE DURATION (HRs) |
|---|---|---|---|---|
| (Popham and Webster 1979) | AGAR | Reproduction Growth | Cd | Growth 2, 6 Rep. 84 |
| (van Kessel et al. 1989) | AGAR | Reproduction Growth | Cd | 24, 48, 72, 96 |
| (Williams and Dusenbery 1990b) | AQUEOUS | Movement | Cd, Be, Pb | 24, 96 |
| (Middendorf and Dusenbery 1993) | AQUEOUS | Reproduction | Flouroacetic acid | 24 |
| (Höss et al. 1997) | Sediment/ Pore Water | Body Length | Cu | 72 |
| (Traunspurger et al. 1997) | Pore Water | Body Length<br># eggs inside worm<br># offspring per worm<br>% gravid worms | Cd | 72 |
| (Hoss et al. 2001) | Sediment/ Pore Water | Body Length | Cd | 72 |
| (Anderson et al. 2001) | AQUEOUS | Movement<br>Feeding<br>Growth<br>Reproduction | Cd, Cu, Pb | Move't & Feed'g 4, 24<br>Growth 24<br>Reproduction 72 |
| (Boyd et al. 2003) | AQUEOUS | Movement<br>Feeding<br>Ingestion | Cd, Cu, Pb | 4, 24 |
| (Cole et al. 2004) | AQUEOUS | Movement | Organophosphate pesticides | 4 |
| (Anderson et al. 2004) | AQUEOUS | Movement | Acetone, DMSO, Al, Cu, Pb, levamisole, mebendazole | 4 |
| (Hasegawa et al. 2004) | AGAR | Growth<br>Reproduction<br>Lifespan | Acrylamide | ~48-96 (Not specified) |
| (Melstrom and Williams 2007) | AQUEOUS | Movement | Carbamate pesticides | 4 |

Table 2.3 - *Summary of C. elegans Toxicity Studies Utilizing Lethality as an Endpoint*.

| REFERENCE | EXPOSURE MEDIUM | CHEMICALS TESTED | EXPOSURE DURATION (HRs) |
|---|---|---|---|
| (Williams and Dusenbery 1987) | AGAR | Hg, Cu | 24 |
| (Williams and Dusenbery 1988) | AGAR | Hg, Be, Al, Cu, Zn, Pb, Cd, Sr | 24 |
| (Williams and Dusenbery 1990a) | AQUEOUS | Ag, Hg, Be, Al, Cu, Zn, Pb, Cd, Sr, Cr, As, Tl, Ni, Sb | 24, 96 |
| (Donkin and Dusenbery 1993) | SOIL | Cu | 24 |
| (Donkin and Dusenbery 1994) | SOIL | Zn, Cd, Cu, Pb | 24 |
| (Hitchcock et al. 1997) | AQUEOUS | -various- (field sample) | 72 |
| (Tatara et al. 1997) | AQUEOUS | Cu, Cd, Ca, Ni, Hg, Mg, Mn, Pb, Zn | 24 |
| (Tatara et al. 1998) | AQUEOUS | Li, Na, Mg, K, Ca, Cr, Mg, Fe, Co, Ni, Cu, Zn, Sr, Cd, Cs, Ba, La, Pb | 24 |
| (Peredney and Williams 2000a) | SOIL | Cd, Cu, Pb, Ni, Zn (Cl vs. $NO_3$ salts) | 24 |
| (Peredney and Williams 2000b) | SOIL | Cu, Cd, Zn, Pb, Ni | 24 |
| (Ura et al. 2002) | AQUEOUS | DMSO, 17β-estradiol, bisphenol A, nonylphenol, benzo[*a*] pyrene, aldicarb, benzophenone, styrene, *trans*-1,2-diphenylcyclobutane, 2,4,6-triphenyl-1-hexene, ponasterene A, Cd | 24 |
| (Yamasaki et al. 2002) | AQUEOUS | Tannins | 24 |
| (Boyd and Williams 2003a) | SOIL | Cu, Ni, Pb, Zn, Cd | 24 |
| (Boyd and Williams 2003b) | AQUEOUS | Cu | 24 |
| (Swatloski et al. 2004) | AGAR | 1-alkyl-3-methylimidazolium | 20 |

Table 2.4 - *Summary of C. elegans Toxicity Studies Utilizing Lethal and Sublethal Endpoints.*

| REFERENCE | EXPOSURE MEDIUM | ENDPOINT MEASURED | CHEMICALS TESTED | EXPOSURE DURATION (HRs) |
|---|---|---|---|---|
| (Donkin and Williams 1995) | AQUEOUS | Lethality Development Reproduction | Cu, H, Pb, Cd, pentachlorophenate | 24, 96 |
| (Dhawan et al. 1999) | AQUEOUS | Lethality Reproduction Movement | Ethanol | Lethality 24 Reproduction 72 Movement 24 |
| (Dhawan et al. 2000) | AQUEOUS | Lethality Movement | Pb, Cu, Cd, Al, Zn | 24 |
| (Williams et al. 2000) | AQUEOUS | Lethality Movement Feeding | Polyamino carb-oxylates of Gd | 24, 48, 72 |
| (Thompson and de Pomerai 2005) | AQUEOUS | HSP-16 reporter Feeding Reproduction Lethality | Methanol, ethanol, iso-propanol, iso-butanool | HSP 6, 20 Feeding 5 Reproduction 3,4,5,6 (days) Lethality 24 |

# CHAPTER 3

# REVERSIBLE ACETYLCHOLINESTERASE INHIBITORS IN *CAENORHABDITS ELEGANS* VS. RATS, MICE[1]

[1] Melstrom PC, Williams PL. 2007. *Biochemical and Biophysical Research Communications*. 357:200-205
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**Abstract**

We are investigating whether *Caenorhabditis elegans*' could be used as a screen for vertebrates by comparing the responses of components of its cholinergic system to well-characterized toxicants. We assessed whether *C. elegans* displays similar toxicity as rats and mice to reversible acetylcholinesterase (AChE) inhibitors, and sought to corroborate that the toxicity mechanism is the same. To determine relative potencies, movement-concentration curves were generated, $50^{th}$ percentiles for movement were located, ranked and compared statistically to rat and mouse oral acute LD50's. The ranking was significantly correlated to rat and mouse rankings ($\alpha = 0.05$). We measured a concentration-dependent decrease in AChE activity correlating to a decrease in movement for each carbamate, suggesting that the mechanism of toxicity is the same. Finally, as seen in mammals, inhibition of AChE activity occurred before a movement decrease. The response of *C. elegans* to carbamate exposure shows significant correlation to rat and mouse data.

Key Words: *Caenorhabditis elegans*; Carbamate; Pesticide; Screen; Toxicity; Acetylcholinesterase.

**Introduction**

We are exploring the feasibility of *Caenorhabditis elegans* to serve as a screen for predicting relative vertebrate neurotoxicity. A sensitive, quantifiable, easily reproduced, fast and inexpensive screen is needed to enable rapid prioritization of compounds most detrimental to the nervous system, as well as to serve as an environmental indicator of exposure to neurotoxicants. There are logistical reasons and advantages of the physiology of *C. elegans* that show promise towards its use as a screening organism for at least some aspects of vertebrate neurotoxicology. *Caenorhabditis elegans* reproduces exponentially, completes its life cycle in approximately 3 days and is well characterized. In addition, large-scale worm culturing and automated high-volume worm sorting techniques such as the Union Biometrica COPAS Biosort (Harvard Biosciences, Boston, MA) have lead to much investigation of *C. elegans* as a potential high throughput organism by pharmaceutical companies (Dengg and van Meel 2004; Jones et al. 2005; Williams et al. 2000) and the National Toxicology Program (http://ntp.niehs.nih.gov). In addition to the practical advantages of using the simple nematode, our current knowledge of the connections from gene to neural circuit to behavior in *C. elegans* makes it an indispensable behavioral model (de Bono and Maricq 2005; Hobert 2003). In this experiment, we continued our analysis of toxicants affecting the acetylcholinesterase (AChE) enzymes of the cholinergic nervous system of *C. elegans* by examining the endpoints of movement and acetylcholinesterase (AChE) activity. Our intent is to explore correlations between the effects of well-characterized toxicants to components of the cholinergic nervous system of *C. elegans* to existing vertebrate data in the hope of defining the strengths and weaknesses of comparison between them.

Past studies have shown significant correlations between the relative toxicity of AChE inhibitors predicted by *C. elegans* and existing mammalian data (Anderson et al. 2004; Cole et

al. 2004). However, both studies were conducted using irreversible AChE inhibitors, organophosphates (OPs). Evidence in the literature suggests reversibility of both the OP and carbamate classes of pesticides in nematodes that is greater than normally seen in vertebrates (Bunt 1975; Cuany et al. 1984; Mulder and Bakker 1988; Opperman and Chang 1992). In view of the reversibility of common AChE inhibitors, coupled with the short lifespan of *C. elegans*, this study addresses the following questions: can a change in *C. elegans'* movement be accurately measured after exposure to a class of reversible AChE-inhibitors, the carbamate pesticides? Do potencies correlate to vertebrate data for this class? And, finally, can we corroborate that the mechanism of toxicity of the reversible carbamates is the same in *C. elegans* and vertebrates?

## Materials and Methods

### Culture of nematodes

We obtained *Caenorhabditis elegans*, wild-type strain N2 from the *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). We raised all developmental stages of all strains of *C. elegans* in 115-mm Petri dishes with K-agar (0.032M KCL, 0.051 M NaCl, 0.1 M $CaCl_2$, 0.1 M $MgSO_4$, 2.5% Bacto-peptone, 0.17% Bacto-agar, and 0.01% cholesterol), a modification of Williams and Dusenbery(1988). We seeded the plates with *Escherichia coli* strain OP50 as a food source and incubated at 20ºC for 24 hours (Brenner 1974.). To age-synchronize the populations, we harvested eggs from adult populations. Two-day juvenile populations were transferred to a plate containing a fresh lawn of OP50 to maintain high nutritional status before testing on day 3 (Boyd et al. 2003). All exposures were carried out using 3-day-old adults.

