Investigations on organophosphorus insecticide-induced neurotoxicity with *Caenorhabditis elegans* as a model system

A Thesis

Submitted to the Department of Bioscience

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In fulfillment of the requirement for the degree of

Doctor of Philosophy

By

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DECLARATION

I hereby declare that the thesis entitled **"Investigations on organophosphorus insecticide-induced neurotoxicity with *Caenorhabditis elegans* as a model system"** *submitted to the* **University of Mysore** *for the award of the degree of* **Doctor of Philosophy in Bioscience** *is the result of the research work carried out by me under the guidance of Dr. P.S.Rajini, Scientist, Food Protectants and Infestation Control Department, Central Food Technological Research Institute, Mysore during the period of 2003-2008.*

I further declare that these results have not been submitted for any other degree or fellowship.

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CERTIFICATE

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Dedicated to my beloved.....

Grand parents &

Parents

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LIST OF SYMBOLS AND ABBREVIATIONS

AChE	Acetylcholinesterase	μM	Micromolar
ACh	Acetylcholine	μmol	Micromole
BSA	Bovine serum albumin	μl	Microliter
cm	Centimeter	M	Molar
° C	Degree celsius	mol	Mole
CaE	carboxylesterase	ml	milliliter
d	Day	min	Minutes
DMSO	Dimethyl sulfoxide	mmole	millimole
DNA	Deoxyribonucleic Acid	ng	nanogram
DFP	Diisopropylphosphorofluoridate	nM	nanomolar
DDVP	Dimethyl dichlorovinyl phosphate	N	Normality
DTNB	5,5' -dithio-bis (2-nitrobenzoic acid)	O.D	Optical density
DTT	Dithiothreitol	ROS	Reactive oxygen species
EDTA	Ethylenediamine tetraacetic acid	rpm	Revolution/minute
GC-MS	Gas chromatography and mass spectrometry	SE	Standard Error
g	Gram	SD	Standard deviation
h	hour	SDS	Sodium dodecyl sulfate
HPLC	Highperformance liquid chromatography	TCA	Trichloroacetic acid
HCl	Hydrochloric acid	UV	Ultra violet
K_a	affinity constant	w/v	Weight by volume
K_p	phosphorylation constant	wk	Week
mg	milligram	v/v	Volume/volume
μg	Microgram	X g	gravity

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ABSTRACT

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ABSRACT

The main objectives of the thesis were to (a) develop a model system employing *Caenorhabditis elegans* for investigating the toxicity of selected organophosphorus insecticides (OPI) using multiple end-points (b) investigate the biochemical mechanisms involved in OPI toxicity in *C. elegans* (c) compare the pattern and extent of cholinesterase inhibition induced by OPI in *C. elegans* and rat brain homogenates *in vitro*. Our experimental approach involved behavioral, physiological and biochemical studies employing *C. elegans* as a model system. Initially we screened three OPI dichlorvos (DDVP), dimethoate (DM) and monocrotophos (MC) for their toxicity in N2 and (PC 72) strain of *C. elegans.* Results revealed that DDVP was most toxic and all the three OPI elicited Hsp response in the transgenic strain. A good correlation was evident between feeding inhibition, AChE inhibition and Hsp induction in dichlorvos treated worms. Dichlorvos at sublethal concentrations induced cessation in feeding and paralysis in *C. elegans* (N2) and these worms also hyper contracted their nose muscles and showed marked decrease in egg laying. The treated worms exhibited significant reduction in the AChE activity and increase in ACh content. Worms exposed to both dichlorvos + atropine showed inhibition in feeding as in worms treated with dichlorvos alone. Worms exposed to dichlorvos + atropine showed a concentration related decrease in the extent of paralysis while worms treated with dichlorvos + atropine followed by treatment with 2-PAM showed 100% recovery from AChE and feeding inhibition. Exposure to dichlorvos significantly decreased the brood size, decreased life span, increased reactive oxygen species and altered antioxidant status in the worms. The results of the qualitative analysis of the fatty acid methyl esters derived from lipids of the worms exposed to dichlorvos showed variations in the fatty acids. Exposure to dichlorvos resulted in significant accumulation of lipofuscin throughout body of the worm. Comparative kinetic studies with AChE of rat brain homogenate and *C. elegans* homogenate revealed inhibition of AChE to be of irreversible in nature in both the systems. AChE of *C. elegans* was more sensitive to inhibition by dichlorvos. A similar pattern of inhibition was also observed with carboxylesterase of *C. elegans*. In conclusion, data obtained from the present series of investigations support the premise that the nematode, *C. elegans* is a good model organism for studying the neurotoxicity of OPI, since this organism offers several simple and easily measurable physiological and biochemical endpoints for studying the mechanistic toxicity of the OPI.

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INTRODUCTION

Organophosphate compounds derived from phosphoric, phosphorous and phosphonic acids find application as pesticides, flame-retardants, therapeutic agents and nerve agents. Organophosphates (OP) being inhibitors of acetylcholinesterase (AChE), an enzyme involved in regulation of neurotransmission by hydrolysis of acetylcholine, induce accumulation of acetylcholine (ACh) at both muscarinic and nicotinic receptors in the central nervous system (CNS) and peripheral nervous system (PNS), leading to initial stimulation and eventually exhaustion of cholinergic synapses and subsequent overstimulation of both nicotinic and muscarinic acetylcholine receptors resulting in cholinergic effects. OP on chronic exposure affects several of the physiological systems, which include central nervous system, neuromuscular junctions, cardiovascular system, metabolic and endocrine systems including reproductive system.

Organophosphate compounds are neurotoxic and exert three types of neurotoxicity: cholinergic neurotoxicity, organophosphorous ester-induced delayed neurotoxicity (OPIDN) and organophophororus ester induced chronic neurotoxicity (OPICN). Cholinergic neurotoxicity results from the ability of organophosphate insecticide (OPI) to inhibit acetylcholinesterase leading to accumulation of ACh and overstimulation of nicotinic and muscarinic ACh receptors. OPIDN is characterized by neurodegenerative disorder with delayed onset of prolonged ataxia and upper motor neuron spasticity and is believed to be caused by inhibition of Neuropathy target esterase (NTE) and protein kinases. OPICN refers to chronic neurotoxicity associated with clinical signs and neurobehavioral abnormalities that persist long after exposure usually to single large toxic dose or small subclinical doses of OP compounds.

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Various *in vitro* models used for assessing neurotoxicity of chemicals provide advantages of direct exposure of cells to the test chemicals, ability to control environment, ease of evaluating intrinsic cell responses and availability of human origin *in vitro* models. Permanent neuronal cell lines are often utilized in assessing delayed neurotoxicity of OPI since these express neuropathy NTE and AChE. Re-aggregating brain cell culture are very promising model as it closely reproduces the in vivo like complexity and is used in detecting acute, chronic and delayed neurotoxic effects. More complex mammalian models such as embryonic stem cell derived embryonic bodies whole rat embryo models are utilized in assessing developmental neurotoxicity. Due to complex nature of *in vitro* model systems, it is often difficult to evaluate interaction of behavior and whole organism responses.

The development of alternate and newer model system of non-mammalian nature is always encouraged, as these can alleviate the complexity of cell models and whole animal responses can be evaluated. Zebrafish and sea urchin embryo are non-vertebrate models, which have been successfully used for assessing developmental neurotoxicity. Adult domestic hens of 8–14 months of age are often used as models for assessing neurotoxicity of OPI. However, toxicological assays using invertebrate species are more rapid and less expensive than traditional mammalian based tests, due to shorter life span and ability to assay in multi-well plate formats.

Caenorhabditis elegans is a free-living nematode with a thin transparent body approximately 1.2 mm in length and is a reliable model organism because it possesses (1) short life cycle (3 d at 20°C), (2) short life span, and (3) ease of rearing in the laboratory on agar medium. In addition, it is a simple multicellular eukaryote whose developmental process and behavior can easily be monitored. *C. elegans* also enables the detection of organism-level end points (feeding, reproduction, life span, and locomotion) and study the interaction of a chemical with multiple targets in an organism. Several studies have compared the toxicity of

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xenobiotics (metallic salts, solvents, and anesthetics) on *C. elegans* with those observed in mammals and reported strong similarities. Genetic and physiologic similarities have been established between the cholinergic nervous system of *C. elegans* and higher organisms.

C. elegans has been recently suggested as a mammalian neurological model based on its behavioral responses to OP and carbamate insecticides, since both have a comparable effect on AChE activity and decline in locomotion, as an indicator of the neurotoxicity. The LC_{50} values in *C. elegans* were closely correlated with LD_{50} in both rats and mice for the OP insecticides. A recent study using OPs found AChE inhibition to be the most sensitive indicator of toxicity and reduction in movement for 10 OPI and correlated to rat and mouse acute lethality data. Thus, *C. elegans* complements both *in vitro* and *in vivo* mammalian models in toxicology / neurotoxicology.

In the present study, attempts have been made to investigate the neurotoxicity of OPI using *Caenorhabditis elegans* as a model system. The basic objectives envisaged at the beginning of the investigations were.

i) To develop a model system employing *C. elegans* for investigating the toxicity of selected OPI using multiple end-points viz., lethality, movement, feeding, reproduction and growth following acute and multiple/ chronic exposure situations.

ii) To compare the pattern and extent of cholinesterase inhibition induced by OPI in *C. elegans* and rat brain homogenates *in vitro*.

iii) To investigate the biochemical mechanisms involved in OPI toxicity in *C. elegans.*

The thesis is presented in *four chapters*, preceded by a *General Introduction.*

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CHAPTER I

Toxicity of organophosphorus insecticides in *Caenorhabditis elegans*.

Organophosphorus insecticides (OPI) constitute one of the most widely used classes of pesticides worldwide. OPI are acutely toxic to all animals due to their effects on the nervous system. AChE inhibition by OPI leads to accumulation of acetylcholine, interfering with the function of the nervous system and depending on the dose may lead to the death of an organism. *C. elegans* has been frequently used in ecotoxicological studies. Toxicity studies with *C. elegans* in general have focused from the beginning not only on mortality but also on numerous physiological parameters such as reproduction, movement, feeding and growth. Currently it is well known that under stressful conditions (viz., exposure to high temperature, free radicals, xenobiotics or metals), cells from both eukaryotes and prokaryotes respond by synthesizing a family of proteins known as heat shock proteins (Hsp) which are believed to buffer cells from damage under adverse conditions.

This Chapter has been presented under two sections. *Section A* focuses on studies to determine the acute toxicity of the selected organophosphate insecticides viz; dichlorvos, dimethoate and monocrotophos in *C. elegans.* The 4h LC_{50} values obtained for dichlorvos, dimethoate and monocrotophos for the strain N2 were 16, 18 and 69 mM respectively. Based on these results, dichlorvos was selected for further mechanistic studies since it was found to be the most toxic among the tested OPI.

Section B describes the potential of dichlorvos to induce stress response in the transgenic *C. elegans* (PC72). This transgenic strain carries an integrated array of lac-z coding sequences, each inserted in frame into the second exon of *C. elegans hsp-16-1* gene allowing qualitative and quantitative assessment of heat shock response by β-galactosidase activity. Initially, we investigated the 4h LC_{50} of dichlorvos (17.0mM), dimethoate (17.4mM) and monocrotophos (66.5mM) in the

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transgenic (PC72) strain. The potential of these OPI to induce heat shock protein was investigated employing equimolar concentrations of the 3 OPI (5, 40 and 80μM). The reporter induction was evident as early as 4h after exposure to $CdCl_2$ (positive control), dichlorvos, dimethoate and monocrotophos. The maximal response obtained for the dichlorvos was after 24h of exposure (1.9 - 3.5 fold) and after 36h for dimethoate (0.25 - 1.91) and monocrotophos (5.44 - 7.42 fold). However, β-galactosidase activity in monocrotophos was consistently higher at all the exposure concentrations and exposure period, compared to that of dimethoate and dichlorvos. Further, dichlorvos induced a significant (69 - 90%) inhibition of AChE activity, accompanied by cessation in feeding (52 – 79%) after 4h, which was confirmed by the acrylic dye feeding. Interestingly, unexposed worms accumulated dye particles in pharynx with 10min, while dichlorvos treated worms showed no pigment particles in the pharynx clearly indicating cessation in feeding. *In situ* histochemical staining revealed β-galactosidase activity in the pharyngeal region after 24h exposure to dichlorvos. Linear regression analysis plots showed a good correlation between feeding inhibition and AChE inhibition, Hsp induction and AChE inhibition and feeding inhibition and Hsp induction. Collectively, these results provide evidences on the complex interrelationships between these three parameters and also on the impact of OPI on the vital physiological functions in *C. elegans*.

CHAPTER II

Mechanisms underlying the toxicity of dichlorvos in *C. elegans*.

OPI are irreversible inhibitors of acetylcholinesterase (AChE), an enzyme that is characteristic of the invertebrate/ vertebrate neuromuscular junctions responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh). ACh interacts with receptors on target cells and elicits a variety of post-synaptic responses. ACh accumulation in the synapses and neuromuscular junctions leads to over stimulation of cholinergic receptors that result in a general pattern of nerve poisoning, hyper excitability, tremors, and paralysis causing neurotoxic effects. In mammals, death is

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caused by asphyxiation, while the action in aquatic organisms including fish and invertebrates is less clear. In view of the above, we have attempted to understand the possible mechanisms underlying the OPI mediated toxicity in *C. elegans* which has been studied using dichlorvos as the model compound.

This Chapter has been presented under three sections. *Section A* focuses on the neurophysiological alterations induced in *C. elegans* following a 4h exposure to dichlorvos (5, 50, 100, 150 and 200μM). Inhibition of AChE was significant between 1- 4h of exposure and ranged between 32-82% at 5-200μM. Accumulation of ACh was also marked at all the tested concentrations (20 - 87%) after 2h of exposure. The extent of feeding inhibition after 4h was 52 – 79% which was also further confirmed by the uptake of red acrylic paint particles. Worms exposed to dichlorvos did not accumulate dye particles in pharynx. Further the percent of worms exhibiting contracted nose significantly increased with increasing concentrations and time of exposure. There was time and concentration related increase in the number of paralyzed worms (6 - 23%) at 1h and at the end of 4h (22 - 81%). The average number of eggs laid in the control group was 3 eggs/ worm, while worms exposed to dichlorvos at the highest dose (200μM) revealed significant inhibition in egg laying (1 or no eggs /worm). The percent decrease in egg laying at 5μM was 50% and at 200 μM was 69%. Taken together, these findings clearly demonstrate the potential of dichlorvos to induce neurophysiological and behavioural alterations in *C. elegans*.

Section B describes studies conducted to assess interactions of atropine and pralidoxime chloride (2-PAM) on *C. elegans* exposed to dichlorvos. Two approaches were adopted during the study. In the first approach, worms were exposed to dichlorvos (100μM), atropine (1, 5, 10 and 15mM) or dichlorvos (100μM) + atropine (1- 15mM) for 4h. After exposure, the worms were washed and observed for the extent of pharyngeal pumping, paralysis, body curvature, AChE inhibition and ACh accumulation. Worms co-treated with dichlorvos + atropine revealed remarkable recovery from paralysis especially at the higher concentrations of atropine. However,

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atropine did not appear to offer any protection against dichlorvos induced inhibition in feeding and AChE inhibition. Worms treated with both the compounds revealed greater body curvature as well as increased accumulation of ACh. These results suggested that atropine could significantly reverse dichlorvos-induced paralysis.

In the second approach, worms were exposed to dichlorvos (100µM) + atropine or atropine alone at concentrations as in earlier study. After 4 h of exposure, worms were washed and then exposed to 2-PAM at 10 and 15mM for 90 min. After the exposure period, the worms were evaluated as before. Treatment with PAM appeared to reverse AChE inhibition, reduce the accumulation of ACh and also reverse the inhibition in feeding. Collectively these findings suggest that the two antidotes together seem to offer significant protection against dichlorvos-induced effects in *C. elegans.*

Section C focuses on investigations aimed to ascertain the potential of dichlorvos to induce oxidative stress in *C. elegans.* Worms exposed to dichlorvos (5 - 200µM) for 4h revealed significant inhibition (45 - 85%) of acetylcholinesterase activity and marked inhibition (17 - 58%) of carboxylesterase activity. Marker of oxidative stress such as significant increase in the elevated levels of (ROS) (13 - 50%), depleted GSH content (14 – 24%) was accompanied by enhanced activities of superoxide dismutase (SOD) (38 - 56%), no alterations in glutathione S-transferase (GST) (6.0 - 24%) and diminished activities of catalase (34- 50%), glutathione peroxidase (GPX) (25 – 36%), glutathione reductase (42 - 57%). L4 stage worms on exposure to above concentrations of dichlorvos for 4h, exhibited decreased brood size (16 - 46%) and decreased lifespan (8 - 15% decrease on day 21). These findings clearly indicate the potential of dichlorvos to induce oxidative stress in *C. elegans* and good correlation with brood size and life span in these worms.

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CHAPTER III

Impact of low doses of dichlorvos in *C. elegans*: Biochemical investigations

OPI cause severe environmental contamination and potential health hazards including acute and sub-chronic cases of human accidental poisoning. The primary mechanism of acute toxic action of these compounds is the inhibition of AChE, which is responsible for degradation of the neurotransmitter acetylcholine leading to cholinergic over stimulation at nicotinic and muscarinic synapses of the peripheral, autonomic, and central nervous system. Besides AChE inhibition, several other biochemical targets have also been considered to contribute towards the acute lethal action and side effects of short- or long-term OPI exposure. Recent research indicated the implication of other systems such as GABAergic, cytokine, or serine hydrolases in the neurotoxic effects of OPI. Most acute symptoms induced by OPI resolve following cessation of exposure, although sometimes they have been documented to persist for days to months. OPI have been associated with an uncommon delayed onset of peripheral neuropathy, which can occur one to several weeks after an acute exposure. More controversial, however, is the question of chronic central nervous system (CNS) effects resulting from repeated exposure to pesticides. Despite their widespread and long-term use in the agricultural community, relatively few studies have investigated the potential health consequences of that use.

This Chapter has been presented under two sections. *Section A* focuses on the determination of LC_{50} values of dichlorvos for various exposure period (24, 36, 48 and 72h) and determination of AChE activity, fatty acid profile, dopamine content and body length as a function of age (0 - 72h) following exposure to dichlorvos (100µM). The LC_{50} values obtained for dichlorvos were: 16.00, 2.99, 1.66, 1.30 and 0.73 mM respectively at 4, 24, 36, 48 and 72h of exposure. Worms exposed to dichlorvos revealed marked decrease (50 - 67%) in body length. Inhibition of acetylcholinesterase increased with exposure duration (40 - 58%). Fatty acid profile

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was distinctly altered in worms exposed to dichlorvos compared to that of the control worms of same age. Similarly, the dopamine content was also significantly increased (97%) among the treated worms at 72 h of exposure. These results suggest the involvement of other biochemical targets such as fatty acids and dopamine, which probably seem to affect the developmental stages directly or indirectly in these worms.

Section B describes the studies on the effect of low doses of dichlorvos on *C. elegans* following chronic exposure (20d). The concentrations of dichlorvos selected for the study were based on their no-effect level on feeding i.e., concentrations in which worms fed normally: 0, 5, 100 and 200nM. The control worms exhibited an increase in basal acetylcholinesterase activity with age untill day 10, followed by a decrease in activity later. Dichlorvos caused inhibition in AChE (12 - 40% and 29 - 73%) on day 1 and day 10 respectively when compared to the activity of the control worms on those days. Maximum inhibition of AChE activity was evident at day 10.

Carboxylesterase (CaE) is a detoxifying enzyme for OPI. An increase in carboxylesterase activity was evident in untreated worms up to day 5, beyond which the enzyme activity decreased drastically. Interestingly, in worms exposed to dichlorvos, the enzyme activity was significantly higher than control worms on day1. On day 5 – 15, the CaE activity in worms exposed to 5 and 100nM was on par with that in control worms. The enzyme activity in worms exposed to 200nM at all the time intervals was lower than that in control worms. Interestingly, the enzyme activity in worms exposed to 5nM dichlorvos was higher than control beyond day 5.