Chemicals tested and exposure conditions

We ordered all compounds from Sigma-Aldrich (St. Louis, MO, USA) as reagent-grade chemicals. For each replicate, we dissolved a known amount of each chemical in K-medium to create a stock solution with further dilutions carried out by adding additional K-medium (0.032 M KCl, 0.051 M NaCl in $dH_2O$) (Williams and Dusenbery 1990). Exposures for assessment of movement used a 12-well sterile tissue culture plate, where we loaded 5 µl (approximately 100 worms) into a single 1-ml well containing K-medium for the controls or a carbamate. We prepared a control population for each exposure. The same exposure routine was used to assess AChE activity with the exception that 40 µl of worm pellet (gravity-settled worms) were distributed into 2 wells containing K-medium with or without a carbamate. In accordance with previous studies, we placed all exposure plates in an incubator at 20ºC for 4h in the absence of food (Anderson 2001; Anderson et al. 2004).

To control for any daily variation between worm populations collected from different egg populations and raised in separate cultures, we normalized all replicates to their control by dividing the movement or AChE activity of a single exposure concentration by its control's measured value, creating a percent of control.

Movement tracking

We patterned the movement tracking after Boyd, et al. (2000). Immediately following the 4-hour exposure, we transferred the worms with a Pasteur pipette into 2-ml glass centrifuge tubes. They were washed by allowing the worms to gravity settle into a pellet, removing the supernatant, adding ~1.5ml of fresh K-medium, gently mixing the worms by creating bubbles with a Pasteur pipette, and repeating the process for a total of 3 washes. We then transferred 5µl

of the settled pellet (~50-80 worms) to a cooled, 2 ml 1% agar pad on a clear glass slide measuring 100mm X 200mm. The worms were allowed to disperse on the agar pad inverted over a Petri dish filled with water to avoid desiccation. We began movement tracking at exactly 1 hour after the end of exposure for each replicate. We placed the individual glass slides in a tracking chamber with a gentle humidified air stream. Using a video camera interfaced with a Macintosh® computer that contains a modification of the NIH tracking software(Dusenbery 1996), the individual worm movements were tracked and recorded to an Excel® spreadsheet. We used a macro to calculate the average μm of movement per worm per second.

Acetylcholinesterase activity assay

After we constructed a movement-concentration response curve for each carbamate, cholinesterase activity was measured at 3 concentrations along the curve in order to construct a cholinesterase activity-concentration response curve for each chemical. We chose concentrations representing the EC50 for movement (concentration required to reduce movement by 50% relative to controls), a value approximating an EC80, and a value termed a 'no-observable-effect-concentration for movement' (NOEC) because the worm population was exposed, but we measured no significant change in movement compared to controls. We measured AChE activity using an adaptation of the Ellman assay most closely resembling Moulton, et al, to measure AChE activity in tissues with low AChE activity (Ellman et al. 1961; Moulton 1996). After a 4-hour exposure, we washed the worms as detailed above, transferred them to 1.5ml plastic centrifuge tubes and centrifuged at 4°C for 10 min. After the diluent was removed, the worms were flash frozen in liquid nitrogen and placed in a –80°C freezer until analyzed. During analysis, we added phosphate buffer 0.05M (pH 8.0, 0.1M potassium

phosphate monobasic, 0.1M potassium phosphate dibasic) to each sample, and then homogenized the sample using a Teflon pestle and a vortexor for 20s. The worms were again centrifuged at 4°C for 10 min and the supernatant was transferred to new 1.5ml centrifuge tubes. We incubated each sample for 5 minutes in a disposable 1.5ml cuvette containing 0.25mM Dithiobis (2-Nitrobenzoic acid). Acetylthiocholine-iodide 156mM was added; it was inverted to mix and placed immediately into a Shimadzu UV-1601 spectrophotometer (Shimadzu Scientific Instruments; Columbia, MD, USA), where we measured the change in absorbance at 405nm over 90s. We quantified protein concentrations for each sample using the Bio-Rad protein assay kit II (Bradford assay) (Bio-Rad Laboratories; Hercules, CA, USA). We used bovine serum albumin as the protein standard, and analyzed the samples at an absorbance of 595nm.

pH measurement

As pH has been shown to affect movement, we measured the pH values for the highest and lowest concentrations of each compound using an Orion Z20A pH meter at room temperature (22-24°C) (Cole et al. 2004; Khanna et al. 1997). (Orion research; Beverly, MA, USA.) No effects of pH on *C. elegans*' movement have been observed within the pH range of those observed for the 11 carbamates used in this study (Cole et al. 2004). Because of this, we did not use buffers or exclude any carbamates from analysis due to confounding pH effects. Measured pH values of all concentrations ranged from 5.8, K-medium, to 3.9.

Statistical analysis

We used the Chi-squared and the Shapiro-Wilk tests for normality (SAS Inc.; Cary, NC, USA.). We modeled movement and concentration for each compound using nonlinear

regression, PROC NLIN, (SAS Inc.; Cary, NC, USA.) and generated EC50 values with 95% confidence intervals. We ranked the EC50 values from most to least potent. We assessed the correlation between the rankings of *C. elegans* and rat or mouse by using Spearman's correlation coefficient. In the event of overlapping confidence intervals for *C. elegans'* EC50 values, we used the mean of the ranks of the tied chemicals to calculate Spearman's correlation coefficient. In the case of dioxacarb, where 2 values of the oral acute LD50 were reported for rats, a mean value was used. We obtained LD50s from the Registry of Toxic Effects of Chemical Substances (RTECS) database. For the AChE activity assay, we performed an ANOVA among the concentrations of a particular chemical, with Tukey's analysis to determine which concentration groups differed at a significance level of 0.05 (SAS Inc.; Cary, NC, USA.).

**Results**

We exposed *C. elegans* to 11 carbamates, a class of environmental contaminants with a well-characterized mode of toxicity in mammals, i.e., reversible AChE inhibition. Following exposure, we generated movement-concentration plots and an EC50 was determined for each pesticide in order to test whether the order of potency corresponded to the ranking order of oral acute LD50s for the rat and mouse. We chose LD50s as a basis for comparison because they are uniformly generated and, unlike a comparable behavioral endpoint, available for all of these chemicals in rats and mice. As the cause of death for the rat and mouse lethality studies is understood to be inhibition of AChE, lethality is an endpoint along a continuum of neurotoxic effects. We last measured the AChE activity to corroborate that the cause of toxicity is the same in *C. elegans* as it is in rats and mice.

Movement

We began by recording movement values for all concentration groups at 30, 45, 60, 75 and 90 minutes to determine reversibility of the compounds as measured by movement. We observed no significant change between 45 and 90 minutes post-exposure. Therefore, we standardized the post-exposure movement analysis to one-hour post-exposure for every subsequent replicate. In this way, we were able to construct 11 movement-concentration plots using the chosen carbamates (Fig. 1). Most movement responses (100% to 0% of control movement) of each carbamate occurred across two orders of magnitude of concentration, while two compounds (ethiofencarb and formetanate) responded over a concentration range of one order of magnitude or less. Overall, the EC50 values fall within approximately 2.5 orders of magnitude of concentration. The ranking of the carbamates using *C. elegans'* movement was significant to $\alpha = 0.05$ when compared to both rat and mouse oral acute LD50 values using Spearman's correlation coefficient (Table 1). The combined values of the control replicates for all 11 carbamates tested were normally distributed with a mean value of 3.52 μm/s (95% confidence Interval 3.41 to 3.62).

Acetylcholinesterase activity

Using ANOVA, concentration was a significant predictor of AChE activity for all carbamates ($p<0.01$). Following exposure to the NOEC-movement concentrations, AChE activity was significantly less than control in 8 of the 11 carbamates. This increased to 9 of 11 for the EC50 concentrations and 10 of 11 for the EC80 concentration groups. The only carbamate not to show a statistically significant reduction between any concentration group and its control was ethiofencarb, which caused both comparatively less and more variable AChE

inhibition than other carbamates. AChE activities among controls were uniformly distributed with a mean value of 11.0 nmol/min∗mg protein (95% confidence Interval 9.2, 12.8).

**Discussion**

We standardized the time interval between the end of the exposure and when either movement was tracked or when the worms were snap-frozen for subsequent AChE activity analysis at one hour. By doing so, we found changes in movement of populations of *C. elegans* to be a valid predictor of mammalian neurotoxicity. Additionally, our findings suggest that carbamate toxicity appears to occur via AChE inhibition in *C. elegans* as in rats and mice.

<u>Movement</u>

The steep slopes of the movement-concentration plots constructed after exposure to carbamates are comparable to the movement-concentration plots generated by Cole, et al (2004), using organophosphates. In addition, the toxicity ranges covered by both classes are similar to mammalian data. The rank correlation performed by Cole, et al. (2004) found EC50 values for *C. elegans'* movement across 4.5 orders of magnitude of organophosphate concentration, which roughly corresponded to the organophosphate toxicity ranges of 3.5 orders of magnitude for rats and 4 orders of magnitude for mice. In the present experiment, the rank correlation found EC50 values for *C. elegans'* movement that spanned approximately 2.5 orders of magnitude of carbamate concentration; this toxicity range also corresponds to the toxicity range of the chosen carbamates in rats and mice, or approximately 2.5 orders of magnitude for both species. Exposure to *C. elegans* is via the oral route, as the cuticle layer of *C. elegans* is thought to be relatively impermeable. This is supported by a recent experiment utilizing synchrotron x-ray

techniques to study metals distributions in *C. elegans* that showed spatial distribution in several internal compartments but none in the cuticle layer following aqueous exposure (Jackson et al. 2005). Because the worms were exposed by ingesting the carbamates and because we desired to maintain an aqueous exposure vehicle, this experiment was subject to the limitations of the water solubilities of the carbamate class. Out of the 25 carbamates that we originally identified for inclusion into the study based upon commercial availability, we prepared the maximum concentrations obtainable in water for the 16 that possessed the best toxicity-to-aqueous-solubility ratios. The highest obtainable concentrations of 5 compounds (Asulam, Carbaryl, Propham, Promecarb, Pirimicarb) in K-medium failed to reduce movement by ≥50%, and were therefore not evaluated further. Therefore, we performed the movement rank comparison over a relatively small toxicity range of carbamates. Despite this, we found a significant correlation between relative toxicities caused by carbamates.

Acetylcholinesterase activity

We measured AChE activity as the 'total AChE activity' normalized to each replicate's control value at concentrations proceeding and corresponding to the constructed movement curves. All 11 carbamates show concentration to be a significant predictor of AChE activity ($p<0.01$). Only ethiofencarb failed to show a significant decline in AChE activity versus control at any exposure group. Although a review of the structure and metabolism of ethiofencarb offers no clues as to why the movement-concentration curve is so uniquely steep, ethiofencarb possesses an unusually steep movement curve (~1/2 order of magnitude), which required us to test ethiofencarb concentrations that differed minimally. Perhaps more importantly, the ethiofencarb movement-concentration curve revealed nematode hyperactivity over a wider

concentration range than was seen among other carbamates. This required us to test a NOEC that was relatively lower than corresponding NOECs of other carbamates tested. Nonetheless, in view of all 11 carbamates showing inhibition of AChE activity directly preceding a decrease in movement, we believe the data suggests that the mechanism of toxicity of carbamates in *C. elegans* is AChE-inhibition, as it is in mammals.

Movement vs. cholinesterase activity

A comparison of inhibition of AChE activity in *C. elegans* to its movement decrease provides an additional basis for comparison to vertebrates. From the pooled carbamate data (Fig. 2), we see an average of 24% inhibition of AChE before an effect on movement is seen (NOEC-movement). This is a similar result to Sheets, et al. (1997), who found that all treatment-related neurobehavioral findings occurred only at dietary levels that produced more than 20% inhibition of plasma, RBC, and brain cholinesterase activity using Fischer 344 rats.

Summary

Rank order correlation coefficients for carbamate potency were significant when compared to either rat or mouse oral acute LD50s. The toxicity range of the carbamates tested was comparable between *C. elegans* and both rat and mouse. A concentration-dependent decrease in AChE activity correlating to a decrease in movement was observed for each carbamate. AChE activity declined before phenotypic effects were observed, as is seen in mammals. In view of the correlation between the actions of neurotoxicants in *C. elegans* and mammals, and because large numbers of *C. elegans* can be raised quickly, inexpensively and

without many of the restrictions of animal welfare issues, we believe *C. elegans* has potential as a sensitive, reproducible, and quantitative screen that is, by its nature, rapid and inexpensive.

**Acknowledgements**

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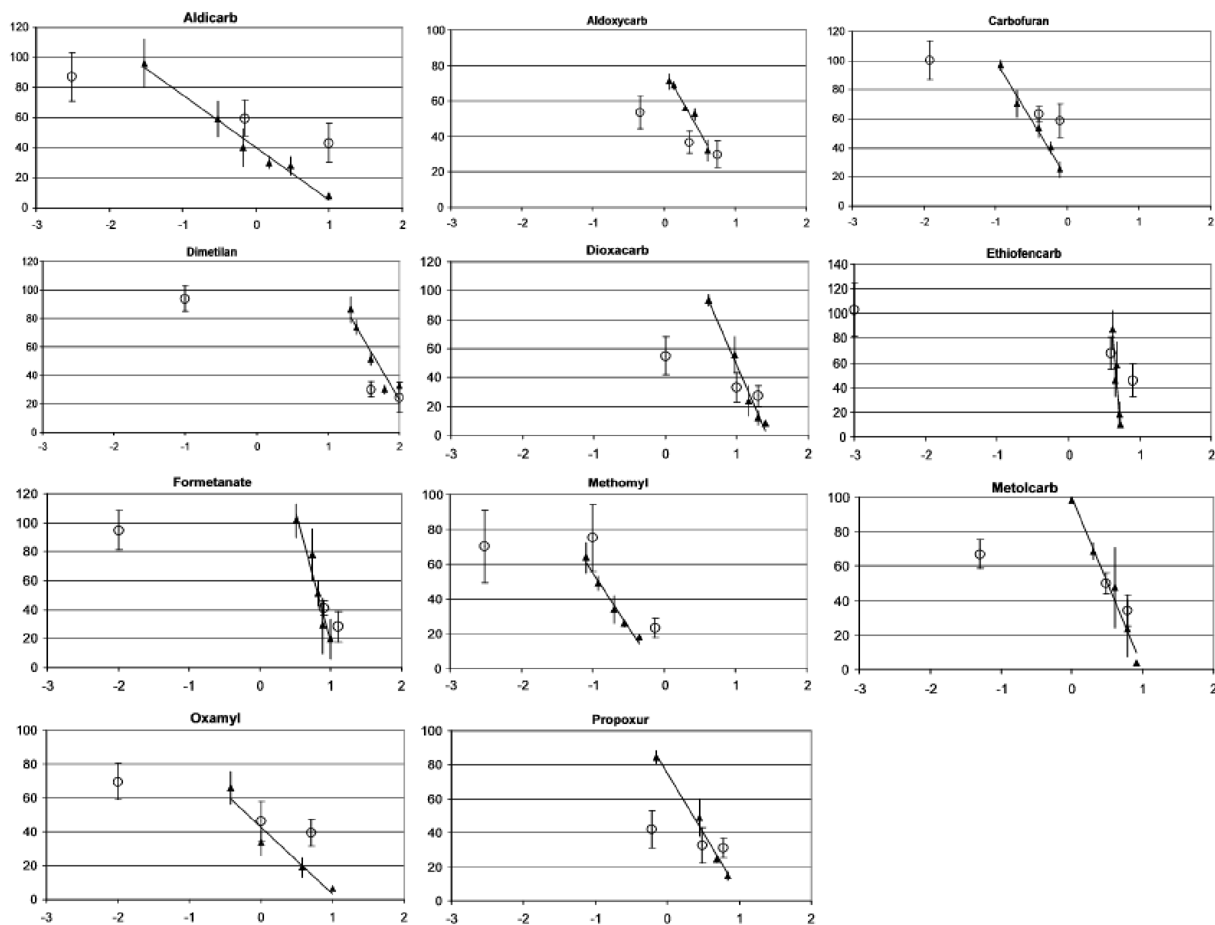


Figure 3.1 -- *In alphabetical order, the individual plots of movement (▲) and AChE Activity (O) shown as percent control against a Log(mM) scale for all 11 carbamates tested are displayed with their corresponding standard error bars. Solid black line denotes least-squares regression line for movement.*

Table 3.1 - *Comparison of Caenorhabditis elegans' EC50s with rat and mouse oral acute LD50s using Spearman correlation coefficient*.

| Chemical | EC50 (mM) | 95% Confidence Interval (mM) | Rat LD50 (mg/kg) | Mouse LD50 (mg/kg) |
|---|---|---|---|---|
| Methomyl | 0.1039 | (0.08390, 0.1285) | 14.7 | 10 |
| Carbofuran | 0.4044 | (0.3504, 0.4668) | 5 | 2 |
| Aldicarb | 0.5263 | (0.3348, 0.8274) | 0.46 | 0.3 |
| Oxamyl | 0.6745 | (0.4006, 1.135) | 2.5 | 2.3 |
| Propoxur | 2.350 | (1.997, 2.764) | 41 | 23.5 |
| Aldoxycarb | 2.541 | (1.961, 3.292) | 20 | NA[a] |
| Metolcarb | 3.161 | (2.404, 4.158) | 268 | 109 |
| Ethiofencarb | 4.529 | (3.781, 5.425) | 200 | 71 |
| Formetanate | 6.683 | (5.461, 8.176) | 20 | 18 |
| Dioxacarb | 9.635 | (8.612, 10.78) | 40 | 48 |
| Dimetilan | 40.18 | (34.56, 46.70) | 25 | 60 |
| | | **Number of comparisons**[b] | 11 | 10 |
| | | Rs[c] | 0.6059[d] | 0.6848[d] |

[a]LD50 data for Aldoxycarb in mice not available in RTECS.

[b] rat ranking data contained 11 comparisons, while mouse data compared 10.

[c] Spearman Correlation Coefficient, (Rs).

[d] Both comparisons are significant to $\alpha=0.05$.

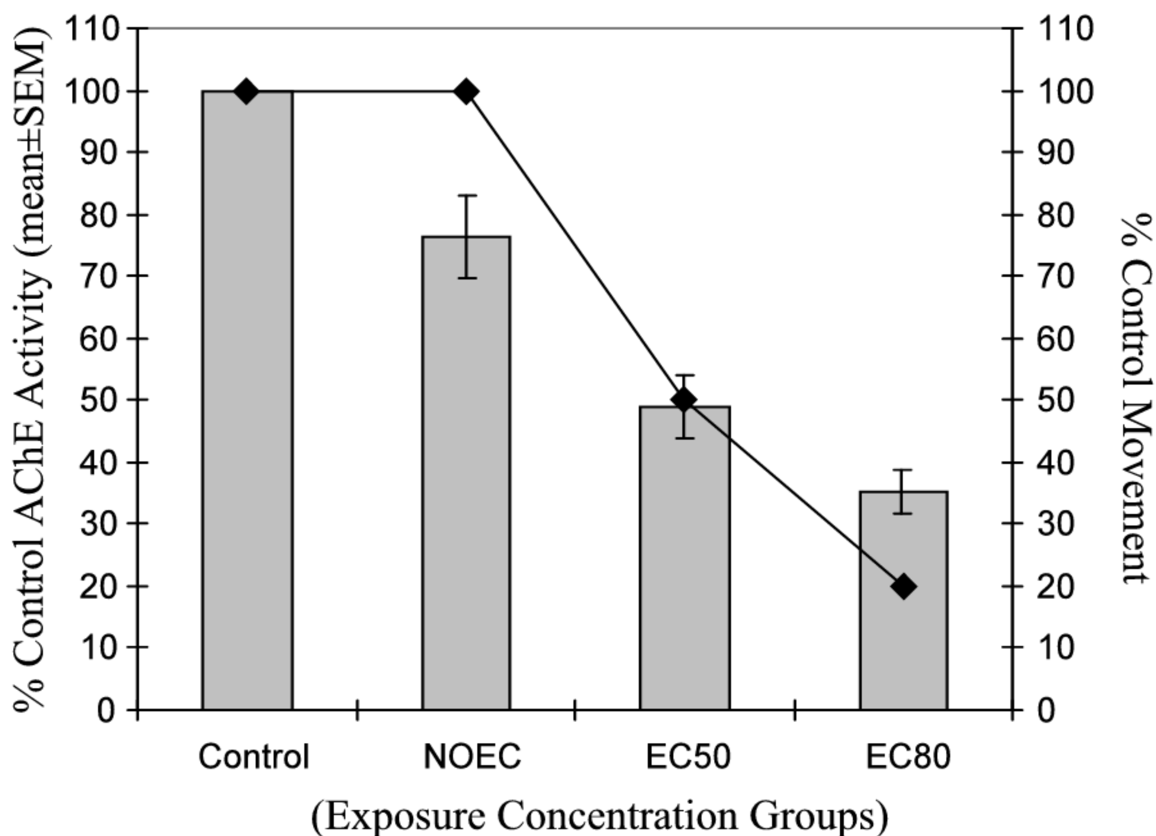


Figure 3.2 -- *Mean values of 'AChE activity as a percent of control' for all carbamates tested at each of the 3 concentrations (NOEC, EC50 and EC80) and control are displayed as bar graphs with standard error bars. The black diamonds connected by a solid line represent the 'movement as a percent of control' at each exposure level: NOEC = 'no difference in movement vs. controls', EC50 = 50% reduction in movement vs. controls, EC80 = 80% reduction in movement vs. controls.*

# CHAPTER 4

# CONTRIBUTIONS OF ACETYLCHOLINESTERASE CLASSES TO MOVEMENT IN *CAENORHABDITIS ELEGANS*[1]

[1] Melstrom, P.C., Williams, P.L. To be submitted to *Journal of Nematology*.