Age-dependent increase in lipofuscin content was observed among the control worms. Lipofuscin accumulation on days 10, 15 and 20 was 2.3, 3.4 and 3.8 fold higher in comparison with 5 d old worms. Interestingly, remarkable concentration and age dependent increase in lipofuscin accumulation was observed on exposure to dichlorvos. Dichlorvos at 5,100 and 200nM after 5d of exposure

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caused 1.3, 4.6 and 5.3 fold increase in lipofuscin levels compared to controls. Similarly lipofuscin accumulation on day 20 at 5,100 and 200nM was 0.6, 1.1 and 1.2 fold higher compared to controls. While lipofuscin accumulation in control worms was restricted to intestine only. Exposure to dichlorvos resulted in significant fluorescence throughout the intestine of *C. elegans*. These data suggest that chronic low-level exposure to dichlorvos leads to inhibition of acetylcholinesterase, carboxylesterase and accumulation of ageing marker lipofuscin in *C. elegans.*

CHAPTER IV

Responses of selected enzymes to dichlorvos: A comparative *in vitro* study in rat brain and / or *C. elegans* homogenates.

Acetylcholine (ACh) is the primary excitatory neurotransmitter involved in *C. elegans* motor function, as in higher animals. While vertebrates have a single AChE gene whose product can undergo alternative splicing post-transcriptionally to yield different forms. *C. elegans* has four separate genes (ACE-1, ACE-2, ACE-3 and ACE-4) yielding four acetylcholinesterase enzymes. Two of these enzymes, ACE-1 and ACE-2, account for roughly 95% of all AChE activity in *C. elegans* and they share 35% identity, ACE-1 and ACE-2 display a high degree of functional redundancy. OPI are well known inhibitors of esterase, and their effects on esterase activity are used as the primary means for classifying these enzymes. Inhibition of CaE by OPI has been documented both *in vivo* and *in vitro* and irreversible inhibition of CaE by OPI leads to sequestration of the inhibitor molecule, which is considered a potential non-catalytic detoxification mechanism. In view of the great interest in the role of carboxylesterases in the detoxification of oraganophosphorus compounds and xenobiotics, several investigators have purified the enzyme from insects and studied its properties. There is a lack of data on toxicity mechanism and detoxification of OPI in *C. elegans*. In this study, we have attempted to study the kinetics of both the enzymes in homogenate systems.

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This Chapter has been presented under two sections. *Section A* focuses on the kinetics of AChE in rat brain and *C. elegans* homogenate system. AChE in both systems exhibited substrate saturation, rat brain at 1.5mM and *C .elegans* at 5mM of substrate acetylthiocholine iodide (ATCI). K_m for rat brain AChE was 59 μM and for *C. elegans* AChE was 725.41μM. V_{max} values for rat AChE and *C .elegans* AChE were 112.4 and 33-nmol/min/mg protein, suggesting the higher efficiency of the former. The IC_{50} for *C. elegans* and rat brain AChE were 45.7 nM and 107.2 nM respectively. Kinetics of enzyme inhibition from Lineweaver – Burk plot, the K_m, V_{max} and V_{max}/ K_m values for rat brain (57.48μM, 42.25 nmol of ATCI hydrolysed/min/mg protein,0.74 min/mg protein) and for *C. elegans* (734μM, 10.1nmole of ATCI hydrolysed/min/mg protein, 0.013 min/mg protein) at IC_{50} concentration of dichlorvos. Inhibitory kinetics from Lineweaver-Burk plot revealed that the inhibition of AChE by dichlorvos in both model systems was irreversible and *C. elegans* AChE was more sensitive to dichlorvos (lower IC_{50} value) compared to rat brain AChE.

Section B focuses studies on the kinetics of CaE in *C. elegans.* The substrate saturation of CaE obtained for p-nitrophenyl acetate (PNPA) was in excess of 350μM. The Km and Vmax of *C. elegans* CaE were 21μM and 4.6 nmol / min / mg protein. The IC_{50} obtained with dichlorvos was 83nM and the Lineweaver- Burk plot for DDVP inhibition at IC_{50} concentration, revealed apparent K_m and V_{max} as 19.57μM and 2.88 nmol/min/mg protein and suggests irreversible inhibition.

Point wise summary has been included at the end of each chapter. A section listing point-wise overall conclusions has also been presented. At the end of the thesis, a comprehensive list of cited references is included.

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AIM AND SCOPE

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AIM AND SCOPE OF THE PRESENT INVESTIGATION

Organophosphorus insecticides (OPI) constitute one of the most widely used classes of pesticides worldwide. OPI are acutely toxic to all animals due to their effects on the nervous system. OPI are irreversible inhibitors of acetylcholinesterase (AChE), there by accumulation of acetylcholine (ACh) in the synapses and neuromuscular junctions leading to over stimulation of cholinergic receptors resulting in general pattern of nerve poisoning, hyper excitability, tremors, and paralysis causing neurotoxic effects. In mammals, death is caused by asphyxiation, while the action in aquatic organisms including fish and invertebrates is less clear.

Several *in vitro* and *in vivo* models are used for the determination of effects of OPI and their neurotoxicity mechanisms. Demerits of *in vitro* models are that they are complex, injures directly to cells, their is no interaction between other systems and effects on behavior studies is not possible and raises concerns about integrated systemic functions that is missing critical cell–cell or gene–environment interactions, metabolism, immune mediators, and endocrine function. The development of alternate and newer model system of non-mammalian nature is always encouraged, as these can alleviate the complexity of cell models and whole animal responses can be evaluated. Zebrafish and sea urchin embryo are non-vertebrate models, which have been successfully used for assessing developmental neurotoxicity. Zebra fish are not sensitive to some of the chemicals, sea urchin embryos lacks transparency and drosophila is not extensively utilized as a toxicity model. Adult domestic hens of 8–14 months of age are often used as models for assessing neurotoxicity of OPI, all the hens are not susceptible targets of neurotoxicity and response is age specific.

Toxicological assays using invertebrate species are more rapid and less expensive than traditional mammalian based tests, due to shorter life span and ability to assay in multi-well plate formats and it is now clear that the fundamental principles underlying these cellular events of neurodevelopment are remarkably conserved across species ranging from the nematode (*Caenorhabditis elegans*) to

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humans. These advances provide the opportunity to develop neurotoxicity alternatives model.

C. elegans which is extensively studied and its genome, cell lineage and nervous system are mapped (302 neurons) and few of its advantages are short life span (20 days at 20º C), life cycle (3days), high rate of brood size, optical transparency, ease of culturing and exposure to toxicants, enables the detection of organism-level end points (feeding, reproduction, life span, and locomotion) and study the interaction of a chemical with multiple targets in an organism. Several studies have compared the toxicity of xenobiotics (metallic salts, solvents, and anesthetics) on *C. elegans* with those observed in mammals and reported strong similarities. Genetic and physiologic similarities have been established between the cholinergic nervous system of *C. elegans* and higher organisms.

Despite these advantages, *C. elegans* is not widely used as a neurotoxicity model. Hence, in this proposal, it is attempted to investigate the neurotoxicity of OPI using *Caenorhabditis elegans* as a model system. Thus the proposal basically addresses three fundamental questions viz: i) To develop a model system employing *C. elegans* for investigating the toxicity of selected OPI using multiple end-points viz., lethality, movement, feeding, reproduction and growth following acute and multiple/ chronic exposure situations. ii) To compare the pattern and extent of cholinesterase inhibition induced by OPI in *C. elegans* and rat brain homogenates *in vitro* iii) To investigate the biochemical mechanisms involved in OPI toxicity in *C. elegans.*

The data obtained from these investigations will provide (i) a comprehensive overview of the effects of OPI on *C. elegans* (ii) *In vitro* experimental evidences that *C. elegans* homogenate confers similar responses as that in mammalian model (iii) various biochemical, behavioral and physiological responses on exposure to OPI (iv) *C. elegans* as a useful model for assessing efficacy of antidotal drugs/molecules/ oximes on exposure to OPI.

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GENERAL INTRODUCTION

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ORGANOPHOSPHORUS INSECTICIDES (OPI)

Pesticides are used world wide to control pests and two-thirds of their total use is in agriculture. The WHO report on pesticides in agriculture estimated 220,000 deaths due to pesticide poisoning in 1990; 99% of these were in the developing world and 20,000 were unintentional (WHO, 1990). Chronic pesticide exposure is often a problem in occupational settings, particularly among poor rural populations, where men, women and children all work and live in close proximity to fields on which chemicals are applied and stored (Food and Agriculture Organization (FAO) of the United Nations/WHO, 2004; World Bank 2002). The pesticides most commonly used in agriculture are organophosphorus compounds (OP). There are more than 100 different OPs, and poisoning due to these chemicals, both intentional and unintentional, is a worldwide health problem; unintentional poisoning may disproportionately affect infants and children (WHO, 2004).

STRUCTURE OF OPI

The organophosphorus insecticides (OPI), which are of major commercial and toxicological interest, are esters or thiols derived from phosphoric, phosphonic, phosphinic or phosphoramidic acid. The basic chemical structure of OP is depicted in **Figure 1.** Usually, R1 and R2 are aryl or alkyl groups that are bonded to the phosphorus atom either directly (forming phosphinates), or through an oxygen or sulphur atom (forming phosphates or phosphothioates). In other cases, R1 is directly bonded to the phosphorus atom, and R2 is bonded to an oxygen or sulphur atom (forming phosphonates or thiophosphonates). In phosphoramidates, at least one of these groups is -NH2. The amino group of the phosphoramidates may be un-, mono- or bi-substituted. The atom double bonded with phosphorus is either oxygen or sulphur. Finally, the -X group may belong to a wide range of halogen, aliphatic, aromatic or heterocyclic groups (Sogorb and Vilanova, 2002). The -X group, also binding to the phosphorus atom through an oxygen or sulphur atom is

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called 'leaving group', because, it is released from the phosphorus atom when the OP is hydrolysed by phosphotriesterases (PTE), or upon interaction with protein targets.

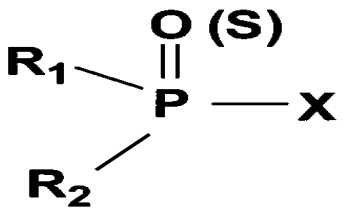

Figure 1. General chemical structure of OP

IMPORTANCE OF OPI

Organophosphorus (OP) compounds are utilized in a variety of household, agricultural, and industrial processes. They are primarily sold as pesticides, but have secondary markets as industrial plasticizers, fuel additives and chemical warfare agents. Insecticidal applications increase both large-scale monoculture crop and smaller-scale domestic garden and orchard yields by destroying herbivorous insect pests such as weevils and moths. Usage of OP compounds also destroys insects that would decrease the aesthetic value of home landscapes (Japanese beetles and Gypsy moths), the structural stability of human residences (termites and carpenter ants), and the quality of interior home life (cockroaches and silverfish). OPI are additionally been used in public health applications to control insect vectors that might cause pathogenic changes in a host species. Veterinarians use many of the OP compounds as anthelmintics and anti-flea and tick treatments (Booth and McDonald, 1982). OP compounds are also used in medicinal applications. Ecothiophate iodide and isofluorophate can relieve the intraocular pressure associated with glaucoma. Following application of these compounds to the conjunctiva of the

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eye, constriction of the ciliary and iris muscle decreases and allows intraocular aqueous to be resorbed more efficiently at the canal of Schlemm thus reducing the intraocular pressure (Koelle, 1994). Investigations into various human neuropathies also benefit from the use of OP compounds. Administration of these compounds to animal models generates a substantial amount of information on axonal transport, their interaction with cytoskeletal elements, and their implications in neurodegeneration.

OPI RESIDUES

Presence of pesticide residues in fruits and vegetables has become a global phenomenon. Frank et al., (1987) reported residues of OC and OP insecticides along with fungicides and herbicides in fruits from Ontario, Canada. Recent studies of Kumari et al., (2006) revealed the extent of pesticide residues in Indian fruits and vegetable samples. Fruit samples processed to determine residues of some commonly used OPI viz. monocrotophos, methyl parathion, chlorpyrifos and quinalphos revealed that among the tested OPI, quinalphos was a dominant contaminant with guava containing maximum residues. The probable reason of more frequent residues of quinalphos was attributed to its use on the tested fruits for controlling fruit fly attack close to harvest stage. However, all OPI detected were well within their respective maximum residue limit (MRL) values. Presence of OPI residues in vegetables and fruits is indicative of change in usage pattern of insecticides in India where a shift has taken place from OC to the easily degradable groups of these insecticides in the last decade.

MODE OF ACTION OF OPI

The OPI exert their main toxicological effects through non-reversible phosphorylation of esterases in the central nervous system (Aldridge and Reiner, 1972), and there by inhibiting the acetylcholinesterase enzyme (AChE), which catalyzes the hydrolysis of the neurotransmitter acetylcholine (Landes and Yu, 1995; Pope, 1999). AChE inhibition by OP insecticides leads to

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accumulation of acetylcholine, interfering with the function of the nervous system and leading to over-stimulation of nicotinic and muscarinic acethylcholine receptors. Depending on the doses, may lead to death (Eto, 1974). Reductions in AChE activity have been widely used to indicate exposure to OP in vertebrate and invertebrate species (Clegg and van Gemert, 1999). A number of studies found significant reductions in AChE activity, which correlated with effects on behavior that may impair subsequent survival of the exposed organisms (Fryday et al., 1996; Brewer et al., 2001).

DETOXIFICATION OF OPI

The OPI are mainly detoxified through oxidation and hydrolysis. The two main enzyme groups involved in the hydrolysis of these compounds are carboxylesterases (CaEs) and phosphotriesterases (PTEs). The OPI exert most important toxic effects through phosphorylation of serine residues in the active centers of CaEs (Aldridge and Reiner, 1972). The phosphorylation of AChE and Neuropathy Target Esterase are linked with toxic effects. However, in mammals (especially in liver and serum) there is a pool of CaEs of unknown physiological role, of which the inhibition does not cause apparent toxic effects. These esterases, defined as B-esterases, may be considered as enzymes involved in the detoxification of OPs and since, each molecule of CaEs is able to ‘scavenge’ a minimum of one molecule of insecticide before this reaches targets in the nervous system. This detoxification system is very efficient since, the resistance to OPs of some strains of cockroaches (Prabhakaran and Kamble, 1996) and flies (Smyth et al., 1994) is associated with an over expression of B-esterases that remove the insecticides from the media through phosphorylation of their active center.

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CHOLINERGIC EFFECTS OF OPI

Organophosphates being inhibitors of esterases particularly the AChE, lead to acute cholinergic crisis in the initial phase. Accumulation of ACh occurs at nerve endings, as AChE is inhibited, leading to initial stimulation and eventually exhaustion of cholinergic synapses. The clinical findings are thereby a mixture of muscarinic effects resulting from the excitation of postganglionic parasympathetic activity, nicotinic effects resulting from accumulation of ACh at neuromuscular junctions and consequent depolarization and central nervous system effects causing initial excitation and subsequent inhibition of all CNS activity (Sharma, 2000).

CLINICAL EFFECTS OF OPI

The symptoms and signs of OP poisoning may be divided into three groups, muscarinic, mediated by muscarinic receptors in parasympathetic effector organs, nicotinic, mediated by nicotinic receptors in autonomic ganglia and the neuromuscular junction, and effects in the central nervous system, which are mediated by receptors of both types (Marrs, 2007).

Muscarinic receptor symptoms

The muscarinic symptoms and signs on exposure to OP are the result of increased activity of the parasympathetic system and include bronchorrhoea, salivation, constriction of the pupil of the eye (miosis), abdominal colic and bradycardia (Grob and Harvey, 1953) and leading to diarrhoea, lachrymation, salivation, bronchospasm and urination. However, depending upon the balance between the nicotinic and the muscarinic effects, the affected may have hypertension and tachycardia occurring due to nicotinic actions rather than hypotension and bradycardia (Mars, 2007).

Nicotinic receptor symptoms

The nicotinic receptors activated during acute intoxication lead to muscle paralysis. Fasciculation may be seen and is a reliable sign of poisoning.

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Progression of paralysis may occur and the muscles of respiration may get affected. The mechanism of action of paralysis is depolarization and desensitization blocks induced by acetylcholine at the neuromuscular junctions. Severe intoxication may cause emotional irritability, mental obtundation, cognitive impairment, coma and convulsions because of CNS effects. In the cholinergic phase, paralysis usually passes off within 48 - 72 hours but complete clinical recovery from all the effects may take up to a week after exposure to these compounds (Chang et al., 1990).

ACUTE TOXICITY

The extent and effects of toxicity due to acute OPI poisoning are well documented in terms of clinical syndromes, assessment of severity and complications (Senanayake et al., 1993; Eddleston et al., 2005) and treatment (de Silva et al., 1992; Buckley et al., 2005), and are the subjects of extensive ongoing research.

Organophosphorus compounds exert acute systemic toxicity by inhibiting the enzyme acetylcholinesterase (AChE) through a process of phosphorylation. This leads to an accumulation of the neurotransmitter acetylcholine at nerve endings, which produces the common signs of OPI intoxication. The harmful effects of acute exposure manifest as an acute cholinergic crisis, an intermediate syndrome consisting of paralysis of the neck, proximal limb and respiratory muscles 24 to 96 h after the cholinergic crisis, and a delayed onset distal polyneuropathy 2-3 weeks after poisoning. Restoration of AChE activity occurs by de-novo biosynthesis of new enzyme, slow spontaneous dephosphorylation of the inhibited enzyme, and as a result of treatment with oximes. The rate and extent of dephosphorylation of the inhibited enzyme and its subsequent reactivation depends on the exact structure of the OPI residue and the treatment given. Besides their inhibitory effects on AChE, there is increasing evidence that OPI also induce oxidative stress through generation of

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free oxygen radicals, leading to lipid peroxidation and DNA damage (Bagchi et al., 1995; Gultekin et al., 2000; Vidyasagar et al., 2004).

Acute intoxication with anticholinesterases produces a complex mixture of muscarinic and nicotinic signs which vary in relative severity with target organ tissue, dose, and agent (Marrs, 1993). An 'intermediate syndrome' has also been described as a late complication of some cases of severe acute poisoning (Senanayake and Karalliede, 1987). It is characterized by a specifically proximal muscle weakness lasting 5-18 days which can lead to respiratory failure. A further effect produced by some, but not all, organophosphorus esters is organophosphorus ester-induced delayed polyneuropathy (OPIDPN). This is characterized by a delayed onset 10-14 days after exposure (Richardson, 1995). The lesion is a highly selective distal axonopathy affecting the distal axons of sensory, motor, and autonomic neurons, and the longer central tracts. The lower motor neuron axonopathy is reversible, but not that of the upper motor neuron (Vasilesceu and Floresceu, 1980). Within the central nervous system excitotoxicity has been described as a consequence of frank poisoning. Pathogenesis probably involves a combination of hypoxia and cholinergically mediated excitotoxicity with secondary recruitment of glutamatergic excitotoxicity (Solberg and Belkin, 1997). This is a high dose effect, which has only been produced by exposures sufficient to produce clear signs of poisoning.

Toxic mechanisms

Any persisting effects of exposures that were high enough to cause frank poisoning but not high enough to cause effects mediated by trauma, excitotoxicity or hypoxia, might involve a lasting down regulation of receptors secondary to cholinesterase inhibition. In addition there is good *in vitro* evidence of a non-anticholinesterase mediated action of some organophosphorus pesticides and carbamates on muscarinic, nicotinic, glutamatergic and GABAergic synapses (Camara et al., 1997). However, the relevance of these findings to the clinical or even the experimental animal situation is far from clear, especially since methamidophos, which does not act

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directly on receptors, still produces the full range of acute,intermediate, and delayed polyneuropathic syndromes in man (Camara et al., 1997).

For peripheral neuropathy, the primary biological target (for OPIDPN) is a protein known as neuropathy target esterase (NTE). This is a protein which, within the nervous system, is specifically localized to neurons (Glynn et al., 1998). Its biological function, other than as a target for neuropathic organophosphates, is unknown but NTE mutations are known to affect brain development in *Drosophila* (Lush et al., 1998). Many studies have shown that the nature of the toxicity (i.e. acute cholinergic or OPIDPN). Produced by an agent can be predicted by its relative ability to interact either with acetylcholinesterase AChE or with NTE (Lotti and Johnson, (1978)

CHRONIC TOXICITY

There is a paucity of data on the possible deleterious effects of chronic exposure to OPIs in occupational and/or environmental settings. In general, the literature brings out three types of 'non-acute' OPI poisoning: occupational exposure with reductions in ChE levels; occupational exposure with no reduction in ChE levels; and environmental exposure. However, the relationships between chronic exposure, ChE inhibition and symptoms do not, as yet, seem to be well established. Available evidence suggests that there is a possibility of adverse effects occurring below OPI concentrations that are generally considered to be safe based on measurements of ChE inhibition; i.e. these effects are not clearly related to the inhibition of cholinesterases (Ames et al., 1989; Popendorf, 1990; Salvi et al., 2003). Studies on health hazards to agricultural workers who handle, store and use OPI pesticides have documented a range of non-specific self-reported symptoms that have been attributed to chronic exposure. These include: burning or prickling of the skin; tingling or numbness of hands and face; muscular twitching or cramps in the face, neck, arms and legs; respiratory symptoms, including chest pain, cough, runny nose, wheezing, shortness of breath, irritation of the throat; excessive

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sweating; nausea, vomiting, diarrhoea; excessive salivation; abdominal pain; lacrimation and irritation of the eyes; difficulty in seeing; restlessness; difficulty in falling asleep; trembling of hands; and irritability (Ames et al., 1989; Ohayo-Mitoko et al., 2000). An increased prevalence of symptoms was found at ChE activities generally considered to be non-adverse (Ohayo-Mitoko et al., 2000). However, detailed, long-term studies of occupational or environmental exposures to OPI are needed to distinguish the effects of the active OPI from the solvent or other components of the OPI. Subtle abnormalities on neurological examination, such as impaired two-point discrimination and vibration sensation, have also been reported in workers chronically exposed to OPIs (Beach et al., 1996; Stokes et al., 1995). More objective investigations into the effects of chronic OPI exposure on nerve conduction and neuromuscular transmission are inconclusive; some report evidence of nerve abnormalities during occupational exposure (Stalberg et al., 1978) while others do not (Enegel et al., 1998).

Mechanisms of chronic toxicity

Chronic toxicity to OPIs may be related to the rate of regeneration of AChE and the speed at which pesticide metabolites are hydrolysed and eliminated from the body. This ‘fast’ or ‘slow’ enzymatic hydrolysis status seems to be determined by gene polymorphisms of hydrolases such as paraoxonase (Lee et al., 2003). However, in some situations where there is chronic exposure to OPI, there seems to be poor correlation between evidence of toxicity and the degree of AChE inhibition (Ray and Richards, 2001). It may well be that toxicity in these situations is mediated more by other mechanisms, such as oxidative stress through OPI-induced generation of free oxygen radicals leading to lipid peroxidation rather than inhibition of AChE (Gultekin et al., 2000; Bebe and Panemangalore, 2003).

A mechanistic explanation of possible longer term neurobehavioural consequences, either of mild poisoning or of low level exposures, is largely lacking at present, but several partially characterized brain proteins

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(Richards et al., 1998), cognitive enhancement appears to be the lowest dose effect conclusively demonstrable for some cholinesterase inhibitors on the brain.

Concerns about major adverse health effects of low level exposure to anticholinesterases in general seem entirely unwarranted on the basis of currently available data, but this is at present insufficient to rule out the possibility of subtle, agent-specific effects.

Neurobehavioural effects in chronic toxicity

Chronic exposure to OPI has been associated with impaired neurobehavioural performance in some, but not all, epidemiological studies (Ray and Richards, 2001). Chronic organophosphate-induced neuropsychiatric disorders (COPIND) occur without cholinergic symptoms, and although the underlying mechanisms are not established, they do not seem dependent on AChE inhibition (Brown and Brix, 1998; Prendergast et al., 1998: Ray and Richards, 2001). Clinical features reported include: anxiety disorder; depression; psychotic symptoms; dysthymic disorder (DSM-III-R); problems with short-term memory, learning, attention, information processing, eye hand coordination and reaction time; autonomic dysfunction; and enduring extra pyramidal symptoms (Salvi et al., 2003). Similar clinical features have also been reported by soldiers suffering from the much-publicized Gulf-War Syndrome, which led to the, so far unproven, hypothesis that the illness was caused by chronic exposure to chemical agents with similar effects to OPIs (Gronseth, 2005). Despite these reports of impaired neurobehavioural patterns following chronic exposure to OPI, other reports indicate that asymptomatic exposure to OPI is not connected with an increasing risk of delayed or permanent neuropsychopathological effects (Eyer, 1995).

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NEUROPATHOLOGICAL ALTERATIONS INDUCED BY OPI

Neuropathological lesions induced by OPI have been characterized by axonal degeneration, seen in the cerebral cortex, basal ganglia, thalamus, sub thalamic region, hypothalamus, hippocampus, fornix, septum, preoptic area, superior olliculus, pretectal area, basilar pontine nuclei, medullary tegmentum, and corticospinal tracts. Neuronal degeneration of hippocampal cells is consistent with organophosphorus compound-induced alterations in behavior, and cognitive deficits such as impaired learning and memory (Bushnell et al., 1991; Kassa et al, 2001), Furthermore, chronic exposure to organophosphorus compounds resulted in long-term cognitive deficits, even in the absence of clinical signs of acute cholinergic toxicity (Prendergast et al, 1997, 1998).

Acetylcholinesterase inhibition and behavior

A frequently studied physiological correlate of behavioral change is brain cholinesterase activity. Beauvais et al. (2000) and Brewer et al. (2001) also found a correlation between changes in swimming speed and brain ChE activity induced by exposure to diazinon and malathion. Correlation between the interference of the organophosphate, DDVP, on feeding behavior and brain acetylcholinesterase was demonstrated in the bream (*Abramis brama*) by Pavlov et al., (1992). There may, however, exist different behavioural correlates if ChE activity is assayed in different tissues. Roex et al. (2003) observed that whole-body acetylcholinesterase inhibition by parathion correlated to increased food consumption in zebrafish (*Danio rerio*).

NON-ANTICHOLINESTERASE TARGETS OF OPI

The most important effects of OP nerve agents are anticholinesterase actions, however, OPs bind to a variety of enzymes, including esterases other than acetylcholinesterase, carboxylesterase, (long-chain fatty acid hydrolase), serine peptidases, amidases and proteases and others (Lockridge and Schopfer, 2005). Some evidence suggests that OP compounds can act directly on muscarinic and nicotinic receptors (Bakry et al., 1988; Silveira et al., 1990;

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Eldafrawi et al., 1992). More complex direct effects at cholinergic receptors may be important. It has also been demonstrated that OPs, affect neurotransmission pathways other than cholinergic ones, for example γ-aminobutyric acid (GABAergic) receptors as well as glutamatergic, dopaminergic, somatostatinergic and noradrenergic systems (Fletcher et al., 1989). It is likely that most if not all such perturbations are secondary to effects on cholinergic systems, but there is evidence that recruitment of non-cholinergic systems is responsible, at least in part, for seizure activity (Solberg and Belkin, 1997). Furthermore, the efficacy of some experimental treatments not directed at the cholinergic neurotransmission system suggests a role for other neurotransmission systems. Thus, Braitman and Sparenborg (1989) found that a potent antagonist at the *N*-methyl-d-aspartate (NMDA)-type glutamate receptor (MK-801), together with other antidotes, protected against seizures induced in guinea pigs by soman. Moreover, Filliat et al.(1999) found that learning deficits produced in rats by soman were reduced by antagonists at α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors and NMDA-type glutamate receptors, together with atropine. It has also been suggested that the protective action of caramiphen against OP poisoning may be, in part, due to the ability of this drug, which is also an anticholinergic agent, to modulate the NMDA receptor (Raveh et al.,1999). Kovacic (2003) suggested that oxidative stress might play a part in the toxic manifestations of OPs. It is possible that some of the CNS effects of nerve agents are secondary to changes in the blood brain barrier.

DEVELOPMENTAL NEUROTOXICITY OF OPI

AChE and other esterases play a role in neural development suggesting a potential for interference from exposure to anti-ChE compounds (Brimijoin and Koenigsberger, 1999; Garry, 2004). Chlorpyrifos, an OPI elicits developmental neurotoxicity at exposure levels below the threshold for lethality (Pope, 1999; Slotkin 1999, 2004; Weiss et al., 2004). Fetal or neonatal CPF exposures at levels below the threshold for systemic effects or even for

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cholinesterase inhibition (Slotkin, 2004), it is likely that CPF has a direct effect on neuronal differentiation, specifically compromising the development of the cholinergic phenotype. Developmental neurotoxicity of these agents represents a separable set of mechanisms that operate at lower exposures (Colborn, 2006).

OPI AND OXIDATIVE STRESS (OS)

Numerous studies have demonstrated enhanced oxidative stress (OS) due to OPs in rats and humans although the most prominent clinical effects of poisoning with OPs result due to inhibition of AChE. Ranjbar et al., (2002) studied OS status and AChE activity in blood samples obtained from OP formulatory pesticide workers with a minimum history of 1 year in the age range of 23 – 55 years. Their results showed marked inhibition of AChE activity and increased TBA reactive substances indicating lipid peroxidation. Subchronic exposure of malathion has been reported to enhance antioxidant enzymes, which were also accompanied by elevated levels of MDA in rat erythrocytes and liver (Akhgari et al., 2003; Abdollahi et al., 2004). Malathion has also been reported to increase plasma MDA content Yarsan et al., (1999) and enhance microsomal TBARS in rats (Pedrajas et al., 1995). Studies of Shadnia et al., (2005) also demonstrated that chronic exposure of humans to OPI was associated with increased activities of antioxidant enzymes such as, CAT, SOD, and GPX in erythrocytes. Gromov et al., (1993) studied the influence of dichlorvos on the activity of antioxidant enzymes such as SOD and CAT in brain and blood of female rats and found that CAT activity was lowered in brain 3 h after administration. Also Altuntas et al., (2002a, b) investigated the effects of methidathion on lipid peroxidation and antioxidant enzymes and ameliorating effects of a combination of vitamin E and C against the toxicity. Their results demonstrated that treatment of rats with methidathion increased the lipid peroxidation and decreased antioxidant enzymes in erythrocytes.

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OPI AND ANTIDOTES

Inhibition of acetylcholinesterase enzyme by OP results in an overabundance of ACh in the synaptic cleft and occurrence of neurotoxic symptoms, including convulsive seizures. The treatment of poisonings produced by AChE inhibitors has remained unchanged for many decades with the muscarinic antagonist atropine (ATR) being used as a primary antidote and AChE reactivators (oximes) are used in addition to Atropine. 2 - PAM is a known antidote for the reactivation of inhibited AChE in organophosphate poisoning. While atropine relieves muscarinic signs and symptoms, oximes are supposed to shorten the duration of respiratory muscle paralysis by reactivation of cholinesterases (Johnson et al., 2000). Reactivation of AChE achieved by oximes is primarily restricted to the peripheral nervous system since the polar nature of oximes makes penetration of the blood–brain barrier difficult (Wilson et al., 1992). In the past decades, many new AChE reactivators have been synthesized and approval of numerous new oximes, which were synthesized and tested concerning their efficacy as antidotes (Bismuth et al., 1992) is hampered by difficulties in drug evaluation. The investigation of oxime efficacy in vivo is not possible in humans for ethical reasons. Therefore, testing of new substances was primarily performed in animal experiments in the last decades Rodents and primates have traditionally been the animal models used for comparative investigations of the benefits of various oximes following OP intoxication (Clement., 1992; Clement and Erhardt., 1994).

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MODELS EMPLOYED FOR NEUROTOXICITY ASSESSMENT OF OPI

The discovery that humans are susceptible to OPIDN resulted from accidental exposure to an organophosphate led to the development of the hen model for OPIDN testing which is the only model for neuropathy used in routine screening studies and, the possibility that pesticide exposures can be risk factors for neurodegenerative diseases needs to be considered in epidemiological models, whether alone or in combination with other factors. Development of animal models to more completely assess for possible relationships between pesticide exposure and neurodegeneration in humans need to be developed and validated to render data useful for neurotoxicity risk assessment.

NON-MAMMALIAN MODELS OF NEUROTOXICITY

Current approaches for assessing neurotoxicity in mammals rely on behavioral, morphological (neurohistopathology) and biochemical assays. These are labor-intensive, require specialized training and are subjective. The application of biochemical markers for neurotoxicity testing is an area of ongoing research within the research community (McGrath and Li, 2008). Although several biochemical markers, including changes in enzyme activity and protein phosphorylation, have been examined, these approaches have not proven useful for detecting toxicity in specific types of neurons. Compared with histopathology, biochemical assays that correlate brain functions with metabolism have not been rigorously tested (Doherty et al., 1983). For the reasons mentioned above, development of rapid assay methods and new animal models to predict neurotoxicity are urgently needed.

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IN VITRO MODELS OF NEUROTOXICITY

In the field of neurobiology, *in vitro* cell culture techniques have been successfully developed and employed to address specific questions of cell biology and nervous system functioning and provide a means to systematically study complex nervous systems (Jacobson,1991). Such systems have two major and critical functions: (1) To understand the complexity of cellular functions of CNS elements (2) They provide a convenient experimental tool for testing possible functions or postulates *in vivo* that otherwise might not be conducted (Jacobson,1991).

Taking into account that the final approach should be easily transferable, reproducible and applicable for high throughput screening, cell lines are proven to be very valuable. They allow screening for specific mechanistic aspects and identification of certain cell death pathways (Westerink and Vijverberg, 2002; Madia et al., 2004). Therefore, they are recommended for the inclusion *In vitro* test batteries. A variety of cell line models have been used extensively due to the relative ease to be genotypically and phenotypically manipulated (Stingele et al., 1999; Coecke et al., 2002).

Promising *in vitro* models

Advantages of *In vitro* models for neurotoxic hazard assessment are the direct exposure of cells to test substances, the ability to control the environment, the possibility to evaluate the intrinsic cell response, and for the most recent models, the possibility to have *In vitro* models of human origin (Atterwill et al., 1994).

DEVELOPMENTAL NEUROTOXICITY

Promising *in vitro* strategies for gaining greater understanding of neurodevelopmental adverse effects are those that capture a broad developmental period and evaluate effects on different cells and regions at different stages of maturation. More complex mammalian models such as

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embryonic stem cell derived embryoid bodies (Spielmann et al., 1997; Bremer and Hartung, 2004) whole rat embryo models (Freeman and Steele, 1986), reaggregating brain cell cultures (Monnet-Tschudi et al., 2000), micromass test (Flint and Orton,1984) and non-mammalian vertebrate models such as zebrafish (Levin et al., 2004) as well as non-vertebrate models such as sea urchin embryo's (Manzo, 2004) are promising models for screening a wide range of potentially developmental neurotoxic compounds (**Table 1**). More recent models such as normal human neural progenitor cells, human stem cell lines and human cord blood derived models (Klemm and Schrattenholz, 2004) could eliminate species differences in toxic responses and proceed towards the development of human based *in vitro* neurodevelopmental toxicity test systems.

Table 1 Characteristics of *in vitro* models for neurotoxicity assessment (Coecke et al., 2006)

	In vivo-like complexity	Reduction of animals	Neuro behaviour	Neuro Pathlogy	Neuro Physiology	Neuro Chemistry	Handling reproducibility
In vivo	+++++	-	+++++	+++++	+++++	+++++	++
slices	++++	+(+)	-	++++	+++	+++++	+++
aggregates	+++(+)	+++	-	+++	+++	+++++	+++
mixed primary cultures	++(+)	+++	-	++	++	+++++	++++
primary cells	+(+)	+++	-	+	+	+++	++++
human cell lines	+	+++++	-	+	+	++	+++++
other cell lines	-	+++++	-	+	+	++	+++++

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ALTERNATIVE MODELS FOR DEVELOPMENTAL NEUROTOXICITY (DNT)

Developmental toxicity testing involves exposing pregnant animals, usually rats or rabbits, to compounds and subsequently assessing toxic effects on fetuses. Several alternative methods for assessing developmental toxicity have been developed, including *in vitro* cell differentiation assays using either primary cell cultures or immortalized cell lines, the *in vitro* rodent whole embryo culture test and the *in vivo* frog embryo teratogenesis assay (FETAX) (Marathe and Thomas., 1990). Unfortunately, *in vitro* tests have been of limited value in predicting the effect of drugs on human embryonic and fetal development, due to complexity of interdependent events in the developing nervous system raises concerns as to whether a DNT test battery based on a few well-defined end points reproduced *in vitro* using cell based models will be predictive of *in vivo* effects. Therefore, systems based models employing simple organisms that enable assessment of integrative effects may offer a more immediately viable and ultimately more powerful approach to DNT alternatives. Simple organisms that have been proposed as candidate DNT alternatives include: sea urchins, nematodes (*C. elegans*), flies (*Drosophila melanogaster*), fish [zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*)], and chick embryos (Lein, 2007).

A significant advantage of all these organisms is their small size, rapid embryonic development, and short life cycle, which will greatly reduce time and space costs. *C. elegans,* Zebrafish and medaka offer an advantage relative to the other models in that their embryos are optically transparent. It is possible to visualize dynamic changes in gene expression (using fluorescent reporter transgenic lines) and detailed morphogenetic movements as they occur in the live developing embryo. This provides the opportunity to study compensatory mechanisms in developing nervous systems exposed to neurotoxicants, and to obtain repeated measures in the same organism over time, there by reducing the number of animals required for testing.

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CAENORHABDITIS ELEGANS AS A MODEL ORGANISM

Active worm research was originated by Sydney Brenner and co-workers in the early 1960s. The reference strain named N2 was dug up from soil in Bristol. The transparency, small size (1 mm long) and ease of maintenance made the nematode *Caenorhabditis elegans* a good target for cellular analysis, completed by Sulston and Horvitz (1977) and Sulston et al. (1983). In nature, the nematode *C. elegans* lives in soil and feeds on bacteria. The worms grown in laboratory feeds on bacteria (*E. coli*) seeded on NGM-agar in a petri dish. The most favorable temperature for worms is 20°C and when grown at +15°C the lifespan of the worms lengthens and at +25°C it shortens. *C. elegans* has two sexes: hermaphrodite and male. Hermaphrodites produce ~300 eggs and when fertilized by males the number of eggs rises to ~1000. The *C. elegans* hermaphrodites have two X chromosomes (designated XX) and the males are hemizygous carrying only one X chromosome (designated XO). While males can arise from self-reproduction of hermaphrodites, mating with males increases the proportion of male progeny.

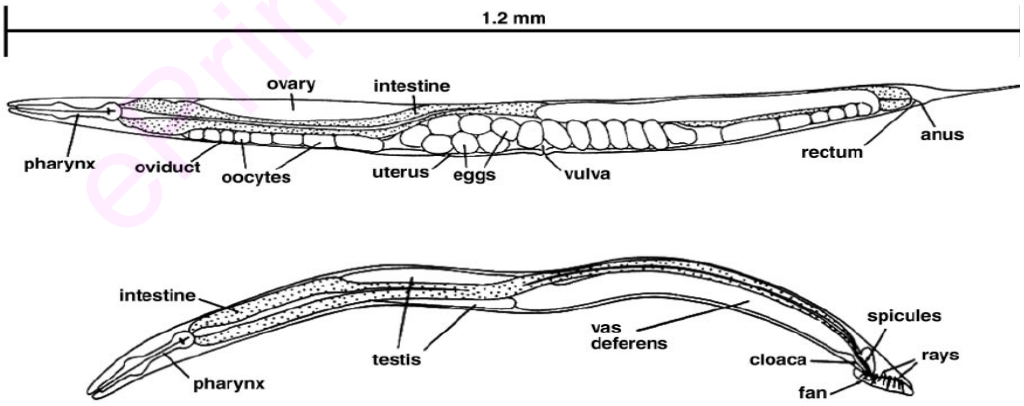

Figure 2 Anatomical features of an adult *C. elegans* hermaphrodite (top) and male (bottom) (Kevin Strange, 2003).