**Abstract**

The use of *Caenorhabditis elegans* as a potential high-throughput screening organism is gaining attention. We are investigating compounds affecting the acetylcholinesterase (AChE) enzymes by measuring movement, a potential high-throughput endpoint. Here, we explored the precision of measuring movement by measuring and comparing movements of strains lacking one of the 2 dominant genes for AChE, and wild-type. We then compared the sensitivities of the 3 strains to an AChE-inhibitor (propoxur) by generating movement-concentration curves, identifying concentrations that decreased movement by 50% (EC50s), and comparing them. The order of movement of the strains is: N2 (wild-type) > $ACE1^-$ > $ACE2^-$; therefore, loss of the *ace-2* gene is more detrimental to movement. EC50s show an order of: N2 ≈ $ACE2^-$ < $ACE1^-$. Therefore, the enzymes encoded by *ace-1* were more susceptible to propoxur than those of *ace-2*. In conclusion, measuring movement was sufficiently precise to record differences following genetic manipulation and further chemical exposure.

Key Words: *Caenorhabditis elegans*; screen; toxicity; acetylcholinesterase; inhibitor; movement; propoxur; carbamate; pesticide.

**Introduction**

Because *Caenorhabditis elegans* reproduces exponentially, completes its life cycle in approximately 3 days, and because the connections from gene to neural circuit to behavior in *C. elegans* are well characterized (de Bono and Maricq 2005; Hobert 2003), pharmaceutical companies (Dengg and van Meel 2004; Jones et al. 2005; Williams et al. 2000) and the National Toxicology Program (http://ntp.niehs.nih.gov) continue to investigate the free-living nematode as a potential high-throughput organism. We are exploring the suitability of *C. elegans* to serve as a screen for predicting relative vertebrate neurotoxicity. A fast and inexpensive screen would be useful to enable rapid prioritization of compounds most detrimental to the nervous system, as well as to serve as an environmental indicator of exposure to neurotoxicants. Our approach has been to compare *C. elegans*' simple behavioral responses to toxicants of known mechanism to that of the responses of mammals. We are currently evaluating compounds affecting the acetylcholinesterase (AChE) enzymes of the cholinergic nervous system of *C. elegans* by measuring movement, a potential high-throughput endpoint.

Many studies have used *C. elegans*' movement to assess phenotypic behavior following genomic modification and/or chemical treatment. Examples include subjective observation, timing the worms' ability to escape from a chemo-repellant, and measuring the frequency and amplitude of the trail left by individual worms on an agar plate. Technological advances now make it possible to measure the individual movements of up to 400 worms simultaneously, increasing the power and precision of these types of measurements.

*C. elegans* has three functioning classes of AChE: AChE A, B, and C. The two main functioning classes are AChE A, which is encoded by the gene *ace-1* on the X chromosome (Johnson et al. 1981), and AChE B, which is encoded by the *ace-2* gene on chromosome I

(Culotti et al. 1981). A third functional class, AChE C, is encoded by the gene *ace-3* on chromosome II (Johnson et al. 1988). However, AChE C accounts for <5% of the total AChE activity measured (but not required for 'normal' functioning) in the N2 wild-type strain (Combes et al. 2000). A fourth gene also exists, *ace-4*, which is thought to encode a non-functional protein (Combes et al. 2003). The three functional classes were originally grouped by their catalytic activity; indeed, *in vitro* data show similar within-class $K_m$s and significantly different between-class $K_m$s (Johnson et al. 1988; Johnson and Russell 1983). Early histochemical staining showed a wide overlap of AChE A, B and C distribution in *C. elegans*' tissues (Culotti et al. 1981; Johnson et al. 1988). Later studies using Green Fluorescing Protein (GFP) expression found *ace-1* in all body-wall and vulval muscle cells (Culetto et al. 1999), *ace-2* almost exclusively in neurons, and *ace-3* in several muscle cells of the pharynx and in the two Canal Associated Neurons (CAN cells) (Combes et al. 2003). Despite the distinct areas of expression, however, loss of either the *ace-1* or *ace-2* gene produces a worm with no observable movement defect, whereas loss of both *ace-1* and *ace-2* produces a mutant whose movement is clearly impaired. Additionally, AChE activity in the *ace-1* deficient strain (VC505, or ACE1$^-$) and the *ace-2* deficient strain (GG202, or ACE2$^-$) are roughly equivalent (Culotti et al. 1981). Possible explanations for the functional redundancy between *ace-1* and *ace-2* despite differing areas of expression include 1) the acetylcholine escapes from a synapse and migrates to a muscle cell and is hydrolyzed, or vice versa, or 2) the AChE migrates between muscle cells and neurons (Johnson et al. 1988). Subtle differences in locomotion might also exist between the ACE-1$^-$ and ACE-2$^-$ strains. Although it has been hypothesized that a difference may occur (Culotti et al. 1981; Johnson et al. 1981), it has never been proven. In this study, we explored the precision of measuring the endpoint of movement by noting whether we could measure a difference between

these two mutants. Furthermore, if a substantial difference between the relative contributions of these 2 main AChE classes exists, exposure to compounds with differing affinities toward the AChE classes might affect the sensitivity of the endpoint of movement as compounds with greater affinity towards one class would produce effects not observed by measuring movement. We also explored how the functional redundancy of *C. elegans*' AChE classes affects the precision by exposing the mutant and the N2 wild-type strains to an AChE inhibitor, the carbamate pesticide propoxur, and noting differences in sensitivity.

**Materials And Methods**

Culture of nematodes

We obtained *C. elegans* N2 wild-type, $ACE1^-$ strain VC505, $ACE2^-$ strain GG202, $ACE1^-2^-$ strain GG201 from the *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). We raised all developmental stages of all strains of *C. elegans* in 115-mm Petri dishes with K-agar (0.032 M KCL, 0.051 M NaCl, 0.1 M $CaCl_2$, 0.1 M $MgSO_4$, 2.5% Bacto-peptone, 0.17% Bacto-agar, and 0.01% cholesterol), a modification of Williams and Dusenbery (1988). We seeded the plates with *Escherichia coli* strain OP50 as a food source and incubated at 20ºC for 24 hours (Brenner 1974.). To age-synchronize the populations, we harvested eggs from adult populations. Two-day juvenile populations were transferred to a plate containing a fresh lawn of OP50 to maintain high nutritional status before testing on day 3 (Boyd et al. 2003). All exposures were carried out using 3-day-old adults.

Exposure chemicals and conditions

We acquired propoxur from Sigma-Aldrich (St. Louis, MO, USA) as a reagent-grade chemical. For each replicate, we dissolved a known amount of propoxur in K-medium to create a stock solution and made further dilutions by adding additional K-medium (0.032 M KCl, 0.051 M NaCl in $dH_2O$) (Williams and Dusenbery 1990). Exposures for assessment of movement used a 12-well sterile tissue culture plate, on which we loaded 5 μl (approximately 100 worms) into a single 1-ml well containing propoxur, or K-medium for the control well. We prepared a control population for each exposure. Pursuant to previous studies, we placed all exposure plates in an incubator at 20ºC for 4hr in the absence of food (Anderson 2001; Anderson et al. 2004). To control for any daily variation between worm populations collected from different egg populations and raised in separate cultures, we normalized all replicates to their control by dividing the movement of a single exposure concentration by its control's measured value, creating a percent of control.

Movement tracking

We patterned the movement tracking after Boyd, et al. and Dhawan, et al. (2000; 1999). Immediately following the 4-hr exposure, we transferred the worms with a Pasteur pipette into 2 ml glass centrifuge tubes. They were washed by allowing the worms to gravity settle into a pellet, removing the supernatant, adding ~1.5 ml of fresh K-medium, gently mixing the worms by creating bubbles with a Pasteur pipette, and repeating the process for a total of 3 washes. We then transferred 5 μl of the settled pellet (~50-80 worms) to a cooled, 2 ml 1% agar pad on a clear glass slide measuring 100mm X 200mm. The worms were allowed to disperse on the agar pad inverted over a Petri dish filled with water to avoid desiccation. We began movement

tracking at exactly 1 hour after the end of exposure for each replicate. We placed the individual glass slides in a tracking chamber with a gentle stream of humidified air. Using a video camera interfaced with a Macintosh® computer that contains a modification of the NIH tracking software (Dusenbery 1996), the individual worm movements were tracked and recorded to an Excel® spreadsheet. We used a macro to calculate the average µm of movement per worm per second.

pH measurement

No effects of pH on *C. elegans*' movement have been observed between the pH range of 5.8 (K-medium, alone) to 3.5 (Cole et al. 2004). For this reason, to facilitate comparisons with historical data, and to prevent any interaction between the buffer and propoxur, we did not use a buffer. We measured the pH values for the highest and lowest concentrations of propoxur using an Orion Z20A pH meter at room temperature (22-24°C). (Orion research; Beverly, MA, USA.)

Statistical analysis

Normality of $ACE1^-$ and $ACE2^-$ distributions was tested using Shapiro-Wilk. We modeled the relationships between movement and concentration for each strain using nonlinear regression, PROC NLIN, (SAS Inc.; Cary, NC, USA.), and we generated an EC50 value along with its 95% confidence interval for each strain.

## Results

Movement

The rates of movement for both the $ACE1^-$ and the $ACE2^-$ deletion mutant strains were normally distributed over slightly different ranges. Results of mean average rates of movement

among ACE1$^-$, ACE2$^-$, and N2 populations were greatest for N2 wild-type, intermediate for ACE-1$^-$, and least among ACE-2$^-$ strains ($p<0.001$; Table 1). Therefore, loss of the *ace-2* gene (AChE class B) is more deleterious to *C. elegans'* movement than loss of the *ace-1* gene (AChE class A).