The lifespan of a worm is approximately three weeks and adulthood is reached in three days. The development of a worm from fertilization to adult is fully characterized and the cell lineage for every cell is known. During their development the worms undergo four larval stages (L1, L2, L3 and L4). *C. elegans* also has an option to go into the dauer stage if living conditions turn unfavorable. The worms can turn into dauers after the L2 stage and can stay in this developmental arrest stage having minimal metabolism for months waiting for the conditions to change. *C. elegans* was the first multicellular animal to have its genome sequenced (The *C. elegans* Sequencing Consortium, 1998). The completion of the *C. elegans* genome sequence without a gap was reached in 2002. The *C. elegans* genome consists of 100 Mb that encode 23,977 genes, 92.9% of these have molecular information, 33.7% are confirmed genes (www.wormbase.org). The genes are organized into five autosomes and one sex chromosome termed X. In addition to transparency, short life span and other advantageous features, the worms are complete organisms with nervous and reproductive system, muscles and digestion, thus providing an opportunity to study genetic and molecular functions and interactions in an easily manageable system (**Figure 2**).

NERVOUS SYSTEM OF *C. ELEGANS*

The nervous system of adult hermaphrodites contains 302 neurons and 56 glial and support cells. Males have 381 neurons and 92 glial and support cells. The nervous system of *C. elegans* is generally divided into the pharyngeal and central nervous systems. Twenty neurons innervate and regulate the activity of the pharynx. These neurons are connected to the central nervous system by two interneurons. Neuronal processes of the central nervous system are oriented as bundles that run along the hypodermis in longitudinal cords or circumferential commissures. The ventral and dorsal nerve cords emanate from the circumpharyngeal nerve ring. Interneurons and motor neurons make up the ventral nerve cord. The dorsal nerve cord is formed largely of ventral cord motor neuron axons that enter via commissures. An important feature of the

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C. elegans nervous system is that only three neurons, CANL, CANR and M4, are required for viability under laboratory conditions. The CAN (excretory canal) neurons run along the excretory canals and may play an important role in regulating systemic salt and water balance. M4 is a pharyngeal motor neuron that controls isthmus peristalsis and thus controls feeding (Avery and Horvitz., 1989). White et al. 1986 have reconstructed and mapped the connectivity of the entire hermaphrodite nervous system using serial electron microscopy (**Figure 3**) and underwent extensive development as a model organism in order to study the nervous system (Brenner, 1974). Its neuronal lineage and the complete wiring diagram of its nervous system are stereotyped and fully described (Sulston, 1983; Sulston et al., 1983; White et al., 1986).

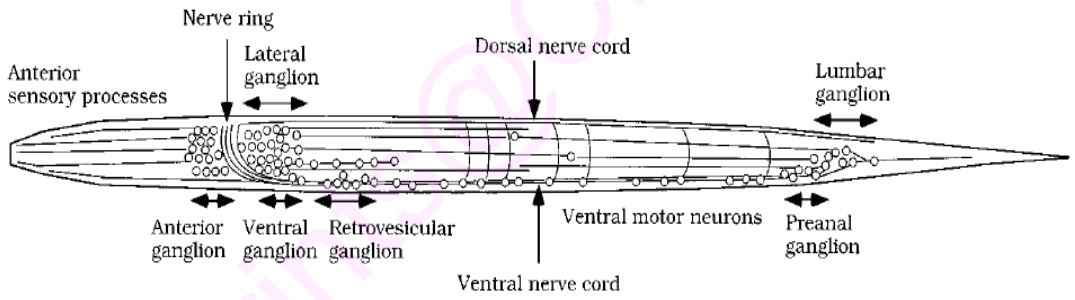

Figure 3 The structure of the *C. elegans* nervous system (Bargmann and Kalpan, 1998).

Nervous system is comprised of 6393 chemical synapses, 890 electrical junctions, and 1410 neuromuscular junctions (Chen et al., 2006). Additionally, the neurotransmitter systems (cholinergic, γ-aminobutyric acid (GABA)ergic, glutamatergic (Glu), DAergic, and serotoninergic) and their genetic networks (from neurotransmitter metabolism to vesicle cycling and synaptic transmission) are phylogenetically conserved from nematodes to vertebrates, which allows for findings from *C. elegans* to be extrapolated and further confirmed in vertebrate systems.

ACETYLCHOLINESTERASE ENZYME (AChE) SYSTEM IN *C. ELEGANS*

Acetylcholine (ACh) is the primary excitatory neurotransmitter involved in *C. elegans* motor function, as in higher animals (Rand and Nonet, 1997). Also conserved is the synaptic degradation of ACh by acetylcholinesterase (AChE) enzymes. While vertebrates have a single AChE gene whose product can undergo alternative splicing posttranscriptionally to yield different isoforms, *C. elegans* has four separate genes yielding four AChE enzymes (Combes et al., 2001). Two of these enzymes, ACE-1 and ACE-2, account for roughly 95% of all AChE activity in *C. elegans*. Though they share only 35% identity, ACE-1 and ACE-2 display a high degree of functional redundancy (Combes et al., 2001). Johnson and Russell (1983) found that dual mutations nullifying the product of both genes were required to produce a phenotype readily distinguishable from the wild type. ACE-3 and ACE-4 are minor forms of AChE in *C. elegans* accounting for approximately 5% and 0.1% of wild-type activity, respectively (Combes et al., 2001). ACE- 3 exhibits enough activity for ace-1, ace-2 double mutants to be viable, but movement and development are severely degraded.

Except for different drug sensitivities, none of the single ace mutants or *ace-3; ace-2* double mutants have a significant behavioral phenotype. Clearly, no single activity is essential. The *ace-2; ace-1* double mutant, however, is characterized by slow, uncoordinated forward movement and by hypercontraction and paralysis when the animals are stimulated to go backward. The phenotype of the triple ace mutant is more severe. When all three mutants are present, paralyzed but otherwise normal appearing larvae are produced that do not seem to be able to escape from the eggshell. When the shell is broken, the animals fail to develop. Interestingly, strains that contain mutations in all three ace genes and in severe alleles of cha-1 or unc-17 are viable (although they grow extremely poorly). The lethality of the triple ace

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mutants that appears to result from excessive buildup of ACh, rather than from the loss of some essential function of the ace genes themselves.

NEUROTRANSMITTERS IN *C. ELEGANS*

ACETYLCHOLINE

Acetylcholine (ACh) was the first substance proven to be a neurotransmitter (Loewi, 1921). It was identified in *Ascaris* and other nematodes in 1955 by Helen Mellanby (Mellanby, 1955), and was subsequently shown to be an excitatory transmitter at nematode neuromuscular junctions (del Castillo et al., 1963; del Castillo et al., 1967).The action of acetylcholine, however, is terminated by direct enzymatic hydrolysis of the neurotransmitter in the synaptic cleft by acetylcholinesterase (AChE). The resulting choline is then transported back into the presynaptic neuron by a high affinity choline transporter (HAChT, or ChT); this choline is then available for the synthesis of additional ACh.

ACh-mediated behaviors in *C. elegans*

Cholinergic transmission is involved, directly or indirectly, in many *C. elegans* behaviors, locomotion, egg laying, feeding, and male mating.

Locomotion

Acetylcholine mediated behavior, involving by far the greatest number of cholinergic neurons. Locomotion (both crawling on surfaces and swimming in liquid) requires the ability to generate smooth, sinusoidal waves (at variable propagation rates) of either anterior-directed or posterior-directed body muscle contractions. The involvement of ACh in locomotion includes not only neuromuscular transmission, but also nerve-nerve transmission which is poorly characterized (Rand, 2007).

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Egg Laying

Egg laying behavior involves the action of several neurotransmitters, and ACh appears to affect egg laying through multiple mechanisms. The major effect of ACh appears to be an inhibition of egg laying, because animals deficient in cholinergic release (*cha-1* and *unc-17* mutants) are constitutive for egg laying, and retain very few embryos (Bany et al., 2003). However, levamisole can stimulate egg laying (Kim et al., 2001), suggesting a possible stimulatory cholinergic mechanism operating through levamisole-sensitive receptors on muscles.

Pharyngeal pumping

The MC neuron is cholinergic and stimulates the pharynx muscle (EAT-2 receptor) to control pharyngeal pumping rate (McKay et al., 2004). Wild-type animals pump >250/min; animals ablated for MC is ~ 45/min (Avery and Horvitz, 1989; Raizen et al., 1995).

DOPAMINE

Dopamine is made in eight neurons in the hermaphrodite and in an additional six neurons located in the tail of the male. Dopamine signaling allows *C. elegans* to respond to changes in its environment by modulating locomotion behavior (Sawin et al., 2000). Dopamine-dependent effects on *C. elegans* behavior have been gleaned primarily from the analysis of: 1) wild-type animals exposed to exogenous dopamine; 2) wild-type animals in which the dopaminergic neurons have been ablated; and 3) mutants for *cat-2*, which encodes a tyrosine hydroxylase responsible for catalyzing the rate-limiting step in the synthesis of dopamine (Bettinger and McIntire, 2004).

Exogenous dopamine has been reported to inhibit motility in *C. elegans* (Schafer and Kenyon, 1995) and it has been observed that 20mM dopamine is capable of completely immobilizing worm populations when preincubated on NGM plates containing dopamine (Sanyal, 2004).

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SEROTONIN

Serotonin is made in eight types of neurons in *C. elegans*. Its role in modulating behavior has been established largely through analysis of mutants for the genes encoding the serotonin biosynthetic enzymes, or through behavioral studies of wild-type animals exposed to drugs thought to alter serotonin signaling. Treatment with exogenous serotonin causes dramatic behavioral effects: it inhibits locomotion and the defecation motor program, but stimulates egg laying and pharyngeal pumping (Weinshenker et al., 1995; Horvitz et al., 1982; Sawin et al., 2000; Niacaris and Avery, 2003; Mendel et al., 1995). Defects in some of the same behaviors are observed in mutants that lack activity of the serotonin biosynthetic enzymes BAS-1 or CAT-4 (Loer and Kenyon, 1993; Sawin et al., 2000). Analysis of mutants serotonin has shed light on the mechanisms by which serotonin modulates locomotion, pharyngeal pumping, and egg laying.

Serotonin mediated egg laying

Serotonin signaling has been studied extensively in the control of egg laying. Serotonin is made in two classes of neurons that directly innervate the vulval muscles: the VC4/5 neurons (Duerr et al., 1999) and the pair of HSN hermaphrodite-specific neurons (Desai et al., 1988; McIntire et al., 1992; Sze et al., 2000). A strong case has been made that HSN-released serotonin stimulates egg laying. Exogenous serotonin causes egg laying, and mutants in which the HSN neurons die (*egl-1* mutants) are strongly egg-laying defective (Egl).

GABA

There are 26 GABAergic neurons in *C. elegans.* These neurons are further divided into six classes: DD, VD, RME, AVL, DVB and RIS (McIntire et al., 1993; White et al., 1986). The DD and VD are D-motor neurons that mediate inhibitory GABA transmission to dorsal and ventral body wall muscles and thereby causing relaxation of the respective muscles. The sinusoidal

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movement of a worm is evoked by contracting muscles on one side of the body and relaxing them on the opposite side. When the GABAergic neurons were ablated, worms displayed movement impairment and hyper contracted when touched (McIntire et al., 1993).

DIGESTIVE TRACT IN *C. ELEGANS*

The digestive tract of *C. elegans* consists of a pharynx, intestine, and rectum. *C. elegans* is a filter feeder, and the pharynx is a muscular organ that pumps food into the pharyngeal lumen, grinds it up, and then moves it into the intestine. A pharyngeal-intestinal valve connects the pharynx to the intestine. The rectum is connected to the intestine via the intestinal-rectal valve. The sphincter muscle, an anal depressor muscle, and two muscle cells that wrap around the posterior intestine control defecation.

MUSCLES IN *C. ELEGANS*

C. elegans muscles are particularly powerful model for defining muscle physiology, structure, molecular biology, and development (Waterston, 1988; Moerman and Fire, 1997). *C. elegans* possesses both striated and nonstriated muscles. Striated body wall muscles are the most numerous muscle cell types. They are arranged in longitudinal bands along the body wall and are responsible for locomotion. Nonstriated muscles are associated with the pharynx, intestine, anus, and the hermaphrodite uterus, gonad sheath, and vulva. These muscles are responsible for pharyngeal pumping, defecation, ovulation and fertilization, and egg laying. Specialized nonstriated muscles are located in the tail of male worms and function in mating.

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UTILITY OF *C. ELEGANS* AS A TOXICOLOGICAL MODEL

C. elegans has a small body size and hence *in vivo* assays can be conducted in a 96-well microplate. The transparent body also allows clear observation of all cells in mature and developing animals. Furthermore, the intensively studied genome, complete cell lineage map, knockout (KO) mutant libraries, and established genetic methodologies including mutagenesis, transgenesis, and RNA interference (RNAi) provide a variety of options to manipulate and study *C. elegans* at the molecular level. *C. elegans* also enables the detection of organism-level end points (e.g., feeding, reproduction, life span, and locomotion) and the interaction of a chemical with multiple targets in an organism and uptake of neurotoxins/substances through three distinct routes: Pharynx, skin and via sensory neuronal endings (Koletta and Hengartner, 2006). Thus, *C. elegans* complements both *in vitro* and *in vivo* mammalian models in toxicology.

The use of *C. elegans* as a predictive model for human toxicity was first proposed in the context of heavy metals (Williams and Dusenbery, 1988). The *C. elegans* assay was validated as a predictor of mammalian acute lethality using eight different metal salts, generating LC_{50} values parallel to the rat and mouse LD_{50} values. A later study investigated the acute behavioral toxicity of 15 OP pesticides in *C. elegans* (Cole et al., 2004). The toxicity of these pesticides in *C. elegans* was found to be significantly correlated to the LD_{50} acute lethality values in rats and mice. Several other studies have also validated a number of *C. elegans*–based assays for predicting neurological and developmental toxicity in mammalian species (Tatara et al., 1998; Dhawan et al., 1999; Williams et al., 2000; Anderson et al., 2004).

Caenorhabditis elegans has been extensively used as a model system to elucidate the toxicity and toxicological mechanisms of various heavy metals, such as Aluminum (Al), Arsenic (As), Barium (Ba), Cadmium (Cd), Copper (Cu), Lead (Pb), Mercury (Hg), Uranium (U), and Zinc (Zn) (summarized in **Table 2**).

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In general, these studies focused on various toxic end points, such as lethality, reproduction, life span, and protein expression. Some studies have also focused on the effects of these metals on the nervous system by assessing behavior, reporter expression and neuronal morphology. Further, by recording short videos and subsequently analyzing them using computer tracking software, the overall movement of *C. elegans* (distance traveled, directional change, etc.), body bends and head thrashes, upon metal treatments has been quantified allowing to further correlate the data with damages to neuron circuites. These computer tracking studies have shown that worms displayed a dose-dependent decrease in locomotory movement upon exposure to Pb (Johnson and Nelson, 1991; Anderson et al., 2001, 2004) and Al (Anderson et al., 2004), while an increase in locomotion was observed on exposure to low concentrations of Hg as compared with Cu (Williams and Dusenbery, 1988). Another study showed that exposure to Ba impaired both body bend and head thrashing rates in a dose-dependent manner (Wang et al., 2008), corroborating mammalian data on the effect of Ba on the nervous system attributed to its ability to block potassium channels (Johnson and Nelson, 1991). Feeding behavior has also been shown to be affected upon heavy metal exposure. Feeding requires a different neuronal circuitry including M3 (involved in pharyngeal relaxation), MC (control of pumping rate), M4 (control of isthmus peristalsis), NSM (stimulate feeding), RIP, and I neurons (Riddle et al., 1997). A decrease in feeding has been reported in worms exposed to Cd or Hg (Boyd et al., 2003; Jones and Candido, 1999). Observation of neuron morphology following heavy metal exposure has also performed using *C. elegans* strains expressing the green fluorescent protein (GFP) in discrete neuronal populations.

Similarity in neural circuitry and the conservation in genetic makeup between *C. elegans* and humans have led to a number of recent studies on pesticide neurotoxicity in this species (summarized in **Table 2**). *C. elegans* showed a remarkable decline in locomotion at a concentration of OPI below

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survival reduction (Williams and Dusenbery, 1990b). Comparison studies using behavioral analyses to assess movement alteration as an indicator of the neurotoxity of 15 OP pesticides (Cole et al., 2004) showed that carbamate pesticides, unlike OP pesticides were reversible AChE inhibitors (Melstrom and Williams, 2007). The LD_{50} values in *C. elegans* closely correlated with LD50 in both rats and mice. Pesticides (vapona, parathion, methyl parathion, methidathion, and funsulfothion) that showed cholinesterase inhibition were associated with pronounced behavioral toxicity (i.e., decrease in movement). A recent study has compared end points using OPs and found AChE inhibition to be the most sensitive indicator of toxicity but also the most difficult to measure (Rajini et al., 2008). Reduction in movement for 10 OPs was found to correlate to rat and mouse acute lethality data. Finally, simulation studies examining the rate of absorption and biodegradation of OP (parathion) (Saffih-Hdadi et al., 2005) establish the relevance and reliability of *C. elegans* as an experimental model and predictor for soil toxicity.

C. elegans has also been widely exploited to study the toxicity of alcohols, steroid and synthetic hormones and several other xenobiotics (summarized in **Table 3**).

Further, several transgenic strains of *C. elegans* have been successfully employed to study the toxicity as well as for biomonitoring of environmental samples (summarized in **Table 4**).

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Table 2 Toxicity studies on pesticides and heavy metals using *Caenorhabditis elegans* as a model organism

Substances	Assay	Strains used	Reference
Carbamate, Aldicarb and Levamisole	Bioassay	N2	Winter, 2002
Organophosphates and Carbamates	Acetylcholinesterase	N2	Combes et al., 2001
Mancozeb	Hsp-16 induction	PC-72	Easton et al., 2001
Captan, Fungicides,captofol and folpet	Stress response	PC-72	Jones et al., 1996
Phosphine	Molecular oxygen LC_{5O}	N2	Cheng et al., 2003
Paraquat	SOD, cytotoxicity	Mutants SOD	Oeda et al., 2001;
15 OPI	AChE assay , EC_{50}, LD_{50}, and Behavior;	N2	Cole et al ., 2004
Captan, N-folpet, Captafol, Tetrahydrophthalimide, Phthalimide, N-phthaloyl-L-glutamic acid	Feeding assay, β-galactosidase staining (Hsp-16)	PC-72	Jones et al., 1996
Cu, Beryllium, Mercury, Lead, Organophosphates (Malathion and vapona)	Computer tracking (Locomotion and frequency of change of direction are reported real-time)	N2	Williams et al., 1990
Pb, Cu, OP, Organic solvents and heavy metals	Locomotor behavior and acute behavioral toxicity	N2	Anderson et al., 2004
Pb,Cu and Cd	EC_{50}, reproduction	N2	Anderson et al.,2001
Gadolinium based magnetic resonance imaging(MRI) compounds	Minimum lethal dose (MLD)	N2	Williams et al., 2000
Li, Na, Mg, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, Sr, Cd, Cs, Ba, La and Pb	LC_{50}, 24h	N2	Christopher et al .,1998

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Table 3 Toxicity studies on alcohols and xenobiotics using *Caenorhabditis elegans* as a model organism

Substances	Assay	Strains used	Reference
Ethanol	LC_{50}, RC_{50} and BC_{50}	N2	Dhawan et al., 1999
Alcohols, heavy metals, sulfahydryl reactive compounds, salicylate and heat	Hsp-16 Protein expression, feeding, pharyngeal pumping	PC-72	Jones et al., 1999
Bisphenol A(Bis A), 4-(1-adamantyl)phenol and 4,4-(1,3-adamantanediyl) Diphenol(ADP)	5-generation test hatching toxicity	N2	Tominaga et al., 2003
Alkyl phenol	ED_{50},LD_{50}	N2	Tominaga et al., 2003
Benzo (α) pyrene, Nonylphenol, Benzophenone, Bisphenol A and Cadmium chloride	24 h mortality	N2	Ura et al ., 2002
Acrylamide	Growth, fecundity, life span	N2	Hasegawa et al., 2001
Glucosinolates	LC_{50} (24 h)	N2	Steven et al., 2003
Colchicine and Chloroquine	P-glycoproteins	N2	Broeks et al.,1995
Cadmium and Arsenite	MRP homologue and P-glycoprotein	N2	Miller et al., 2001
Hydrogen phosphide, EMS Mutagenesis	LC_{50}	Pre-1,Pre-7, Pre-33	Cheng et al.,2003

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Table 4 Transgenic *Caenorhabditis elegans* used in toxicology research

Target tagged	Reporter used	Applications	Reference
Heat shock proteins	GFP; β-galactosidase	Widely used for measuring stress response associated with toxicity of heavy metals, fungicides, pharmaceuticals	Dengg and van Meel , 2004; Easton et al., 2001 Mutwakil et al., 1997; Roh et al. 2006
Metallothionein	β-galactosidase	Specifically used for monitoring the bioavailability of heavy metals	Cioci et al., 2000

Clearly, *C. elegans* will be a valuable toxicity model only if its results were predictive of outcomes in higher eukaryotes. There is increasing evidence that this is the case both at the level of genetic and physiological similarity and at the level of actual toxicity data. Many of the basic physiological processes and stress responses that are observed in higher organisms (e.g., humans) are conserved in *C. elegans.*

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CHAPTER I

Toxicity of organophosphorus insecticides in *Caenorhabditis elegans*

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CHAPTER I

Toxicity of organophosphorus insecticides in *Caenorhabditis elegans*.

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PREFACE

The main objective of this study was to establish the toxicity of selected organophosphorus insecticides in the two strains of *Caenorhabditis elegans* – N2 (wild type) and PC72 (transgenic).

This Chapter has been presented under **two** sections:

Section A describes the lethality profile of the three OPI – dichlorvos, dimethoate and monocrotophos, in terms of their 4h-LC_{50} in N2 strain of *C. elegans.* Based on the results, we selected dichlorvos for further mechanistic studies in N2 strain.

Section B describes the toxicity of the three OPI - dichlorvos, dimethoate and monocrotophos in the transgenic strain, PC72. This section includes the lethality profile as well as the heat shock protein induction potential of the three OPI and further the causal relationship between endpoints in worms exposed to dichlorvos.

SECTION A

Toxicity of organophosphorus insecticides (OPI) to N2 strain of *Caenorhabditis elegans*

1.0 INTRODUCTION

Pesticides are known neurotoxicants and are widely used in the world agricultural community. Studies of pesticide neurotoxicity have typically evaluated the long-term effects of pesticides. A large number of studies support the notion that long-term low-level exposure to organophosphorus insecticides (OPI) may cause neurological and neurobehavioural effects (Ray, 1998; De Silva, 2006). Prediction of neurotoxic effects is a key feature in the toxicological profile of compounds (Crofton et al., 2004). Neurotoxicity can occur following both acute and chronic exposures. Acute effect can be observed after a single exposure to a compound either immediately or delayed while chronic effects can be observed due to repeated, low-dose exposures (Coecke et al., 2006).

The free-living soil nematode, *Caenorhabditis elegans* (*C. elegans*) has been employed in testing environmental contaminants known to have neurotoxic effects. It is a thoroughly characterized multicellular organism with regard to genetics, development, behavior and anatomy. *C. elegans* has been vastly employed in molecular biology for studying nervous system and in aging studies due to its ease of culture and maintenance, short life span and life cycle, cellular simplicity and genetic tractability (Barr, 2003; Lee, 2004). It is one of the simplest organisms with a centralized nervous system. Neurotransmitter-mediated transduction of the synapse in *C. elegans* has been demonstrated for acetylcholine, serotonin, dopamine and GABA (Rand and Nonet, 1997). Recent studies have strongly recommended the utility of *C. elegans* as a neurological model for studying neurotoxicity of pesticides (Williams and Dusenbery, 1988; Cole et al., 2004; Rajini et al., 2008; Leung et al., 2008; Peterson et al., 2008). It has been suggested that *C. elegans* may react to neurologically active chemicals with enough similarity to mammals and thus serve as a model for

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neurotoxicity. It has also been suggested that comprehensive studies are necessary to clarify similarities between OPI toxicity in *C. elegans* and animals (Leung et al., 2008; Peterson et al., 2008).

Hence, comphrensive studies are scarce on the neurophysilogical impact of OPI in *C. elegans.* The objective of the first series of present investigations was to employ *C. elegans* as a model to test its response to selected OPI. Initially, we wished to establish the 4h-LC_{50} of three selected OPI, viz., dichlorvos, dimethoate and monocrotophos, in N2 strain of *C. elegans* in order to select the pesticide and the concentrations of the pesticide for further detailed mechanistic investigations.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Agar, Cholesterol, Hypochlorite solution, Proteose Peptone and Yeast extract were purchased from Hi Media (Mumbai, India). Cadmium chloride ($CdCl_2$), di-Sodium hydrogen orthophosphate (Na_2HPO_4), Magnesium sulphate ($MgSO_4$), Magnesium chloride ($MgCl_2$), Potassium chloride (KCl), Sodium chloride (NaCl), Sodium Dihydrogen ortho-phosphate (NaH_2PO_4), Sodium hydroxide (NaOH) and Sodium dodecyl sulfate (SDS) were procured from Sisco Research Lab., (Mumbai, India).

Dichlorvos (DDVP; Technical grade; 97.6%) and Dimethoate (Technical grade; 94%) were gift from Hyderabad Chemical Supplies Ltd., (Hyderabad, India). Monocrotophos (Technical grade; 95%) was kindly provided by United Phosphorous Ltd., (Mumbai, India).

2.2 Pesticide solution

Stock solutions (1M) of pesticides (Dichlorvos, Dimethoate and Monocrotophos) were prepared in double distilled water and further diluted to obtain working solutions for the assays.

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2.3 Worms and culture conditions (Brenner, 1974)

Worms used in this study were wild type hermaphrodite *Caenorhabditis elegans* (Bristol strain N2) obtained from Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA) which is funded by the NIH National Center for Research Resources (NCRR). All developmental stages of *C. elegans* were maintained in 70 mm petriplates on Nematode growth medium (NGM) that had been seeded with *Escherichia coli* (*E.coli*) at 20ºC as described below.

2.3.1 Nematode growth medium (NGM) (Brenner, 1974)

NGM agar (0.032M KCl, 0.051M NaCl, 2.5% Bactopeptone, 0.17% Bacto-agar, in distilled water) was autoclaved and cooled to 50-60°C. To the warm medium the following were supplemented: 0.01% cholesterol, 0.1M $CaCl_2$, 0.1 M $MgSO_4$. The agar was poured on petridishes and allowed to solidify and then stored at 4°C.

2.3.2 Preparation of bacterial food source (Brenner, 1974)

The worm is fed with a special strain of bacteria *Escherichia. Coli (E.coli)* known as OP50, which is auxotrophic for uracil and therefore has limited growth on the plates and this allows for easy observation of the worms on the plate.

The starter culture of *E.coli* (OP50) was obtained from the Caenorhabditis Genetics Center (CGC) (Minneapolis, MN, USA) and sub-cultured in our laboratory. OP50 was grown in 'Liquid Broth' (LB: 0.25g NaCl and 0.5g Proteose peptone in 50ml of double distilled water) autoclaved at 121˚C for 15 min and then cooled. Using an inoculation needle, an inoculum of OP50 was aseptically introduced into the autoclaved LB medium and the cultures were incubated overnight at 37°C in shaking condition.

2.3.3 Worm culture and maintenance

A culture of overnight grown E.coli (OP50) was seeded on solidified plates in laminar hood. Approximately 100µl of *E. coli* (OP50) was dropped on NGM plates and the drop was spread using an L-shaped glass rod taking care

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not to spread the lawn till the edge of the plate. Plates were incubated overnight at 37ºC to allow the *E. coli* (OP50) lawn to grow. Seeded plates were stored in an air-tight container so that they remain usable for 2-3 weeks. Worms were transferred under laminar airflow chamber to the OP5O seeded NGM plates and maintained at 20ºC.

2.3.4 Egg preparation/ age synchronization (Fabian and Johnson, 1994)

Gravid worms were washed out from the NGM plates using K-medium, then synchronized according to the alkali-bleaching method. Briefly, eggs and gravid worms were obtained by washing from 8-10 plates by rubbing with glass rod and rinsing plates with K-medium (50 mM NaCl, and 30 mM KCl in distilled water). The resultant suspension was centrifuged for 4min to pellet the worms and eggs, the excess K-medium was removed, and 5ml of fresh hypochlorite solution (20ml Hypochlorite solution, 1g NaOH, 80ml double distilled water) was added and mixed at intervals for 4min. The suspension was centrifuged at 3500rpm for 6min, supernatant was discarded, and pellet was washed three times with K-medium and finally centrifuged at 3,500rpm for 4 min. The egg pellet was transferred to NGM agar plates with overnight grown lawn of *E.coli* (OP50). The plates were incubated at 20°C. (*The eggs hatch and progress through four larval stages, Ll, L2, L3 and L4 with each stage lasting for about 12h after which they reach adult (gravid) stage* (**Figure 1.1**). Two-day old larvae were transferred to a fresh plate containing a lawn of OP50 to maintain high nutritional status before testing on day 3 or 4.

The worms were prepared for the various assays by washing the adult worms with K-medium from the petriplates into a centrifuge tube and allowing them to settle by gravity. The worms were rinsed three times with K-medium and then transferred to a glass perti dish for counting and loading into test wells.

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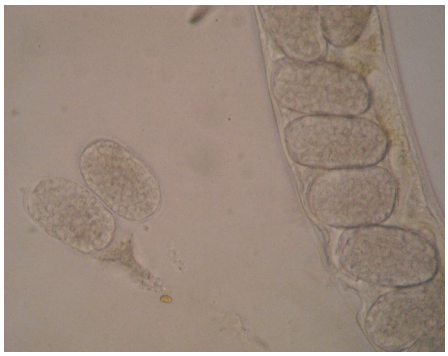
Mixed stages



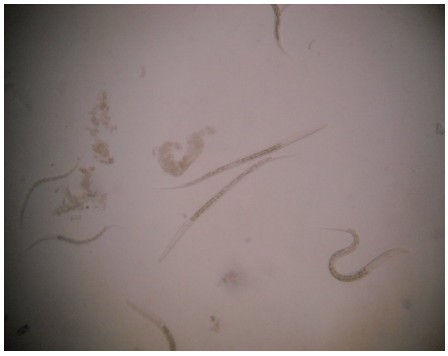
Eggs

L1

L2 / L3

L4 (Young adult)

Gravid



Dauer

Figure 1.1 Various stages of *C. elegans*

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3.0 EXPERIMENTAL PROCEDURE

3.1 Toxicity test: Lethality assay

The nematodes were tested in 24-well tissue culture plates (Tarsons Products Pvt. Ltd., Kolkata, India) containing 1 ml of test solution per well. Test solutions consisted of six pesticide concentrations each (dichlorvos: 5, 10, 15, 20, 25 and 30mM; Dimethoate: 5, 10, 15, 20, 30 and 40 mM; monocrotophos: 20, 40, 60, 80, 100 and 120 mM) and a control with each replicated three times. Using a dissecting microscope, 10 ± 1 L4 stage worms (2 day old) were transferred into each test well with a 10 μl pipette. The worms were incubated for 4h at 20ºC.

4.0 ASSAY METHOD

The number of survivors was counted at the end of the treatment period. The worms were scored dead if they did not respond to a touch stimulus. The concentration-response experiment was repeated three times on three different days. The LC_{50} values, representing 50% lethality at a given exposure period were calculated using regression analysis.

5.0 RESULTS

Untreated worms (Control) in all the replicates showed 100% survival (no mortality). Exposure to the three OPI for 4h induced a concentration-dependent reduction in survival among the worms (**Figure 1.2 A, B, C).** The treated worms in general exhibited initial hyperactivity followed by decreased motility. Worms exposed to higher concentrations of the OPI exhibited twitching and paralysis prior to death. Of the three OPI tested, dichlorvos was the most toxic (4h-$LC_{50:}$ 16.0 mM) followed by dimethoate (4h-$LC_{50:}$ 18.2 mM) and monocrotophos (4h-LC_{50} : 69.0 mM).

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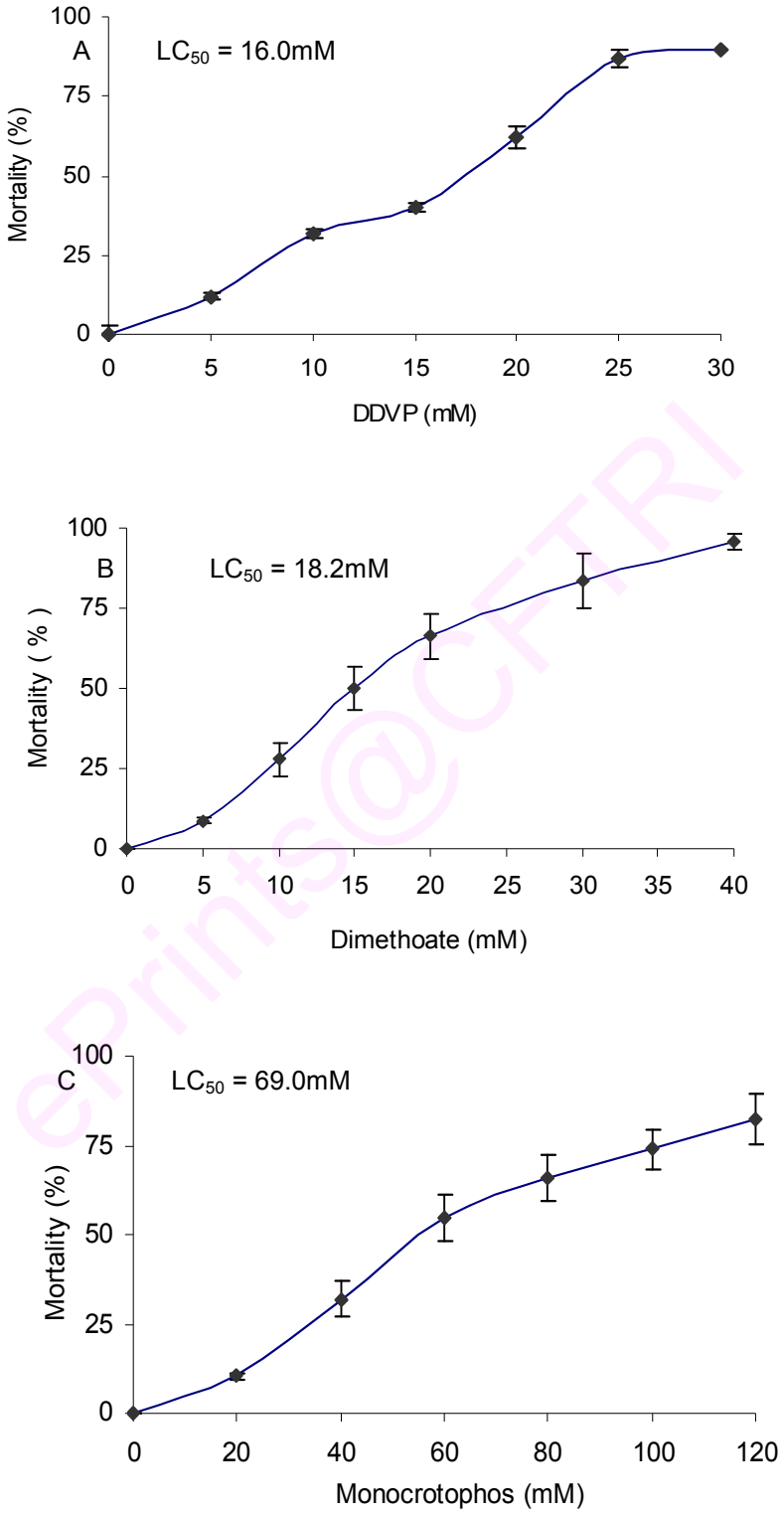

Figure 1.2 Survival profile of *C. elegans* exposed to the OPI for 4h: A-Dichlorvos (DDVP); B-Dimethoate; C-Monocrotophos. Each data point is the mean ± S.E.M of three replicates from three independent experiments.

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6.0 DISCUSSION

We determined lethality of the wild-type strain N2 (Bristol) as a simple signal for toxicity after treatment with dichlorvos or dimethoate or monocrotophos. Among the three OPI, dichlorvos was the most toxic to *C. elegans* in terms of the 4h-LC_{50} values than dimethoate and monocrotophos.

C. elegans has been used in testing environmental contaminants, which are known to have neurotoxic effects. Several studies comparing the effects of xenobiotics in *C. elegans* to those observed in mammals have found strong similarities. By developing a neurotoxicity index from lethality data, Williams and Dusenbery (1990a) concluded that two organophosphate pesticides (malathion and dichlorvos) displayed concentration–response dynamics consistent with neurotoxicity. Williams and Dusenbery (1988) also found that acute lethality values in *C. elegans* exposed to eight heavy metallic salts predicted overall mammalian acute lethality as consistently as tests in rats and mice individually. Studies have also (Anton et al., 1992; Morgan and Sedensky, 1995) found the influence of ethanol on *C. elegans* to be qualitatively parallel to its action in mammals. These studies also suggest *C. elegans* as a useful model for studying the biologic response to volatile anesthetics.

Earlier, there were attempts to use interspecies correlation to develop a predictive model for OP toxicity (Wallace, 1992; Chambers and Carr, 1995). Cole et al., (2004) for the first time reported significant correlation in toxicity of OP in *C. elegans,* rats, and mice. That study was followed by similar findings in *C. elegans* exposed to carbamates (Melstrom and Williams, 2007). A significant correlation was also reported between LC_{50} values in *C. elegans* and the LD_{50} in rats for the 10 OPI (Rajini et al., 2008). As has been reported earlier in other invertebrates (Eisler, 1969) and in *C. elegans* (Cole et al., 2004), in the present study, dichlorvos was the most toxic. Some of the differences in acute toxicity following exposure to OPI reside in differential rates of biotransformation (Costa et al., 1990; Pond et al., 1995). Little is known about drug-metabolizing

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enzymes (cytochrome P-450s) from invertebrates such as the nematode *C. elegans* or specifically the metabolism/disposition of OP pesticides in *C. elegans.*

Further in the present study we obtained a vastly different LC_{50} values for the three OPI compared to that reported earlier in literature (Rajini et al., 2008). While the order of toxicity in the earlier study was: Dichlorvos > Monocrotophos > Dimethoate, the order of toxicity in the present study was: Dichlorvos > Dimethoate > Monocrotophos. Although dichlorvos was most toxic in both the studies, we obtained an LC_{50} value which was about 400 folds higher than reported earlier (Rajini et al., 2008). This could be attributed to the difference in source and purity of the dichlorvos employed.

Based on the LC_{50} values obtained in the present study, we selected dichlorvos for further mechanistic studies in *C. elegans.*

7.0 SUMMARY

1. Initially, three OPI were randomly selected viz., dichlorvos, dimethoate and monocrotophos.
2. They were screened for their toxicity in *C. elegans* (N2) in terms of 4h-$LC_{50.}$
3. Of the three OPI tested, dichlorvos was relatively the most toxic (4h-$LC_{50:}$ 16.0 mM) followed by dimethoate (4h-$LC_{50:}$ 18.2 mM) and monocrotophos (4h-$LC_{50:}$ 69.0 mM).

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SECTION B

Toxicity and heat shock protein induction potential of OPI in transgenic strain of *C. elegans* (PC 72)

1.0 INTRODUCTION

Organophosphorus insecticides (OPI) are one of the most widely used classes of insecticides in the world (Fulton and Key, 2001). The toxicity of OPI results from inhibition of cholinesterase enzyme and hence reduction in cholinesterase (ChE) activity has been widely employed to detect exposure to OPI in vertebrate and invertebrate species (Fulton and Key, 2001). A number of studies have found significant reduction in ChE activity due to exposure to OPI, which correlated with effects on behaviour, survival and growth (Fryday et al., 1996; Dell'Omo and Shore, 1996; Brewer et al., 2001).