We also compared N2, ACE1$^-$ and ACE2$^-$ strains' changes in movement following exposure to an AChE inhibitor, propoxur, by generating movement-concentration curves (Fig. 1). EC50 values were indistinguishable between N2 and ACE2$^-$ populations and least among ACE1$^-$ populations when movement values were normalized to each strain's respective control values (Table 2). Therefore, the movements of worms possessing only AChE class A (ACE2$^-$) were more susceptible to the effects of propoxur than the movement of worms possessing only AChE class B.

**Discussion**

We measured the movements of unexposed samples of the ACE1$^-$ and ACE2$^-$ strains to determine whether AChE type differentially affect movement. Because all three functional AChE genes are independently expressed and *ace-3* constitutes <5% of AChE activity of the wild-type N2 strain, the ACE1$^-$2$^-$ strain was not considered in movement analyses. By using deletion mutants lacking one of the two major forms of AChE, we determined that ACE1$^-$, possessing AChE B class expressed almost exclusively in neurons, moved at 83% the rate (2.91/3.52 μm/sec) of N2 wild-type, while ACE2$^-$, possessing AChE A class expressed almost exclusively in body wall and vulval muscle cells, moved at 54% the rate (1.90/3.52 μm/sec) of N2 wild-type. Therefore, the neuronal AChE B class more greatly affects movement than does

the AChE A class. To our knowledge, this is the first attempt to quantitatively determine whether a difference exists between the movements of these two mutants.

To gain insight into the significance of this difference and the effect of functional redundancy on the precision of measuring movement, we constructed movement-concentration response curves to compare the effect of propoxur-induced inhibition of movement among the ACE1$^-$, ACE2$^-$ and wild-type strains. EC50 values generated for both the ACE1$^-$ and ACE2$^-$ strains show the movement of worms possessing only the neuronal AChE B class (ACE1$^-$) to be less sensitive to inhibition of movement by propoxur than worms possessing only the AChE A class (ACE2$^-$) despite AChE B having a greater effect on movement. In fact, the decline in the movement rate of the ACE2$^-$ strain was approximately equal to that of the N2 wild-type strain, while a decline in the movement rate of the ACE1$^-$ strain did not occur until approximately 40% inhibition of the N2's movement. Therefore, inhibition of the AChE A class likely drove the initial decrease in movement of the N2 stain.

Several conclusions can be made from these data. First, the AChE classes' different innate contributions to movement will not always dictate which class has a greater affect on movement decrease when exposed to inhibitors. In this respect, the innate difference in contributions to movement is less important than the kinetics (in particular, distribution and binding affinity) of the chemical inhibitor. Secondly, the precision of movement as an endpoint for compounds affecting the AChE enzymes of *C. elegans* is limited by the functional redundancy of this enzyme system. However, the extent to which it is limited is a function of the preferential affinity of an inhibitor towards one AChE class over the other. The significance of this preferential affinity is very small with propoxur, as a difference in estimated EC50s of ~2mM is well within the error tolerance for a screen. In past studies, we examined the effects of

reversible and irreversible inhibitors (24 compounds in total) on *C. elegans*' AChE enzymes and found significant correlations to mammals (Anderson et al. 2004; Cole et al. 2004; Melstrom and Williams 2007). This suggests that the preferential affinities of inhibitors towards one AChE class over the other are not significant for many compounds, although they may contribute to the error of statistical correlation calculations. Finally, this experiment was, essentially, a chemistry-to-gene screen that successfully used movement as the measured endpoint. Chemical treatment followed genomic modification, and in this way, we were able to discern the effects of propoxur on both enzyme classes. We believe using movement as the measured endpoint may be useful for other chemistry-to-gene screens using *C. elegans*.

Summary

Measuring average rates of movement across *C. elegans* populations was sufficiently precise to show a difference in the contributions of the two main classes of AChE towards movement in *C. elegans*. Following exposure to propoxur, we were able to deduce that the kinetics of propoxur are more important than this difference. Measuring movement may be a useful endpoint in *C. elegans* to evaluate compounds for toxicity or pharmacological efficacy, particularly through the use of chemistry-to-gene screens.

**Acknowledgements**

We would like to thank Dr. David Dusenberry for the use of his tracking program; the Interdisciplinary Toxicology Program at the University of Georgia for partial funding of this project; the *Caenorhabditis* Genetics Center (CGC), which is funded by the NIH National Center for Research Resources (NCRR), for supplying some of the nematode strains used in this

research; In addition, the strain VC505 was provided to the CGC by the *C. elegans* Reverse Genetics Core Facility at UBC, which is part of the international *C. elegans* Gene Knockout Consortium.

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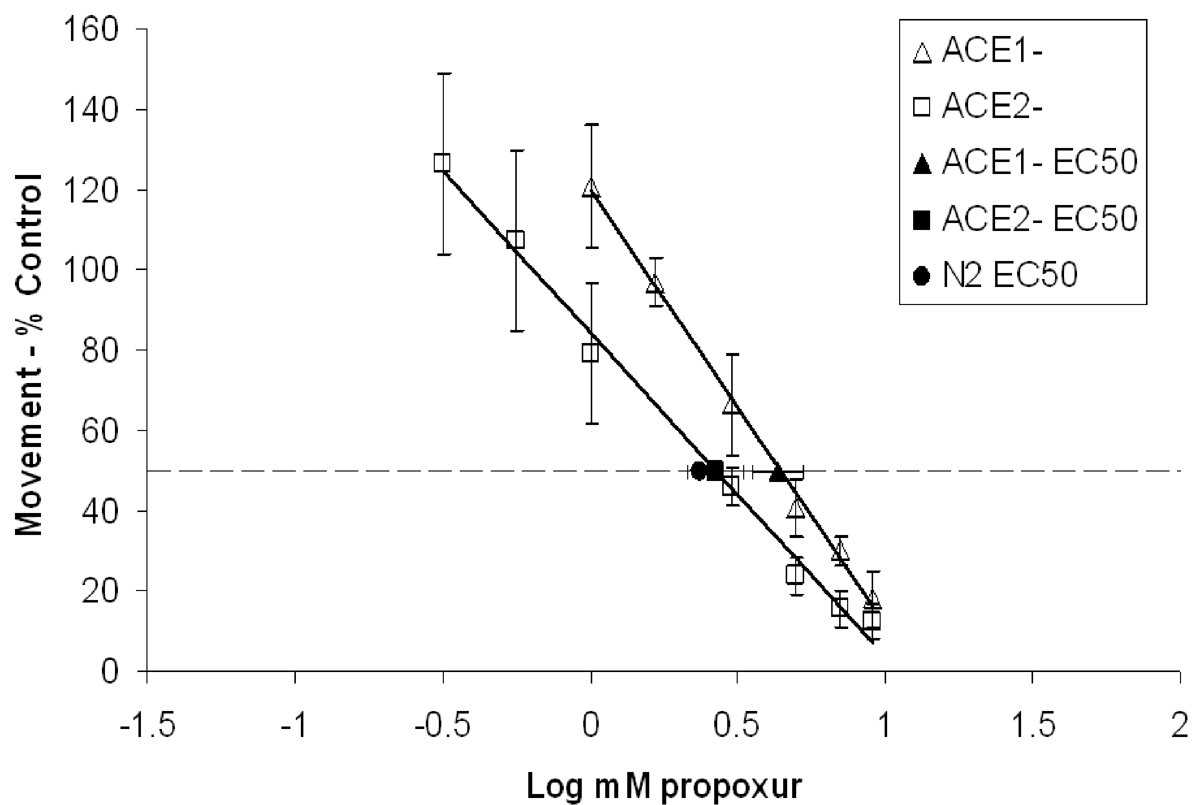


Figure 4.1 -- *Movement rate as a percent of control is shown for ACE1- and ACE2- strains versus log mM propoxur. The EC50 of ACE2- (■) shows greater sensitivity to propoxur than the EC50 of ACE1- (▲), and is nearly identical to the N2 wild-type EC50 value (●).*

Table 4.1 - *Mean average rates of movement of N2 wild-type, ace-1 deficient and ace-2 deficient strains*.

| Strain | Mean Movement Rate (μm/sec) | 95% Confidence Interval |
|---|---|---|
| N2 | 3.52[a] | (3.41, 3.62) |
| ACE1$^{-}$ | 2.91[a] | (2.53, 3.30) |
| ACE2$^{-}$ | 1.90[a] | (1.55, 2.25) |

[a] Difference significant to $p<0.001$

Table 4.2 - *Effective concentrations of propoxur required to decrease movement of N2 wild-type, ace-1 deficient and ace-2 deficient strains by 50% (EC50).*

| Strain | EC50 (mMolar) | 95% Confidence Interval |
|---|---|---|
| N2 | 2.35 | (2.00, 2.77) |
| ACE1- | 4.34[a] | (3.58, 5.24) |
| ACE2- | 2.66 | (2.14, 3.30) |

[a] Difference significant to $p<0.05$

# CHAPTER 5

# LOCOMOTORY RESPONSE OF CHOLINERGIC AGONISTS AND ANTAGONISTS IN THE NEMATODE, *CAENORHABDITIS ELEGANS* [1]

[1] Melstrom, P.C., Williams, P.L. To be submitted to *Parasitology*.

**Abstract**

We are investigating a new method of measuring the effects of compounds exposed to *Caenorhabdits elegans* by measuring its movement following exposure and then comparing these responses to the responses of well-characterized toxicants in vertebrates and other nematodes. In this study, we focused on compounds affecting the acetylcholine (ACh)-activated receptors of *C. elegans*. To determine relative potencies, concentration-movement curves were generated using: the nicotinic agonists, nicotine and levamisole; the nicotinic antagonist, hexamethonium; and the muscarinic antagonist, atropine. We then co-exposed each compound to an acetylcholinesterase inhibitor, aldoxycarb (aldicarb-sulfone), and generated concentration-movement curves. Each compound produced a decrease in movement with increased concentration. Using the concentration-movement relationship, we were able to record that both antagonists lessened the toxicity of aldoxycarb exposure at lower concentrations, while both agonists acted synergistically with aldoxycarb to increase the toxicity at higher concentrations. A comparison of the toxicities of nicotine and levamisole shows the latter to be approximately 4 orders of magnitude more potent at the $50^{th}$ percentile. Following exposure to these compounds, the response of *C. elegans,* as measured by movement, was similar to results seen in testing other nematodes and vertebrates measuring endpoints that are more traditional.

**Introduction**

The detailed characterization of *Caenorhabditis elegans*, its practical advantages as a laboratory model, combined with large-scale worm culturing and automated high-volume worm sorting techniques such as the Union Biometrica COPAS Biosort (Harvard Biosciences, Boston, MA) have lead to the investigation of *C. elegans* as a potential high-throughput screening organism for neuroactive compounds by pharmaceutical companies (Dengg and van Meel 2004; Jones et al. 2005) and the National Toxicology Program (http://ntp.niehs.nih.gov). Our laboratory is evaluating one potential high-throughput endpoint, movement, by studying the changes in *C. elegans*' movement following exposure to compounds with well-characterized effects on components of the cholinergic nervous system. Although movement has been used qualitatively to assess phenotypic effects following genomic modification and/or chemical treatment, advances in motion-tracking technology enable us to measure the movements of up to 400 worms, simultaneously. This allows us to measure an endpoint that is quantitative, reproducible and automated.

Like many nematodes, *C. elegans* possesses an unusually complex cholinergic system for such a simple organism. Three independently-assorted genes encode functioning acetylcholinesterase (AChE) enzymes (Culotti et al. 1981; Johnson et al. 1981; Johnson et al. 1988). Twenty-nine nicotinic acetylcholinergic receptor (nAChR) subunits have so far been identified (Brown et al. 2006). Three acetylcholine-activated G protein-coupled receptor genes (*gar-1*, *gar-2* & *gar-3*) have been isolated (Hwang et al. 1999; Lee et al. 1999; Lee et al. 2000), and 5 additional splice variants have been identified; therefore, 8 muscarinic acetylcholinergic receptors (mAChRs) are known to exist in *C. elegans* (Park et al. 2003; Park et al. 2000; Suh et al. 2001). We began our investigation of the ACh-activated receptors of *C. elegans* by an initial

characterization of their *in vivo* responses to well-characterized mammalian agonists and antagonists.

## Materials and Methods

### Culture of nematodes

We obtained *Caenorhabditis elegans* wild-type N2 strain from the *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). We raised all developmental stages of *C. elegans* in 115-mm Petri dishes with K-agar (0.032M KCL, 0.051 M NaCl, 0.1 M $CaCl_2$, 0.1 M $MgSO_4$, 2.5% Bacto-peptone, 0.17% Bacto-agar, and 0.01% cholesterol), a modification of Williams and Dusenbery (1988). We seeded the plates with *Escherichia coli* strain OP50 as a food source and incubated at 20ºC for 24 hours (Brenner 1974.). To age-synchronize the populations, we harvested eggs from adult populations. Two-day juvenile populations were transferred to a plate containing a fresh lawn of OP50 to maintain high nutritional status before testing on day 3 (Boyd et al. 2003). All exposures were carried out using 3-day-old adults.

### Chemicals tested and exposure conditions

We ordered all chemicals from Sigma-Aldrich (St. Louis, MO, USA) as reagent-grade chemicals. For each replicate, we dissolved a known amount of each chemical in phosphate buffer (pH 8.0, 0.1M potassium phosphate monobasic, 0.1M potassium phosphate dibasic) to create a stock solution with further dilutions carried out by adding phosphate buffer. Exposures for assessment of movement used a 12-well sterile tissue culture plate, where we loaded 5 µl (approximately 100 worms) into a single 1-ml well containing buffer for the controls or a dilution concentration. We prepared a control population for each exposure. In accordance with previous studies, we placed all exposure plates in an incubator at 20ºC for 4h in the absence of food (Anderson 2001; Anderson et al. 2004).

To control for any daily variation between worm populations collected from different egg populations and raised in separate cultures, we normalized all replicates to their control by dividing the movement of a single exposure concentration by its control's measured value, creating a percent of control. Every replicate had a corresponding control group.

Movement tracking

We patterned the movement tracking after Boyd, et al. (2000). Immediately following the 4-hour exposure, we transferred the worms with a Pasteur pipette into 2-ml glass centrifuge tubes. They were washed by allowing the worms to gravity settle into a pellet, removing the supernatant, adding ~1.5ml of fresh K-medium (0.032 M KCl, 0.051 M NaCl in $dH_2O$) (Williams and Dusenbery 1990), gently mixing the worms by creating bubbles with a Pasteur pipette, and repeating the process for a total of 3 washes. We then transferred 5µl of the settled pellet (~50-80 worms) to a cooled, 2 ml 1% agar pad on a clear glass slide measuring 100mm X 200mm. The worms were allowed to disperse on the agar pad inverted over a Petri dish filled with water to avoid desiccation. We began movement tracking at exactly 1 hour after the end of exposure for each replicate. We placed the individual glass slides in a tracking chamber with a gentle humidified air stream. Using a video camera interfaced with a Macintosh® computer that contains a modification of the NIH tracking software(Dusenbery 1996), the individual worm movements were tracked and recorded to an Excel® spreadsheet. We used a macro to calculate the average µm of movement per worm per second.

<u>pH measurement</u>

We measured the pH values for the highest and lowest concentrations of propoxur using an Orion Z20A pH meter at room temperature (22-24°C). (Orion research; Beverly, MA, USA.)

**Results**

We used nicotine as an N-type nAChR agonist, levamisole as a L-type nAChR agonist, hexamethonium as a nAChR antagonist, and atropine as a mAChR antagonist. Although water insoluble compounds have been successfully tested in *C. elegans*, we desired to maintain a single exposure vehicle (aqueous) to facilitate comparison; therefore, B-type receptors were not evaluated, as currently-identified compounds selective for the B-type nAChR receptor are water insoluble. We increased the concentration of each compound until we observed a decrease in movement in order to verify that the compounds cause an effect in *C. elegans* following an aqueous exposure and to provide a basis of comparison among the compounds tested.

All four compounds displayed a concentration-dependent decrease in movement (Figure 1). The slopes of levamisole and nicotine reveal a difference in toxicity between these two compounds (Figure 2). Levamisole's slope shows a toxicity range (100% control movement to its nadir at approximately 18%) that covers approximately 1.5 orders of magnitude. In contrast, nicotine's toxicity was limited by its solubility in water and was unable to reduce the movement lower than approximately 55% of the control value, yet this smaller decline also occurred over a toxicity range of approximately 1.5 orders of magnitude. A comparison of the effective concentrations required to decrease movement by 50% (EC50s) shows levamisole decreased movement at concentrations approximately 4 orders of magnitude lower than nicotine.
We then co-exposed samples of *C. elegans* to the AChE-inhibitor aldoxycarb (aldicarb-sulfone) at a concentration known to reduce movement by 50% (Melstrom and Williams 2007) and to each compound. Our intent was to determine whether the mechanism of action of the compounds tested were the same in *C. elegans* and whether the co-exposure scenarios would be as predicted by results obtained in previous experiments with other nematodes and mammals.

Co-application of aldoxycarb and either nicotine or levamisole caused an increase in an aldoxycarb-induced decline of movement (Figure 3). Co-application of aldoxycarb and either hexamethonium or atropine caused a reversal of an aldoxycarb-induced decline in movement at lower concentrations (Figure 4).

**Discussion**

In previous experiments, we have used movement as an endpoint to demonstrate correlations between the potencies of AChE inhibitors (24 compounds total) in *C. elegans* to those recorded in mammals (Cole et al. 2004; Melstrom and Williams 2007). We have also demonstrated the precision of measuring movement by recording a difference in the movement of mutants lacking one of the two principle AChE genes, ace-1 and ace-2, and by measuring a difference in the sensitivity of these two mutants to an AChE inhibitor (Chapter 4). This experiment evaluated the similarities in response following exposure to compounds affecting the ACh-activated receptors of *C. elegans*.

Because mAChRs are not as well characterized as the nAChRs, our emphasis in this experiment was on characterizing the responses of the nAChRs. However, we did test one common mAChR antagonist, atropine, and found similar results to mammals and other nematodes. Atropine was able to reduce the decrease in movement caused by aldoxycarb, which may suggest a role in movement of at least some of the mAChRs. When co-exposed with aldoxycarb, nicotine - a former nematicide, increased aldoxycarb's toxicity at higher concentrations, as measured by synergistic movement decline. Levamisole, a currently-used nematicide, also caused increased toxicity via synergistic movement decline after co-exposure to aldoxycarb, but was approximately 4 orders of magnitude more potent than nicotine. Given the

magnitude of this difference, this may suggest that the L-type receptors are more involved with movement even considering kinetic differences between the compounds, such as absorption, distribution, and binding affinity. Finally, the fact that co-exposure to aldoxycarb and either nicotine, levamisole or hexamethonium produced effects consistent with those observed in vertebrates and other nematodes suggests that these compounds act, mechanistically, in a similar manner in *C. elegans*, and we were able to record these effects using movement as an endpoint.

## Summary

Measuring movement was sufficiently precise to assess the effects of compounds on the ACh-activated receptors of *C. elegans*. Because measuring movement is automated, quantitative and reproducible, it may be a useful method in high or medium-throughput assays involving the cholinergic system.

**Acknowledgements**

We would like to thank Dr. David Dusenberry for the use of his tracking program; the Interdisciplinary Toxicology Program at the University of Georgia for partial funding of this project; the *Caenorhabditis* Genetics Center (CGC), which is funded by the NIH National Center for Research Resources (NCRR), for supplying the nematode strain used in this research.