During the past four decades, it has been observed that under stressful conditions such as exposure to high temperature, free radicals, xenobiotics or metals, cells from both eukaryotes and prokaryotes respond by synthesizing a family of proteins known as heat shock proteins (hsp) (Nover, 1991). This response has been recognized to represent a universally conserved cellular defense program and was originally observed in *Drosophila melanogaster* (Ritossa, 1962). These 'stress proteins' are believed to buffer cells from damage under adverse conditions by maintaining the proteins prior to assembly into multimolecular complexes in the cytosol and by guiding the newly formed as well as denatured proteins to attain their proper conformation, thereby protecting the cells from further damage (Morimoto et al., 1994). The stress proteins are classified into families according to their apparent molecular weights and respective inducers and include the hsp60, hsp70 and hsp80 family and the low molecular weight or small Hsps, which range in molecular mass from approximately 16-kDa (Morimoto et al., 1990). The small heat shock proteins (sHsps) are found in most organisms where they are induced upon stress (Van Montfort et al., 2001). They function as molecular chaperones since

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they are able to prevent thermal and chemical-induced aggregation of a variety of targets both *in vitro* and *in vivo* (Basha et al., 2004). sHsps interact with various components of the programmed cell death machinery upstream and downstream of the mitochondrial apoptotic events, and can prevent apoptosis in different lethal stress situations (Arrigo, 2005). Because any agent causing conformational damage (proteotoxicity) can induce such a heat shock response, HSP-based methods provide a general "catch-all" biomarker that can respond to xenobiotics. In this respect they are similar to classical ecotoxicological endpoints such as reduced feeding, impaired fecundity/reproduction and eventually, lethality (Easton et al., 2001).

Induction of heat shock proteins in response to pesticide exposure has been amply demonstrated in *Drosophila* (Nazir et al., 2001; Mukhopadhyay et al., 2002 ; Gupta et al., 2005 a, b). Heat shock protein expression on exposure to fungicides, maneb and mancozeb (Guven et al., 1999; Easton et al., 2001), heavy metals (Stringham and Candido, 1994), and diethylhexyl phthalate (Roh et al., 2007) has been reported earlier in transgenic *C. elegans.* Hence, in the present study, we have investigated the detrimental effects of selected OPI employing the transgenic *C. elegans* as an assay model. The potential of three OPI to induce heat shock protein was investigated initially. Further, the casual relationship between the endpoints such as feeding behavior, hsp induction and inhibition of acetylcholinesterase activity were determined in the worms exposed to sublethal concentrations of dichlorvos.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Acetylthiocholine iodide, BSA (fraction V), 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB) and *β*-mercaptoethanol were procured from Sigma Chemical Co., (St. Louis, MO, USA). 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), Folin's reagent, *O*-nitrophenyl *β-D*-galactosidase pyranoside (ONPG), Potassium ferrocyanide, Potassium ferricyanide and Sodium dodecyl sulfate

(SDS) were procured from Sisco Research Lab, (Mumbai, India). All other chemicals used were of analytical grade. Genticyn and Acrylic red paint were procured from local pharmacy/ market.

Dichlorvos (Technical grade; 94%) and Dimethoate (Technical grade; 94%) were gifts from Hyderabad Chemical Supplies Ltd., (Hyderabad, India). Monocrotophos (Technical grade; 95%) was kindly provided by United Phosphorous Ltd., (Mumbai, India).

2.2 Pesticide solution

Stock solutions (1mM) of dichlorvos (DDVP), dimethoate and monocrotophos were prepared in double distilled water and diluted further to obtain working solutions for the assays.

2.3 Worms and culture conditions (Brenner, 1974)

Transgenic strain of *Caenorhabditis elegans* (PC 72) employed in these studies were obtained from Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA) which is funded by the NIH National Center for Research Resources (NCRR). All developmental stages of *C. elegans* were maintained in 70 mm petriplates on Nematode growth medium (NGM) that had been seeded with *Escherichia coli* (*E.coli*) at 20ºC as described earlier (*Chapter I A Sections 2.3.1 – 2.3.3*).

Caenorhabditis elegans strain PC72 contains a fusion of the *E. coli lacZ* gene in frame with the coding region of a 16-kDa heat shock gene, hsp16-1. The fusion gene includes the entire regulatory region of hsp16-1 and is expressed only under conditions of heat or chemical stress. The PC72 strain contains approximately 70–80 integrated copies of the transgene per haploid genome. β-galactosidase produced by PC72 nematodes is sequestered in cell nuclei due to the inclusion of an SV40 nuclear localization signal at the amino terminus of the enzyme, simplifying the identification of cells expressing the transgene.

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3.0 EXPERIMENTAL PROCEDURE

3.1 Toxicity of OPI: Lethality assay

Each test consisted of varying concentrations of dichlorvos (5, 10, 20 and 30mM) or dimethoate (5, 10, 20, 30 and 40mM) or monocrotophos (20, 40, 80,100 and 120 mM) and a control. 10±1 L4 stage worms were transferred onto 24-well tissue culture plates containing 1 ml of test solution in each of the wells. The worms were exposed for 4h to dimethoate and monocrotophos and to varying intervals for dichlorvos - 4, 12, 24, or 36 h at 20ºC. The number of survivors was counted at the end of the treatment period. They were scored dead if they did not respond to a touch stimulus. Data were calculated from three independent experiments conducted on three different days. The LC_{50} values, representing 50% lethality at a given exposure period were calculated using regression analysis.

3.2 Induction of heat shock protein by OPI

To examine the potential of dichlorvos, dimethoate and monocrotophos to induce heat shock protein in the transgenic *C. elegans*, the worms were exposed to varying concentrations of the insecticides (0, 5, 40 and 80 µM) for different time intervals (4, 12, 24, and 36 h) at 25 ± 1ºC. After exposure, the worms were washed three times with K-medium and subsequently used for Hsp16 expression assays-quantitative spectrophotometric and qualitative (histochemical staining) -as described below (*Section 4.1 & 4.2*).

3.3 Effect of dichlorvos on feeding rate

Aliquots of K-medium with OP50 suspended at a concentration of 1.0 OD (at 550 nm) were dispensed into the wells of 12 well plates. Dichlorvos was added to the wells at different concentrations (0, 5, 40 and 80µM). The worms suspended in K-medium were added to the wells such that each well had 5,000 worms. The total volume in each well was 1ml. The plate was incubated at room temperature with constant shaking. Each 1ml sample was drawn at hourly intervals, spun gently for a few seconds at 800 rpm, and the absorbance of the

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supernatant was determined at 550 nm to note the decrease in bacterial density. The samples were returned to the culture plate for further incubation (Jones and Candido, 1999).

3.4 Effect of dichlorvos on pharyngeal pumping

Exposure to dichlorvos was conducted as for the heat shock protein assay. Aliquot (20 µl of 20 mg/ml solution) of acrylic polymer emulsion (Tristar, Mumbai, India) was added to the worm suspension after 4 h of exposure to dichlorvos. The worms were observed after 10 min for the pharyngeal localization of the dye (Jones and Candido, 1999).

3.5 Effect of dichlorvos on acetylcholinesterase (AChE) (EC 3.1.1.7)

To examine the effect of dichlorvos on the AChE activity in transgenic PC72 strain of *C. elegans*, they were exposed to varying concentrations of dichlorvos (0, 5, 40, and 80µM) for 4h at 20 ± 1°C. After exposure, worms were washed thrice with K-medium and subsequently processed for assay.

4.0 ASSAY METHODS

4.1 Quantitative assay for heat shock protein (Stringham and Candido, 1994)

The worms washed thrice with K-medium were pelleted and permeabilized with acetone for 5 min after which the acetone was allowed to evaporate at 37°C. The worm pellet was incubated for 2 h at 37°C in 600 µL of ONPG staining buffer (60 mM Na_2HPO_4, 40mM NaH_2PO_4, 0.01M KCl, 1mM $MgSO_4$, 50mM β-mercaptoethanol and 842 µg/ml ONPG). Following incubation, the reaction was stopped by adding 300 µl of 1M Na_2CO_3 and the absorbance was measured at 420 nm. The β-galactosidase activity was calculated based on the molar extinction coefficient of *o*-nitrophenol (4.5×10^3 L mol^{-1} cm^{-1}).

4.2 Qualitative assay for heat shock protein (Jones and Candido, 1999)

The worms after being washed thrice with K-medium were pelleted and permeabilized with acetone for 5 min. The acetone was evaporated by

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incubating the tubes at 37°C for 5min. The nematodes were then incubated overnight at 37°C in a solution consisting of 0.2M Phosphate buffer (pH 7.5), 1mM $MgCl_2$, 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 0.004% sodium dodecyl sulfate (SDS), 75mg/ml Genticyn and 0.04% 5-bromo-4-chloro-indolyl-β-D-galactoside. The worm samples were mounted with a cover slip and observed for β-galactosidase staining under light microscope.

4.3 Acetylcholinesterase (AChE) (EC 3.1.1.7) (Ellman et al., 1961)

Worms (50,000/well) were exposed to dichlorvos (0, 5, 40 and 80 μM) for 4 h. After the exposure period, worms were washed thrice with K-medium and then homogenized with 50 mM Tris buffer (pH 7.4). The homogenate was centrifuged at 10,000 rpm for 10 min, and the supernatant (equivalent to 500 μg proteins) was used for AChE assay. To the supernatant in a 1ml cuvette were added DTNB (0.3mM) and ATCI (6.24mM). The contents were rapidly mixed and the rate of change in absorbance was measured at 10s intervals for 90s in a Shimadzu UV spectrophotometer at 405nm. Kinetic measurements were recorded and converted to total cholinesterase activity using the extinction coefficient for the colored product, 5-thio-2-nitro-benzoic acid.

4.4 Protein estimation (Lowry et al., 1951)

The worm pellet was washed thrice with K-medium, digested with 2M NaOH at 80ºC for 2h. 10 μl of digested sample was added to 490 μl of distilled water and incubated with 2.5ml alkaline copper sulfate solution for 10 min at room temperature. 250 μl of Folin's reagent (1:1 diluted with water) was added and the color was read after 30min at 670nm. Bovine serum albumin (BSA) was used as the standard.

4.5 Statistical Analysis

All the data are presented as mean ± standard error of three separate experiments performed in triplicate on three different days, using the computer program Excel software (1999). The data were analyzed employing analysis of variance (ANOVA) using SPSS software 10 (Chicago, IL, USA). Duncan's

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multiple regression test (DMRT) for multiple comparisons was performed to determine the significant differences among the groups. *P* Values <0.05 were considered significant.

5.0 RESULTS

5.1 LC_{50} of the OPI in transgenic *C. elegans*

The 4h-LC_{50} values of dimethoate and monocrotophos in the transgenic worms were 17.4 mM and 66.5 mM respectively (*data not shown*). The survival profile of the worms exposed to dichlorvos for varying time periods is depicted in **Figure 1.3.** The LC_{50} values of dichlorvos for all the four exposure periods are presented in **Table 1.1.** Based on the results, three concentrations of dichlorvos, corresponding to approximately 1/20, 1/40, and 1/360 of 4h-LC_{50} - 5, 40 and 80 µM—were selected for further studies. Equimolar concentrations (5, 40 and 80 µM) of the three OPI were employed for heat shock protein induction studies.

5.2 Heat shock protein induction by the OPI

In situ histochemical staining for β-galactosidase activity revealed that 99% of the control worms did not show any β-galactosidase activity (**Figure 1.4A**). Worms exposed to $CdCl_2$ (positive stressor) reflected a strong β-galactosidase activity in the pharyngeal region (**Figure 1.4B**). β-galactosidase staining was evident only in the pharyngeal region of the worms at 24h when exposed to all the three OPI (**Figure 1.4C**), a pattern similar to that elicited by $CdCl_2$.

The extent of reporter (β-galactosidase) induction in the transgenic (PC72) worms in response to the positive stressor, cadmium chloride and the OPI, dichlorvos, dimethoate and monocrotophos was evident as early as 4h after exposure. The induction was significantly high and concentration-dependant on exposure to dichlorvos (0.4 -1.1fold, **Figure 1.5**), dimethoate (0.08 - 0.44 fold, **Figure 1.6**) and monocrotophos (0.02 - 2.29 fold, **Figure 1.7**) at 5-100µM. The

maximal response obtained for dichlorvos was at 24h of exposure while for dimethoate (0.25 – 1.91 fold) and monocrotophos (5.44 – 7.42 fold) the maximum induction was seen at 36h of exposure. Worms treated with dimethoate showed increase in reporter induction with increasing time (**Figure 1.6)**. While worms exposed to monocrotophos showed no concentration response at 12h, they revealed marked increase at 24h and a further increase at 36h (**Figure 1.7)**. Maximal response (1.9 - 3.5 fold increase) in dichlorvos-exposed worms was evident after 24h exposure and at this time point, the response was greater than that elicited by $CdCl_2$. A drop in response was however evident at 36h at all dichlorvos concentrations (**Figure 1.5)**.

Comparatively, at the highest concentration (80μM) after 24h, all the three OPI elicited marked induction of heat shock protein. The order of induction was: monocrotophos (6.4 fold) > dichlorvos (4 fold) > dimethoate (1.6 fold) (**Figure 1.8**). Hsp induction by the OPI appeared to be inversely related to their toxicity (LC_{50}).

5.2 Feeding inhibition

There was a gradual and time-related decrease in bacterial density in the control worm suspension indicating normal feeding by these worms. Dichlorvos at all the tested concentrations induced cessation in feeding in *C. elegans* after 3h of exposure. While the extent of feeding inhibition after 4h in worms exposed to 5μM was 52%, worms exposed to 40 μM and 80 μM showed 77 – 79% inhibition in feeding **(Figure 1.9)**. Feeding inhibition after 4h exposure to dichlorvos was confirmed by uptake of red acrylic paint particles by the treated worms. Untreated worms took up the pigment particles, which accumulated in the pharynx within 10 min, while dichlorvos treated worms showed no pigment particles in the pharynx (**Figure 1.10 A, B**) clearly indicating cessation in feeding.

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5.4 Effect dichlorvos on AChE activity

Table 1.2 shows the AChE activity in control and dichlorvos treated worms. Worms exposed to dichlorvos for 4h exhibited significant, concentration-related inhibition in the enzyme activity. Dichlorvos at the lowest concentration (5μM) induced 69% inhibition of AChE activity while it induced 87 – 90% inhibition at the higher concentrations **(Figure 1.11)**.

5.5 Correlation between feeding inhibition, Hsp induction and AChE inhibition

Linear regression analysis plots showed a good correlation between feeding inhibition and AChE inhibition (R^2= 0.997, **Figure 1.12A**) in dichlorvos treated worms. Correlation was also significant between Hsp induction and AChE inhibition (R^2= 0.994, **Figure 1.12B**) and a good correlation was also observed between feeding inhibition and Hsp induction (R^2= 0.982, **Figure 1.12C).**

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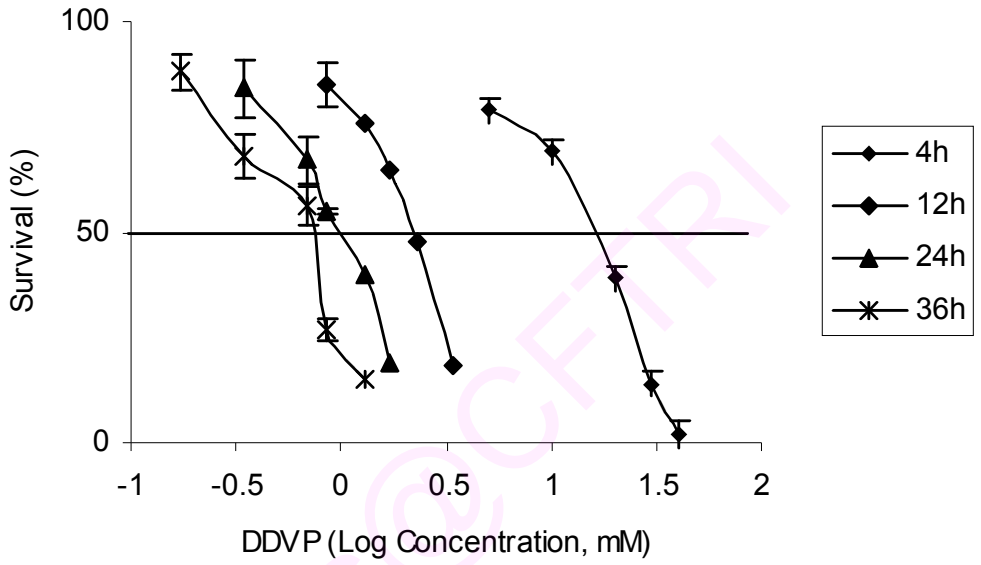

Figure 1.3 Survival profile of *C. elegans* after exposure to various concentrations of dichlorvos (DDVP) for different time intervals.
Each data point is the mean ± S.E.M of three replicates from three independent experiments (some error bars are smaller than the symbol size).

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Table 1.1 The lethal concentration (LC_{50}) of dichlorvos in *C. elegans* after various exposure intervals

Time of exposure (h)	LC_{50} (mM)
4	17.00 ± 0.36
12	2.20 ± 0.13
24	1.04 ± 0.07
36	0.70 ± 0.01

Values are mean ± S.E of three replicates each from three independent experiments

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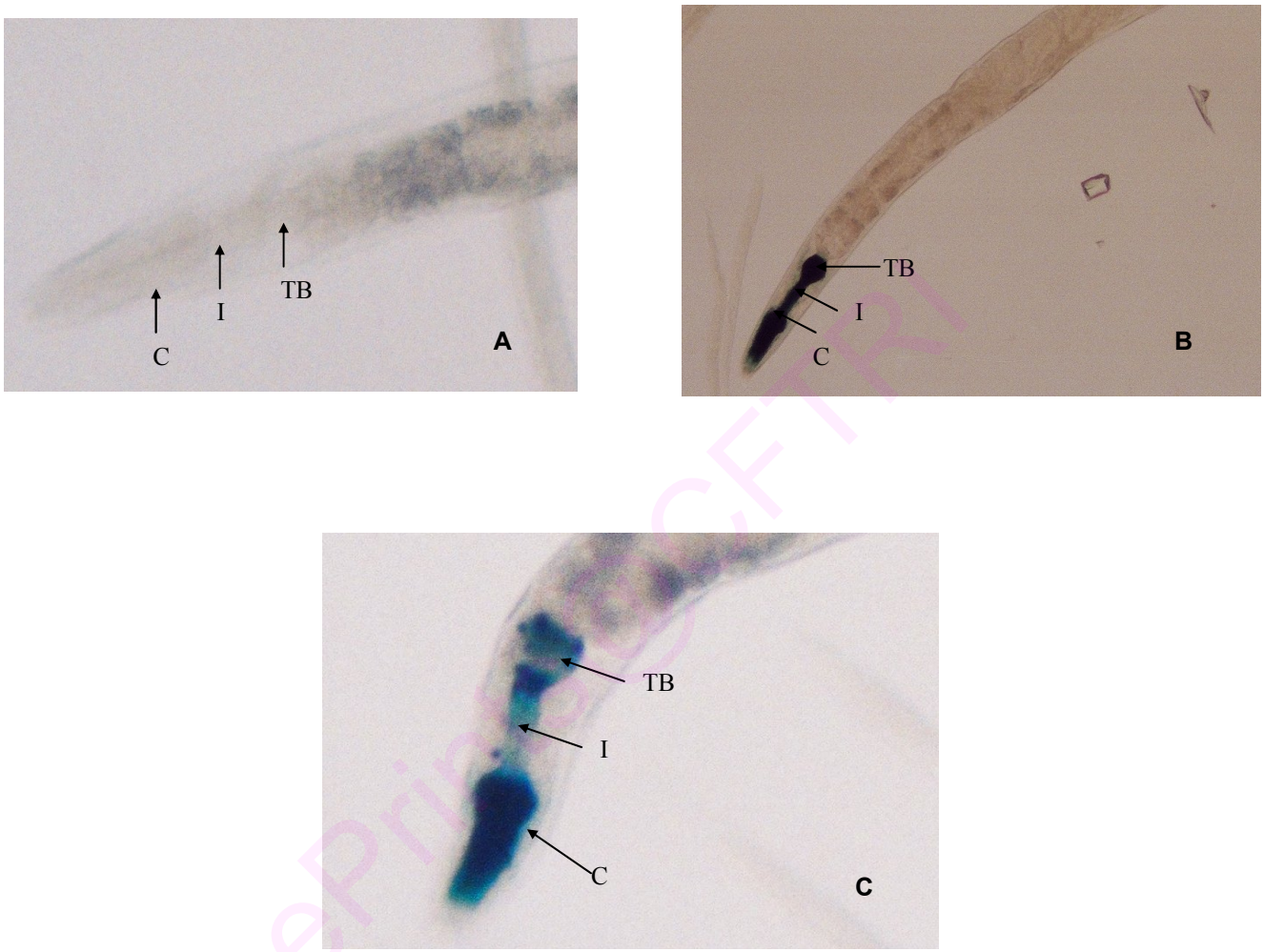

Figure 1.4 *In situ* β-galactosidase staining pattern in pharyngeal region (black arrow) of transgenic *C. elegans* (hsp16 lacZ)
A: Control worms
B: worms exposed to $CdCl_2$ (0.03 mg /ml)
C: worms exposed to dichlorvos (80 μM) for 24h.
The anatomical regions of the pharynx are the corpus (C), isthmus (I) and terminal bulb (TB).