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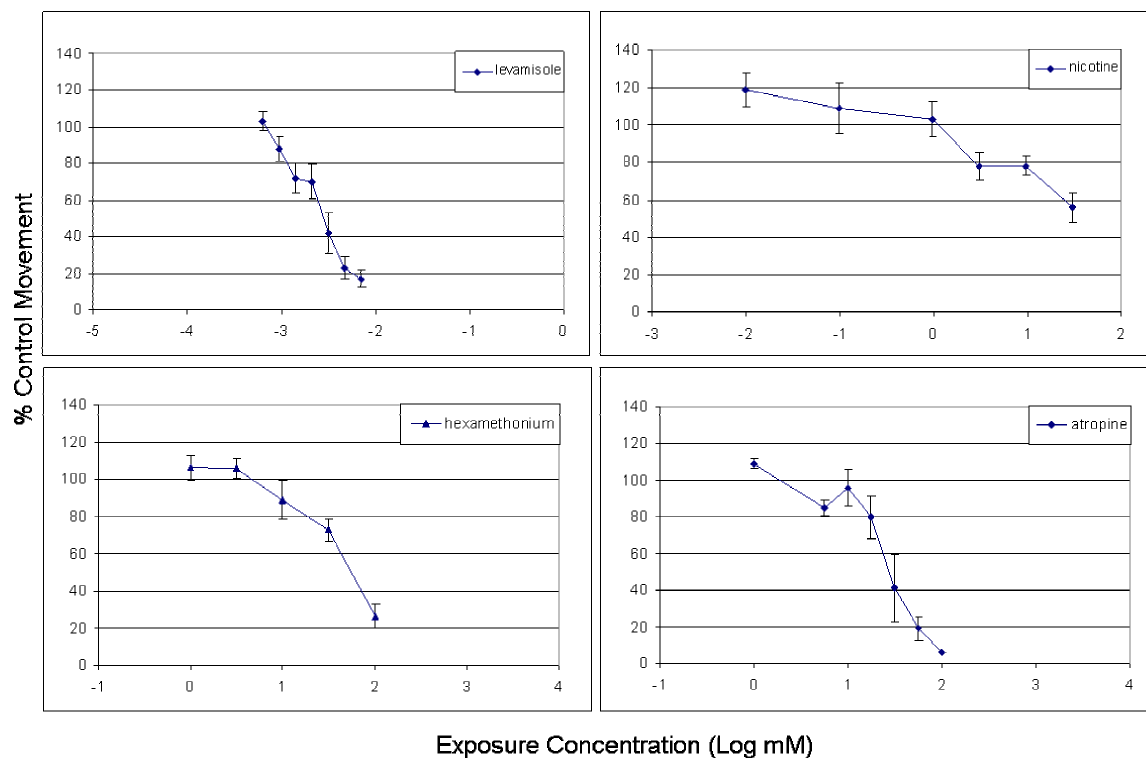


Figure 5.1 – *Plot of % Control movement vs. Log mM of each compound. Each showed a concentration-dependent decrease in movement. The potency of nicotine was limited by its water solubility, as the highest concentration obtainable in water was tested. Although the concentration range differs for each plot, the % movement scales are identical for comparison.*

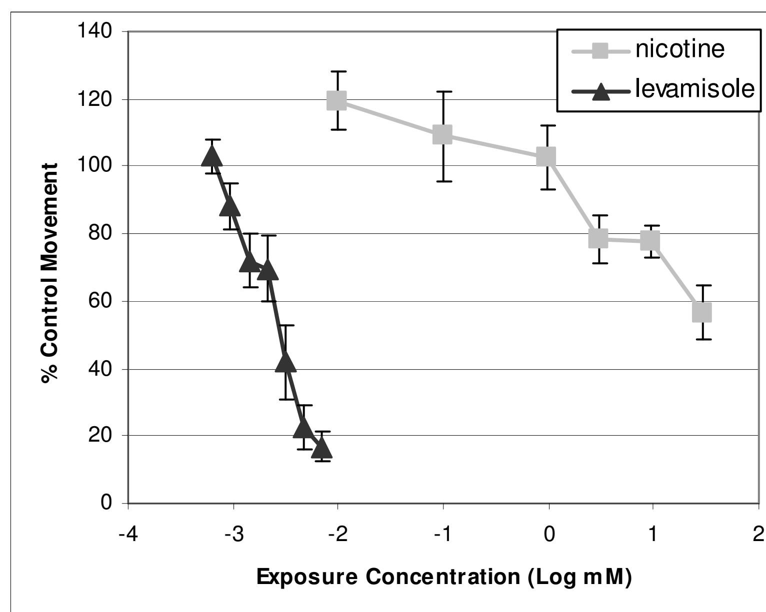


Figure 5.2 -- *A comparison plot of % Control movement vs. log mM for nicotine and levamisole.*

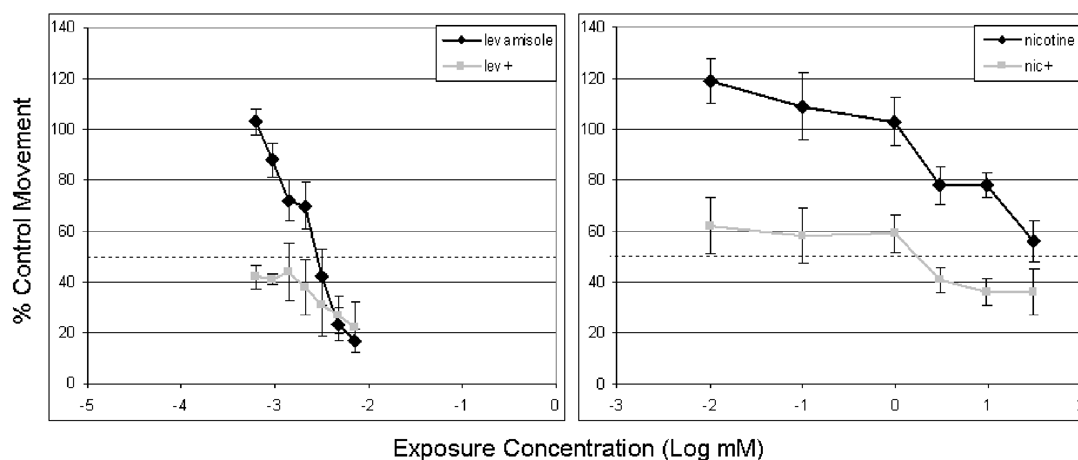


Figure 5.3 -- *Plots of % control movement vs. log mM for agonist compounds (black diamonds) alone and in combination with aldoxycarb (gray squares). The dashed line represents 50% movement, where the concentration of aldoxycarb reduces movement by itself. Both agonists caused synergistic toxicity.*

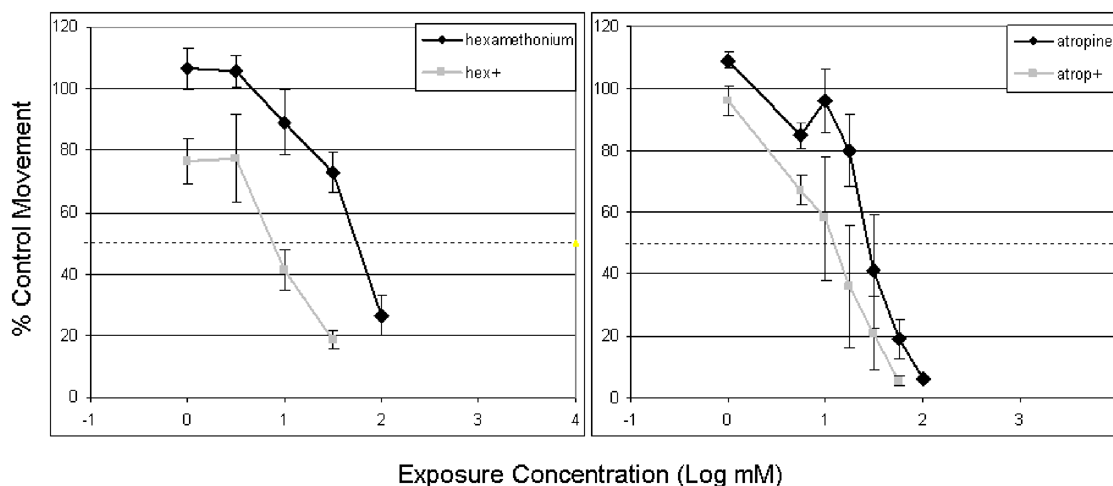


Figure 5.4 -- *Plots of % control movement vs. log mM for antagonist compounds (blue diamonds) alone and in combination with aldoxycarb (pink squares). The dash line represents 50% movement, where the concentration of aldoxycarb reduces movement by itself. Both agonists reduced the toxicity of aldoxycarb at lower concentrations.*

# CHAPTER 6

# ADAPTATION OF MOVEMENT ASSAY TO SOIL-BASED EXPOSURE[1]

[1] Melstrom, P.C., Williams, P.L. To be submitted to *Environmental Toxicology and Chemistry*.

**Abstract**

*Caenorhabditis elegans* is currently used as a toxicity model for soil-based exposures using lethality. Although movement has been successfully used as an endpoint for behavioral toxicity in an aqueous medium for a number of chemical classes, it has not been adapted to a soil medium. This chapter describes a preliminary attempt to adapt the method of measuring movement to a soil-based exposure using a newly developed approach to recover the worms. The new method of recovery was accomplished by a low-pressure, dilution of soil samples, i.e. flooding the worms out using ludox – a solvent that increases the buoyancy of worms and causes them to float out of the soil. We have managed to extract clean samples of worms from soil that are suitable for measurement of movement. Results obtained using unexposed worm samples show average movement rates between 1-1.25µm/s, which is below the average control rate of 3.52µm/s obtained following a 4 hour aqueous exposure in K+ medium. The next step in determining whether behavioral toxicity testing can be performed in a soil-based exposure is to determine whether average control movement rates can be raised high enough to distinguish effects at higher concentrations of compounds tested. We will begin by assessing whether a lack of food is responsible for relatively low control movement rates, then conduct a direct comparison of *C. elegans*' populations exposed' to ludox versus K-medium. If successful, the final phase will use organophosphate (OP) pesticides to establish movement-concentration curves following a soil-based exposure.

Key Words: *Caenorhabditis elegans*, soil, neurotoxicity, sub-lethal.

**Introduction**

*Caenorhabditis elegans* is currently used as a toxicity model for soil-based exposures (Boyd et al. 2000). The endpoint recorded in this ASTM method is lethality. Although movement has been successfully used as an endpoint for behavioral toxicity in an aqueous medium for a number of chemical classes, it has not been adapted to a soil medium. The biggest obstacle in doing so is the recovery of the worms. The samples must be clean in order to track their movements, yet repeated washings can stress the worms and alter their behavior. This chapter describes a preliminary attempt to adapt the method of measuring movement to a soil-based exposure using a newly developed approach to recover the worms.

Developing a method to recover worms from soil in order to employ the automated endpoint of movement would allow the measurement of a more sensitive endpoint vs. lethality, and perhaps more importantly, it could simplify the current method of placing exact counts of worms in soil and then having to recover exact counts in order to calculate a percentage of lethality. The number of worms recovered for movement tracking would not be important as long as a sufficient number of worms were recovered. Therefore, a main obstacle of learning and conducting the current ASTM soil toxicity assay could be circumvented.