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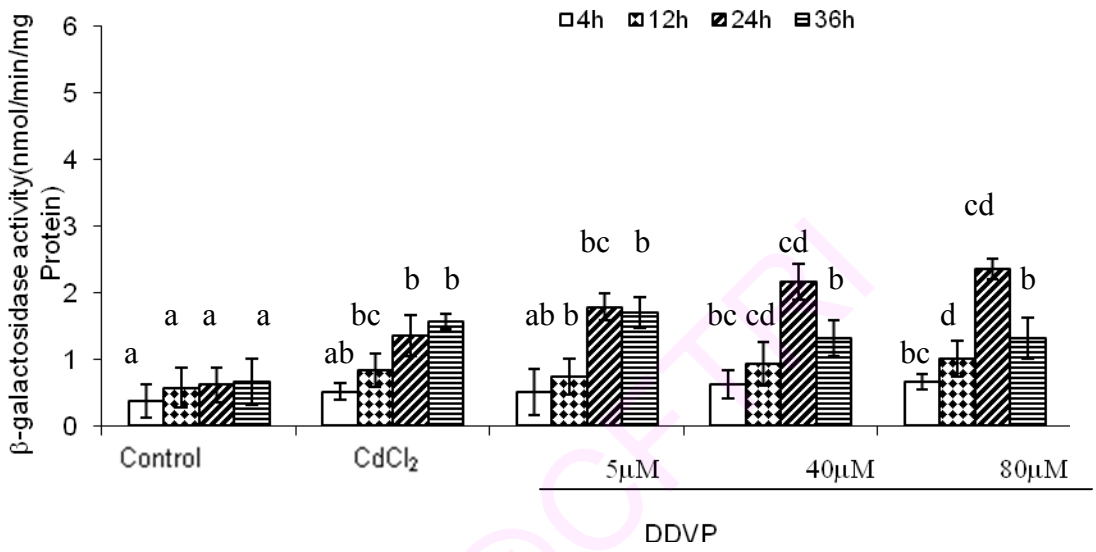

Figure 1.5 Time and concentration response of reporter (β-galactosidase) induction in PC72 worms by dichlorvos (DDVP) and $CdCl_2$ (0.03 mg /ml; positive control).

All values are mean ± S.E.M (n=9). Groups with different letters are significantly different (ANOVA, post hoc DMRT, P<0.05).

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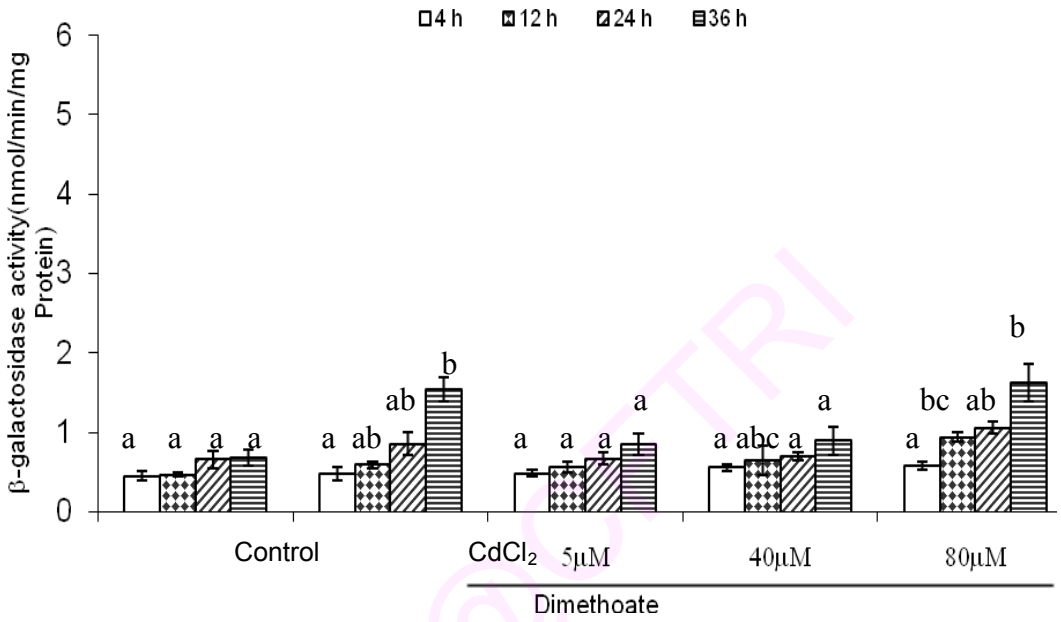

Figure 1.6 Time and concentration response of reporter (β-galactosidase) induction in PC72 worms by dimethoate and $CdCl_2$ (0.03 mg /ml; positive control).

All values are mean ± S.E.M (n=9). Groups with different letters are significantly different (ANOVA, post hoc DMRT, P<0.05).

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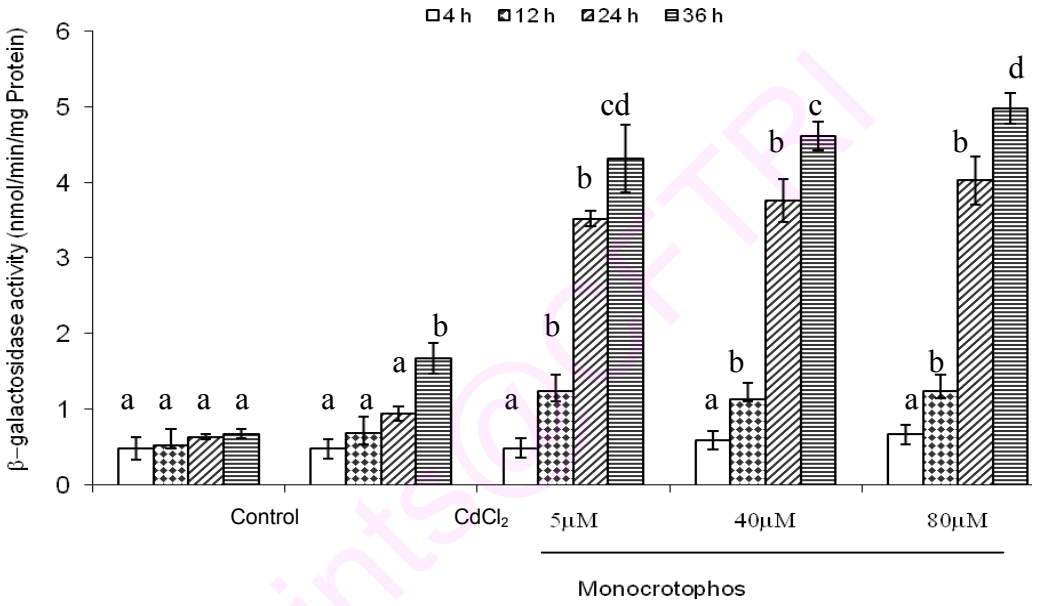

Figure 1.7 Time and concentration response of reporter (β-galactosidase) induction in PC72 worms by monocrotophos and $CdCl_2$ (0.03 mg /ml; positive control).

All values are mean ± S.E.M (n=9). Groups with different letters are significantly different (ANOVA, post hoc DMRT, P<0.05).

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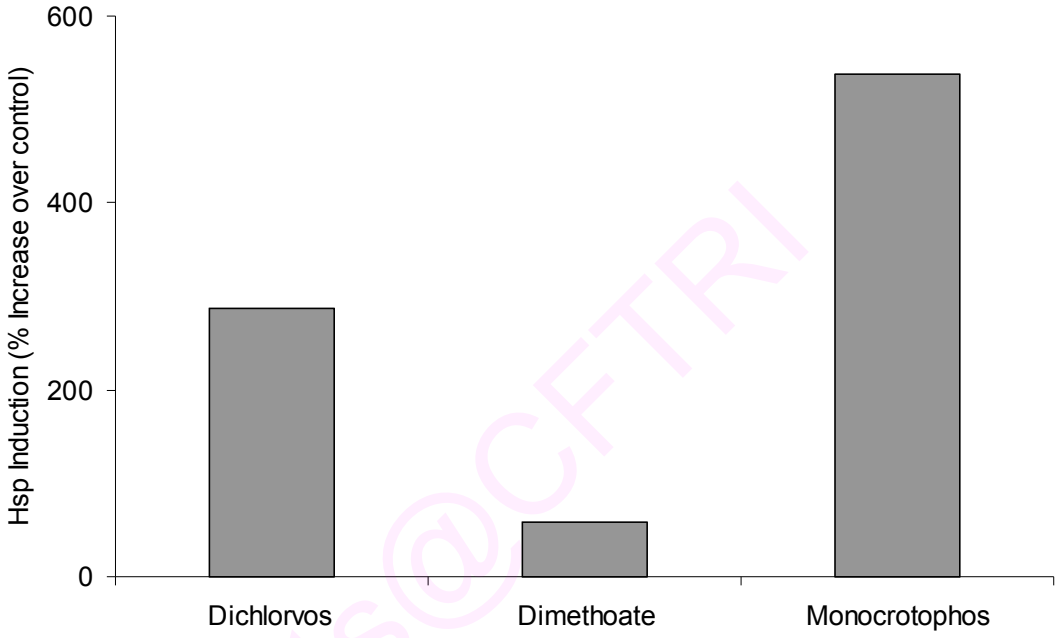

Figure 1.8 Comparative induction of Hsp by the OPI (all at 80 μM after 24h).

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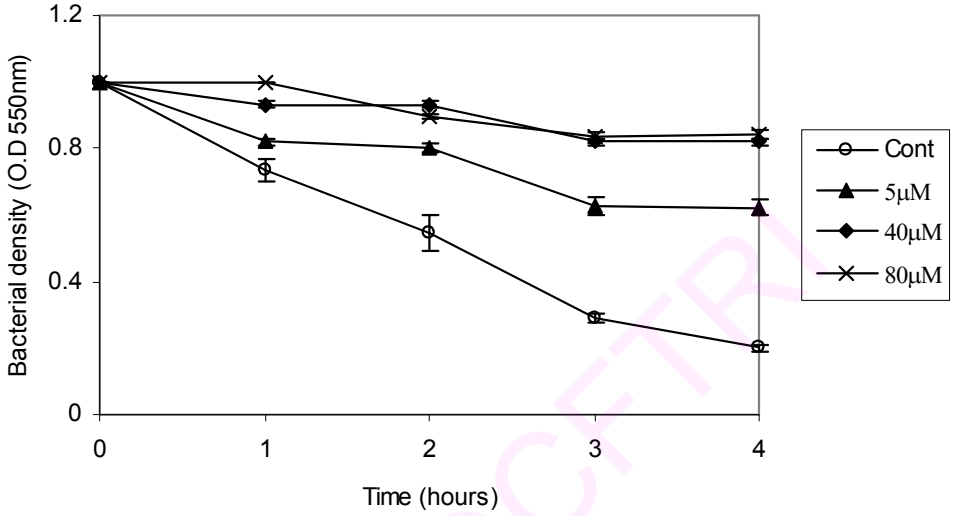

Figure 1.9 The effect of dichlorvos on feeding rate in *C. elegans*.
Each data point is the mean ± S.E.M of three replicates from three independent experiments (some error bars are smaller than the symbol size).

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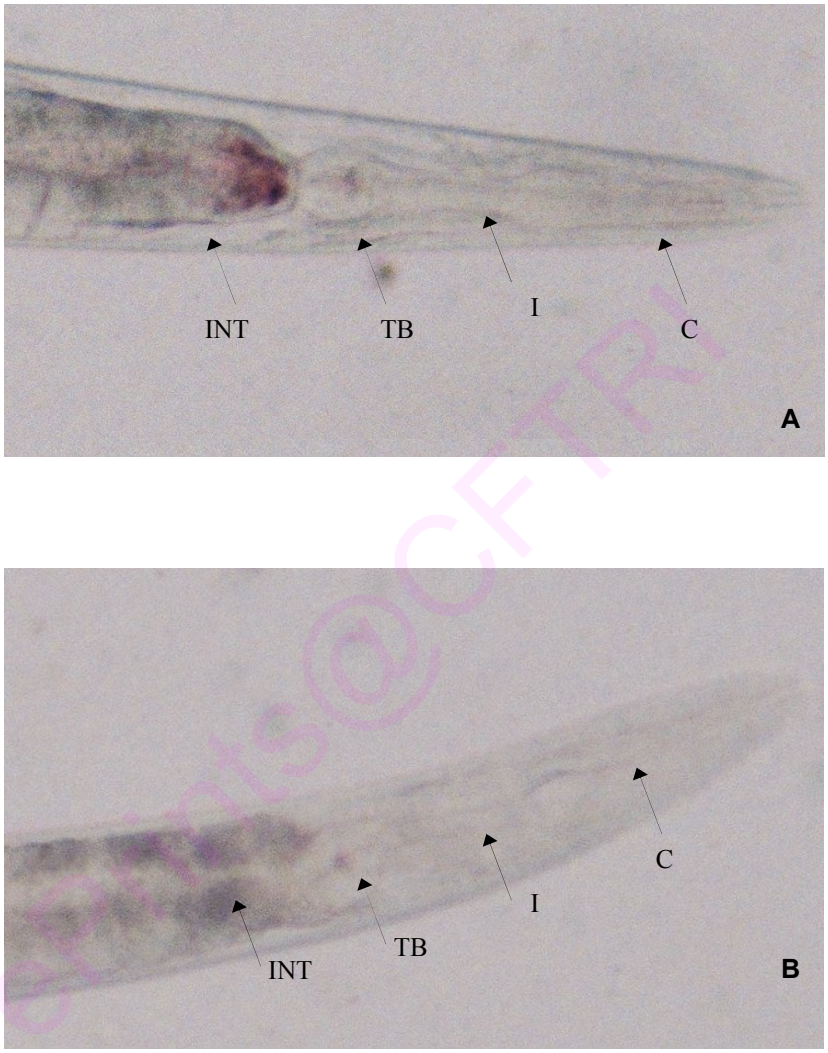

Figure 1.10 Effect of dichlorvos on pharyngeal pumping in *C. elegans* after 4h of exposure; A- Control worms showing particles of red acrylic paint below the pharynx; B – Dichlorvos-treated (80 μM) worms showing absence of pigment particles below the pharynx.
The anatomical regions of the pharynx are the corpus (C), isthmus (I) and terminal bulb (TB); below the terminal bulb is the intestine (INT).

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Table 1.2 Activity of acetylcholinesterase in transgenic *C. elegans* (PC72) exposed to dichlorvos for 4h.

Dichlorvos (μM)	AChE (nmol substrate hydrolysed/min/mg protein)
0	16.35 ± 2.02
5	5.03 [a] ± 0.51
40	2.07 [a] ± 0.04
80	1.61 [a] ± 0.24

Values are mean ± S.E of 3 observations with 3 replicates each.
Data analyzed by Student's *t* test; [a] $p < 0.001$

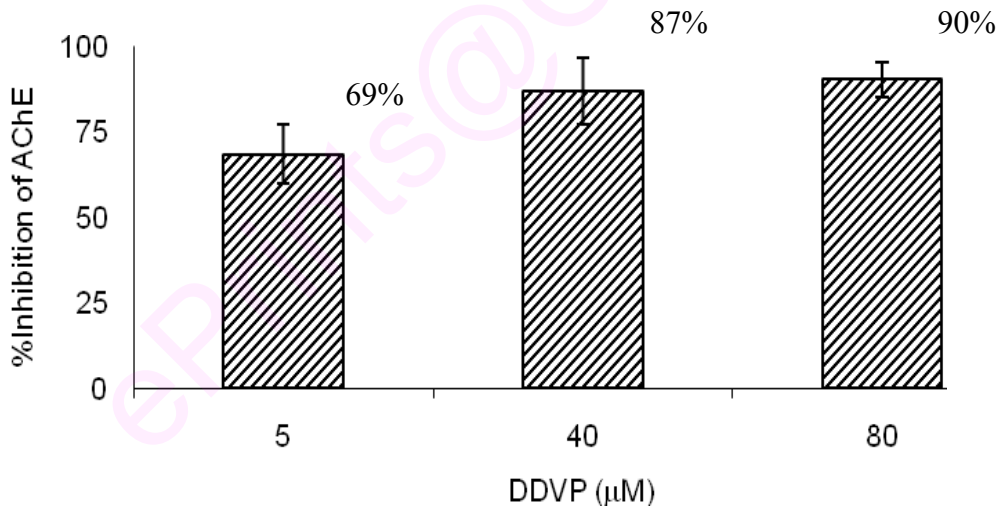

Figure 1.11 AChE inhibition in *C. elegans* exposed to varying concentrations of dichlorvos for 4h.

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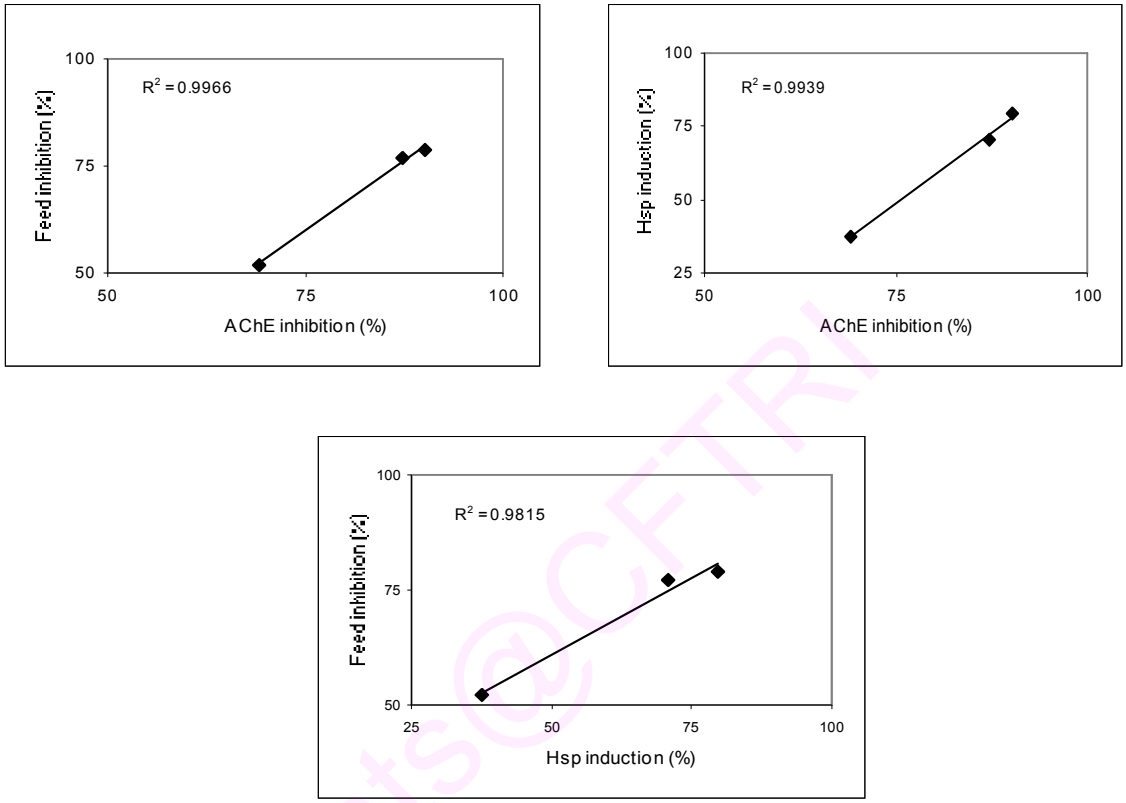

Figure 1.12 Results of regression analysis (three points per parameter) of the effects of sublethal concentrations of dichlorvos on *C. elegans*; for all comparisons p< 0.05.