The new method of recovery was accomplished by a low-pressure, dilution of soil samples, i.e. flooding the worms out using ludox – a solvent that increases the buoyancy of worms and causes them to float out of the soil (detailed below). In this way, the ludox solvent containing the worms can be removed without most of the denser soil particles, and then subsequently diluted in relatively larger volumes of aqueous solvent (dH2O or K-medium). This allows the worms to gravity settle and separates them from the lighter debris found in soil that floats to the top of the concentrated ludox solution.

**Preliminary Results and Discussion**

We have managed to extract clean samples of worms from soil following 24-hour incubations in K-medium that are suitable for measurement of movement (movement tracking). Encouragingly, the number and duration of washings necessary to prepare the samples for movement tracking are comparable to those of aqueous exposures. Preliminary results obtained using unexposed worm samples show average movement rates between 1-1.25µm/s. This is below the average control rate of 3.52µm/s obtained (Melstrom and Williams 2007) following a 4-hour aqueous exposure in K-medium. It is also unacceptably close to the background 'noise' of 0.06µm/s, which is the movement rate of solvent without worms that the program records.

One multi-concentration exposure in soil was performed using levamisole to determine whether a concentration-dependent decrease could be observed. A control (K-medium) and 5 concentrations relating to the concentration-movement curve obtained following an aqueous-based 4-hour exposure (Chapter 5) were used. Control movement rates were similar to those observed in previous experiments. Overall recorded movement rates were too low to show a pattern beyond the lowest concentration, which showed a marked decrease relative to control. Subsequent higher concentration groupings exhibited movement levels near the background level. The low movement values as a percentage of control could be the result of differences between concentrations required to decrease movement using a 24-hour exposure versus a 4-hour exposure that was used as the basis for concentration selection. Another potential factor may be that although 24-hour exposure testing is used in soil-tests measuring lethality, it is possible that there is insufficient food to conduct a movement test following a 24-hour exposure in soil.

Future research

The next step in determining whether behavioral toxicity testing can be performed in a soil-based exposure is to determine whether average control movement rates can be improved. Movement values as low as 1.5µm/s would reduce the percentage of background noise to <5%, and may allow for sufficiently precise comparison between concentration groups. We will begin by assessing whether a lack of food is responsible for relatively low control movement rates. To test this, we will concentrate an OP50 broth and add it to the soil samples to ensure an abundance of food is available for populations of *C. elegans*.

A successful outcome of this study is dependent upon the ability to measure control movement rates high enough to distinguish effects at higher concentrations of compounds tested. Movement rates of worms left in soil for 24 hours may prove to be less than those in worms left in an aqueous medium for 4 hours, however, since background tracking noise has been measured at a relatively low value of 0.06um/s, it would seem that only a modest improvement in control movement would be necessary to enable a soil-based adaptation of the behavioral toxicity test measuring movement. Should the adaptation to a soil-based exposure prove successful, the final step will use organophosphate pesticides to establish movement-concentration curves following a soil-based exposure.

**Materials and Methods**

Chemicals tested and exposure conditions

*Day 0*

Measure 2.33g of soil into a 35mm Petri dish. Add 0.816ml of fluid (either K-medium or chemical) for a combined weight of 3.146g STP. This is 35% moisture - dry weight. Cover with parafilm and incubate @ 20°C for 7 days to allow equilibrization to occur.

*Day 7*

Re-weigh each sample and add diluent to reach 3.146g total, minus the 0.040ml, which will is the volume of diluent used to add the worms. Eg. If the weight of the contents of the Petri dish after 7 days is 3.000, then you need to add 0.146ml of fluid. So, add 0.146 - 0.040 = 0.106ml of fluid. Then add the worm pellet of 0.040ml, cover with parafilm and incubate for 24 hours @ 20°C.

*Day 8*

Place soil-containing 35mm Petri dish in the middle of a clean, empty 115mm Petri dish. (figure 1) Extract worms by dripping ludox on to the center of the soil and gently swirling the Petri dish. When see worms floating in ludox solution and at least half of the volume of the 35mm Petri has been filled, add K-medium continuously until worm-containing solution is overflowing into larger Petri dish and the volume of the 35mm Petri dish is diluted out several times over, non-stop, to ensure capture of as many worms as possible. Remove 35mm Petri dish.

Using a pipette, transfer contents of 115mm Petri dish into a 50ml centrifuge tube containing approximately 30ml of dH2O or K-medium. Allow worms to gravity settle to the bottom. Then, pipette worms directly from the bottom of the tube, removing as little extra

solvent as possible and place them in a 15ml centrifuge tube containing ~10ml fresh diluent. Again, allow worms to gravity settle to the bottom. Again, pipette worms directly from the bottom of the tube, removing as little extra solvent as possible and place the worms into a 3ml centrifuge tube for final wash and subsequent placement on agar pad for tracking.

Culture of nematodes

We obtained *Caenorhabditis elegans*, wild type strain N2 from the Caenorhabditis Genetics Center (Minneapolis, MN, USA). All developmental stages of *C. elegans* were raised in 115-mm Petri dishes with K-agar (0.032M KCL, 0.051 M NaCl, 0.1 M $CaCl_2$, 0.1 M $MgSO_4$, 2.5% Bacto-peptone, 0.17% Bacto-agar, and 0.01% cholesterol), a modification of Williams and Dusenbery (1988) prior to exposure in soil. The plates were seeded with *Escherichia coli* strain OP50 as a food source and incubated at 20ºC for 24 hours (Brenner 1974.). Eggs were harvested from adult populations. Two-day age-synchronized juvenile populations were transferred to a plate containing a fresh lawn of OP50 for testing on day 3 to maintain high nutritional status (Boyd et al. 2003). All exposures were carried out using 3-day-old adults.

Movement tracking

The movement tracking was patterned after Boyd, et al (2000). Immediately following the soil exposure, we washed the worms as detailed above and transferred 5µl of the settled pellet (~50-80 worms) to a cooled, 2 ml 1% agar pad on a clear glass slide measuring 100mm X 200mm. The worms were allowed to disperse on the agar pad while inverted over a Petri dish filled with water to avoid desiccation. Movement tracking began at exactly 1 hour after the end of exposure for each replicate. The individual glass slides were placed in a tracking chamber with a gentle humidified air stream. Using a video camera interfaced with a Macintosh® computer that contains a modification of the NIH tracking software (Dusenbery 1996), the

individual worm movements were tracked and recorded to an Excel® spreadsheet. We calculated the average µm of movement per worm per second.

pH measurement

No effects of pH on *C. elegans*' movement have been observed between the pH range of 5.8 (K-medium, alone) to 3.5 (Cole et al. 2004). A buffer was not used for this reason, and to ensure environmental relevancy. We measured the pH values for the highest and lowest concentrations of each compound using an Orion Z20A pH meter at room temperature (22-24°C). (Orion research; Beverly, MA, USA.)

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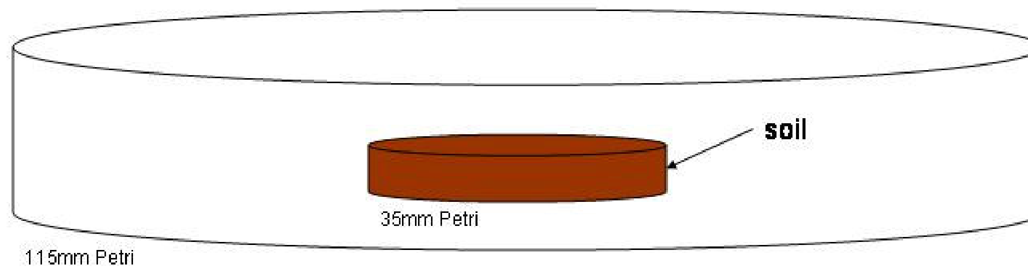


Figure 6-1 – *Diagram of worm recovery following soil exposure*.

# CHAPTER 7

# CONCLUSIONS

This dissertation explored using movement as an endpoint of toxicity to assess the feasibility of *C. elegans* as a toxicological model for compounds affecting molecular components of the cholinergic nervous system. The following steps were taken to test this hypothesis. First, *C. elegans* was exposed to the reversible AChE-inhibitor carbamates in order to determine whether movement could be used with reversible compounds by predicting their relative potencies as determined in mammals. Second, given the functional redundancy of *C. elegans*' AChE enzymes, movement was measured to assess whether there was a difference in the contributions of either of the two main classes of AChE enzymes, and whether this difference influences the sensitivity of measuring movement. Third, agonists and antagonists that are well-characterized in ACh-activated receptors of vertebrates and some nematode species were exposed to *C. elegans*, and measuring movement was used to determine whether their actions would be the same in *C. elegans*. Finally, a new method to recover worms from soil for subsequent measurement of movement was developed.

The following list summarizes the major results and conclusions of the dissertation.

1. <u>Conclusion</u>: by standardizing the post-exposure times when we measure movement, we can assess relative potencies of compounds inhibiting the AChE enzymes of *C. elegans*.

2. Conclusion: measuring movement decline in *C. elegans* is a dependable predictor of the relative potencies of AChE inhibitors.
3. Conclusion: there is a small, statistically-significant difference between the involvement of AChE A and AChE B classes towards movement, but this difference will not always dictate the sensitivity of measuring movement following exposure to an inhibitor.
4. Conclusion: initial evidence was presented that agonists and antagonists that are well-characterized in larger, parasitic species of nematodes and in vertebrates act in the same way, mechanistically, in *C. elegans*, and these actions can be recorded measuring movement.
5. Conclusion: Preliminary data suggests that *C. elegans* can be recovered from soil and assessed for movement using the new method illustrated in this dissertation. However, it is not yet known whether sufficient control movement values can be measured following a 24 hour exposure.

In view of these conclusions, my suggestions for future research include the following:

1. Studies directly comparing the sensitivities of different endpoints to movement.
2. Blinded studies to directly evaluate the predictive power of *C. elegans* as a model for vertebrate neurotoxicity.
3. Studies aimed at evaluating appropriate vehicles for testing more hydrophobic compounds.

4. Evaluation of mutant strains possessing different nAChR subtypes to determine whether movement could be used to assess differences in movement between them, as well as differences in their movement following exposure to a compound of interest.
5. Studies aimed at determining the cause of low control movement values in soil-recovered worms as detailed in chapter 6.
6. A soil-based exposure using organophosphate pesticides to compare to existing aqueous-based data.