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6.0 DISCUSSION

In the present study, the potential of three OPI viz., dichlorvos, dimethoate and monocrotophos to induce heat shock protein (hsp) was evaluated in transgenic *C. elegans* (hsp16-lacZ) by measuring β-galactosidase activity and by *in situ* histochemical staining. We further examined the causal relationship between three endpoints – hsp induction, AChE inhibition and feeding in the worms exposed to sublethal concentrations of dichlorvos. Firstly, we determined the lethality of the strain as a simple signal for toxicity after treatment with the three OPI. The toxicity response in terms of the LC_{50} values was comparable to that of the N2 strain suggesting the equal sensitivity of the strain PC72. We then evaluated the *hsp* inducing potential of the OPI at sublethal concentrations.

Stress genes in the recent years have been considered as potential biomarkers for toxicity because of their early expression and tissue specificity (Ryan and Hightower, 1996). Heat shock proteins (*hsp*) function at the cellular level to protect cells against a wide array of acute and chronic stress conditions. Sub acute activation of heat shock response is reported to result in stress tolerance and cytoprotection against otherwise lethal exposures to stress-induced molecular damage. This protective and adaptive response ensures that the cell responds rapidly to repeated subacute challenges by diverse conditions of cell stress.

Stress response in general is considered a valuable indicator of sublethal toxicity in environmental risk assessment. A clear positive correlation was observed between cytotoxicity of substances and their HSP-inducing capacity (Steinmetz and Rensing, 1997). Despite the universality of heat shock gene expression as a response to many kinds of stresses resulting in damaged proteins, detecting HSPs is difficult requiring either metabolic labeling or immunoblotting (de Pomerai, 1996), both of which are too laborious and expensive for routine toxicity testing. This difficulty can be overcome in

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genetically amenable transgenic strains of *C. elegans* (PC72) that carry HSE-containing heat shock promoters linked to bacterial reporter genes encoding an easily detectable product (e.g., β-galactosidase). The small hsps in *C. elegans* are strictly stress-inducible and act as molecular chaperones (Ding and Candido, 2000). It is believed that *hsp16* play important roles in enhancing survival of the animal under conditions of chemical and physical stress.

Transgenic strain, PC72 carries an integrated array of lacZ coding sequences, each inserted in-frame into the second exon of the *C. elegans hsp-16-1* gene and thus β-galactosidase expression is inducible under stress conditions that activate the endogenous *hsp-16-1* genes (Stringham and Candido, 1994). Stressors will activate reporter expression along with HSP production in such strains, and the resultant product can be quantified or localized using much simpler assay or staining methods (Guven et al., 1999). Strain PC72 has been used as a biomonitor for qualitative assessment of stress responses to a wide range of stressors such as reactive oxygen species (ROS), beta amyloid peptide, heavy metals and pharmaceutical compounds (Jones et al., 1996; Mutwakil et al., 1997; Deng and van Meel, 2004).

In the present study, exposure of the transgenic *C. elegans* to dichlorvos, dimethoate and monocrotophos resulted in a concentration – and time-dependent induction of hsp-16. Exposure to cadmium chloride produced a more generalized pattern of pharyngeal expression of the transgene as reported earlier (Stringham and Candido, 1994). The concentrations of the OPI tested were sublethal and caused no symptoms of toxicity in the worms even after 36h of exposure. Maximum induction of hsp16 was observed following 24h exposure on exposure to dichlorvos and 36h exposure to dimethoate and monocrotophos, although no significant change in the induction level was evident after 4h. As reported with other toxicants (Easton et al., 2001), slow uptake of the pesticide may be a key factor limiting the reporter response observed. Our feeding assay confirms this finding.

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The responses of transgenic worms to various pesticides have been earlier listed (Guven et al., 1999). It has been reported that hsp induction by certain toxicants is generally correlated with early cytotoxic events and is a secondary consequence of damages that affect cellular integrity (Gupta et al., 2005a). The lipophilic nature of the compounds easily allow them to pass the plasma membrane, alter vital cellular functions before interacting with cellular proteins, denaturing them and triggering stress protein induction (Ait-Aissa et al., 2000).

Interestingly, a decline in hsp16 induction was observed in all the treatment groups after 36 h in dichlorvos-exposed worms. In this context, the question of whether such a decline is due to instability of the reporter gene product after 36 h or due to a reduction in the number of cells available for hsp16 assay in the treated worms following 36 h exposure is to be investigated further. It has been reported that as toxicity approaches lethal levels, the efficiency of the reporter induction declines and even where mortality is low, there may still be a substantial drop in reporter expression as has been observed with longer exposure times with the dithiocarbamate fungicide (Easton et al., 2001).

Over expression of proteins of the HSP70 family has been extensively used as a marker of an increasing flux of nonnative or misfolded proteins resulting from heat shock, oxidative stress, or chemical aggressions (Croute et al., 2000). Over expression of HSP70 was also found to occur after exposure to several pesticides including organochlorines (Snyder and Mulder, 2001; Gaubin et al., 2002) and organophosphates (Yang et al., 2002). Further, diverse pesticides (organochlorines and organophosphates) were found to enhance HSP synthesis in cultured neuroactive PC-12 cells and in rat organs (Bagchi et al., 1996) and in Drosophila (Nazir et al., 2001; Gupta et al., 2005a,b). In 1980, Hightower proposed that *hsps* are synthesized in response to the generation of abnormal proteins. Several lines of evidences have shown that the mere presence of abnormally folded proteins can induce the stress response.

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Organisms respond to proteotoxicity with the induction of HSP, which are able to repair partly denatured proteins (Hallare et al., 2004).

OPI are potent neurotoxicants and are known for their inhibitory effect on acetylcholinesterase, leading to the production of symptoms due to accumulation of acetylcholine in synaptic cleft resulting in synaptic dysfunction (Lund, 1985). Several OPI have the potential to phosphorylate a wide variety of proteins, affecting the function of a number of esterases, proteases, cytoskeletal proteins and protein kinases involved in cell signaling (Abou-Donia, 1993; Pope, 1999) as well as other protein targets (Ray, 1998; Flaskos et al., 2007). Hence, one of the possibilities that dichlorvos, dimethoate and monocrotophos may be causing synaptic dysfunction as well as protein damage thereby stimulating hsp16 induction cannot be ruled out.

Previous studies showed that concomitant with a significant induction of hsp70 in primary cultured skeletal muscle cells following dimethoate exposure, significant inhibition in AChE enzyme activity was observed (Yang et al., 2002). Though a direct mechanistic link between the chemical exposure and hsp16 induction in these worms could not be established, it is likely that the inhibition of AChE activity, a key component of nerve function triggering damage to many cellular components including protein thereby stimulating hsp16 induction cannot be ruled out.

The effects of the test chemicals on expression of stress proteins are known to be tissue specific (Stringham and Candido, 1994). It has also been proposed that xenobiotic-induced stress protein expression may reflect tissue injury (Gonzalez et al., 1989; Goering et al., 1992). In the present study, hsp16 was found to be induced in the pharyngeal region at all the concentrations of the OPI tested.

Jones and Candido (1999) have described a response in *C. elegans* where a cessation of feeding occurs in response to stress. This effect could be observed by microscopy as a shut down of the pumping action of pharynx. The

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rate of nematode feeding could be quantified by measuring the rate of reduction of the optical density (at 550 nm) of the bacterial suspension. On addition of a toxicant, the rate of reduction of the optical density was markedly reduced, as a consequence of feeding inhibition (Jones and Candido, 1999). A physiological mechanism that inhibits feeding may be essential for survival when animals are exposed to severe chemical stress. Such a mechanism would serve both to limit the intake of toxins. Thus feeding inhibition also has implications for toxicological studies in *C. elegans* since it imposes a limit on the dose of stressors that can be ingested. Inhibition in feeding in *C. elegans* in response to alcohols, heavy metals, sulphahydryl reactive compounds salicylate and heat has been earlier reported (Jones and Candido, 1999).

Interestingly, feeding inhibition occurred much earlier than hsp induction in worms exposed to dichlorvos. All the concentrations of dichlorvos inhibited feeding (52 – 81%) by 3 – 4h, did not induce significant hsp at this time point. Perhaps the proteins required for maintenance of pharyngeal pumping might themselves be particularly susceptible to damage by dichlorvos. (Jones and Candido, 1999). A correlation between interference of dichlorvos on feeding behaviour and brain AChE was demonstrated in the bream (*Abramis brama*) by Pavlov et al., (1992) while Roex et al., (2003) observed that whole body AChE inhibition by parathion correlated with increased food consumption in zebra fish (*Danio rerio*). The association between AChE inhibition and the activation of a toxic process for invertebrates is not as well understood as it is for vertebrates. However, a number of studies have found significant reductions in ChE activity that correlated with effects on behaviour and feeding that may impair subsequent survival of the exposed organisms (Fryday et al., 1996; Kumar and Chapman, 1998; Brewer et al., 2001; Cooper and Bidwell, 2006). From the parameters investigated in this experiment, both AChE and feeding rate in *C. elegans* were significantly affected by dichlorvos. Several studies with various animal models have demonstrated that decreased food consumption is a common effect upon exposure to OPI (McLoughlin et al., 2000; Roex et al.,

2003). In *Drosophila melanogaster*, dichlorvos is reported to induce increased expression of hsp70 in brain ganglia of the exposed larvae, as well as significantly inhibit AChE activity (Gupta et al., 2005a, b). Dimethoate has been reported to induce significant levels of hsp70 in primary cultured skeletal muscle cells along with marked inhibition in AChE activity (Yang et al., 2002). Although a direct mechanistic link between exposure to dichlorvos and hsp induction cannot be established, it can be presumed from the other reports as well as our data that inhibition of AChE activity, a key component of nerve function may trigger damage to many cellular components including protein (Ray, 1998; Flaskos et al., 2007) thereby leading to hsp induction. Small HSPs are often found to be up regulated in neurodegenerative diseases (Dabir et al., 2004) and also in motor neuron cell injury (Benn et al., 2002).

In conclusion, our data clearly demonstrated a correlation between a well validated toxicity endpoints and upstream induced gene expression. This is quite relevant for ecotoxicological purposes. Our study also provides evidences on the interrelationships between the toxicity parameters and also on the impact of dichlorvos exposure on the vital physiological functioning in *C. elegans*. The correlated endpoints may provide a sensitive assessment of the detrimental effects of dichlorvos as well as other organophosphate pesticide contaminants in water. Initial identification of a stress response induction in transgenic worms due to a suspected OPI contamination may have practical advantage in risk assessment in the aquatic ecosystem. This is likely to further aid in foreseeing the hazards posed by the toxicant to other sensitive organisms inhabiting the aquatic system.

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7.0 SUMMARY

1. The 4h-LC_{50} values of dimethoate and monocrotophos in the transgenic worms were 17.4 mM and 66.5 mM respectively.
2. The LC_{50} values of dichlorvos for all the four exposure periods, 4, 24, 36 and 72h were: 17.00, 2.20, 1.04 and 0.70 mM respectively.
3. Based on the results, three concentrations of dichlorvos, corresponding to approximately 1/20, 1/40, and 1/360 of 4h-LC_{50} — 5, 40 and 80 μM—were selected for further studies. Equimolar concentrations (5, 40 and 80 μM) of the three OPI were employed for heat shock protein induction studies.
4. β-galactosidase staining was evident only in the pharyngeal region of the worms at 24h when exposed to all the three OPI and worms exposed to $CdCl_2$ (positive stressor) also reflected a strong β-galactosidase activity in the pharyngeal region.
5. The extent of reporter (β-galactosidase) induction in the transgenic (PC72) worms in response to the positive stressor, cadmium chloride and the OPI, dichlorvos, dimethoate and monocrotophos was evident as early as 4h after exposure.
6. The induction was significantly high and concentration-dependant on exposure to dichlorvos (0.4 -1.1fold), dimethoate (0.08 - 0.44 fold) and monocrotophos (0.02 - 2.29 fold) at 5-100μM.
7. The maximal response obtained for dichlorvos was at 24h of exposure while for dimethoate (0.25 – 1.91 fold) and monocrotophos (5.44 – 7.42 fold) the maximum induction was seen at 36h of exposure.
8. Maximal response (1.9 - 3.5 fold increase) in dichlorvos-exposed worms was evident after 24h exposure and at this time point, the response was greater than that elicited by $CdCl_2$ and a drop in response was however evident at 36h at all dichlorvos concentrations.

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9. Comparatively, at the highest concentration (80μM) after 24h, all the three OPI elicited marked induction of heat shock protein. The order of induction was: monocrotophos (6.4 fold) > dichlorvos (4 fold) > dimethoate (1.6 fold).
10. Dichlorvos at all the tested concentrations induced cessation in feeding in *C. elegans* after 3h of exposure.
11. While the extent of feeding inhibition after 4h in worms exposed to 5μM was 52%, worms exposed to 40 μM and 80 μM showed 77 – 79% inhibition in feeding.
12. Feeding inhibition after 4h exposure to dichlorvos was confirmed by uptake of red acrylic paint particles by the treated worms. Dichlorvos treated worms showed no pigment particles in the pharynx clearly indicating cessation in feeding.
13. Worms exposed to dichlorvos for 4h exhibited significant and concentration-related inhibition in the enzyme activity. Dichlorvos at the lowest concentration (5μM) induced 69% inhibition of AChE activity while it induced 87 – 90% inhibition at the higher concentrations.
14. Linear regression analysis plots showed a good correlation between feeding inhibition and AChE inhibition and between Hsp induction and AChE inhibition in dichlorvos treated worms.

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CHAPTER II

Mechanisms underlying the toxicity of dichlorvos in *C. elegans*.

PREFACE

From our earlier study (chapter IA) it is evident that the toxicity of dichlorvos was relatively higher in *C. elegans*, based on 4h-LC_{50} values. Hence we selected dichlorvos for further mechanistic studies. In the present study, we employed sublethal concentrations of dichlorvos.

This chapter has been presented under **three** sections:

Section A describes investigations on the neurophysiological effect of dichlorvos in *C. elegans* when exposed at sublethal concentrations for 4h.

Section B describes the interactions of the antidotes atropine and pralidoxime chloride (2-PAM) on *C. elegans* exposed to dichlorvos at sublethal concentrations.

Section C describes the effect of sublethal concentrations of dichlorvos on oxidative stress parameters as well as brood size and life span in *C. elegans*.

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CHAPTER II

Mechanisms underlying the toxicity of dichlorvos in *C. elegans*

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SECTION II A

Neurophysiological alterations in *C. elegans* exposed to sublethal concentrations of dichlorvos

1.0 INTRODUCTION

Organophosphorus insecticides (OPI) are among the most commonly used pesticides in the world. Their success relies on their high toxicity and rapid environmental degradation (Eto, 1974). Unfortunately, OPI lack target specificity and can cause severe, long lasting population effects on terrestrial and aquatic non-target species, particularly invertebrates (Schulz and Liess., 1999, Fulton and Key., 2001). Most OPI are irreversible inhibitors of acetylcholinesterase (AChE), an enzyme that is characteristic of the neuromuscular junctions responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh). ACh, a major neurotransmitter in both vertebrate and invertebrate nervous systems interacts with receptors on target cells and elicits a variety of post-synaptic responses. AChE is the primary target of OPI, which by phosphorylating the serine hydroxyl group located in the active site of the AChE, inhibits its activity (Ozmen et al., 1999). As a consequence, ACh accumulation in the synapses and neuromuscular junctions leads to over stimulation of cholinergic receptors that result in a general pattern of nerve poisoning, hyper excitability, tremors and paralysis and causes neurotoxin effects (O'Brien, 1960**)**. In mammals, death is caused by asphyxiation (Murphy, 1966**)**, but the action in aquatic organisms including fish and invertebrates is less clear (Day et al., 1990). However, the manifestation of cholinergic signs of toxicity in non-target species exposed to OPI suggests a mechanism common to that in insects. Since an organism's behavior is the cumulative interaction of many factors, the assessment of behavioral changes after environmental exposure could serve as a sensitive indicator of adverse effects. One of the important issues in performing behavioral studies is the identification of specific endpoints and monitoring changes in movement.

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The nematode *C. elegans* has become an important model organism for the study of development, aging, host-pathogen relationship, and certain diseases (Leung et al., 2008). This organism possesses simple nervous system, consisting of 302 neurons and facilitates the study of the organism's behavior. Considerable toxicity testing has been performed employing *C. elegans* as a bioassay system and various endpoints assessed have been assessed in both aquatic and soil environments. Most widely used end points include lethality (Leung et al., 2008), fecundity and motility (Williams and Dusenbery, 1990; Leung et al., 2008; Rajini et al., 2008), reproduction and development, growth rate and inhibition of feeding (Leung et al., 2008). Locomotion, feeding (pharyngeal pumping) and defecation have also been extensively evaluated and the two former parameters have been considered as sensitive behavioral end points in toxicity testing.

Dichlorvos is reported to be neurotoxic due to its irreversible inhibitory effect on AChE (Wang et al., 2004). There is a lack of data pertaining to invertebrate exposure to dichlorvos as non-target organism. Recent studies have reported the toxicity of dichlorvos in *C. elegans* with special reference to comparing its LC_{50}, EC_{50} with that of rat LD_{50} (Cole et al., 2004 ; Rajini et al., 2008). In our earlier study (Jadhav and Rajini, 2009), we showed that sublethal concentrations of dichlorvos induce heat shock proteins, inhibit feeding and AChE activity in transgenic *C. elegans*. We also reported significant correlation between the three evaluated endpoints, which suggested their possible interrelated role in the physiological dysfunctions evoked by sublethal concentrations of dichlorvos. In the present study, we have addressed two major issues i) the pattern of AChE inhibition and accumulation of ACh in *C. elegans* exposed to sublethal concentrations of dichlorvos and ii) possible correlation between AChE inhibition, ACh accumulation and the incidence of neurobehavioral responses. The concentrations of dichlorvos selected for the present study were based on the 4h-LC50 (16 mM) obtained in a preliminary study (Chapter IA).

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2.0 MATERIALS AND METHODS

2.1 Chemicals

Acetylthiocholine iodide, BSA (fraction V) and 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB) were procured from Sigma Chemical Co., (St. Louis, MO, USA). Folin's reagent, Hydroxylamine hydrochloride and Ferric chloride were procured from Sisco Research Lab. (Mumbai, India) while acetylcholine iodide was purchased from Hi Media (Mumbai, India). All other chemicals used were of analytical grade. Acrylic red paint was procured from local market.

Dichlorvos (DDVP; Technical grade; 97.6%) was a gift from Hyderabad Chemical Supplies Ltd., (Hyderabad, India).

2.2 Pesticide solution

Stock solution (1mM) of Dichlorvos (DDVP) was prepared in double distilled water and further diluted to obtain working solutions for the assays.

2.3 Worm culture and maintenance (Brenner, 1974)

The wild-type *Caenorhabditis elegans* Bristol strain N2 was obtained from Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA) which is funded by the NIH National Center for Research Resources (NCRR). The worms were cultured and maintained as described in Chapter IA (*Section 2.3.1 – 2.3.3*). Adult worms (3.5 d old) were used for the studies.

3.0 EXPERIMENTAL PROCEDURE

3.1 Effect of sublethal concentrations of dichlorvos on feeding

Feeding was quantified in worms exposed in K-medium (*E.coli* – OP50-suspended in the medium with or without the pesticide) by monitoring the changes in the optical density of the suspension in a spectrophotometer at 550 nm. Aliquots (I ml) of K-medium with OP50 suspended (after dilution at roughly 1 O.D at 550 nm) were dispensed into the wells of 12 well tissue culture plates. Dichlorvos was added to the wells at different concentrations (0, 5, 50, 100, 150

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and 200μM). The worms suspended in K-medium were added to the wells such that each well had 5,000 worms. The plate was incubated at room temperature with constant shaking. Each 1ml sample was drawn at hourly intervals, spun gently for a few seconds at 800 rpm. The absorbance of the supernatant was determined to note the decrease in bacterial density and the samples were returned to the culture plate for further incubation (Jones and Candido, 1999).

3.2 Effect of dichlorvos on pharyngeal pumping

Cessation of pharyngeal pumping was confirmed by including red acrylic paint in the exposure medium. After 4h exposure to dichlorvos, aliquot (20μL of 20 mg/ml solution) of acrylic polymer emulsion (Tristar, Mumbai, India) was added to the worm suspension. The worms were observed under 10X magnification after 10 min for the pharyngeal localization of the dye particles (Jones and Candido, 1999).

3.3 Induction of paralysis and nose contraction by dichlorvos

For paralysis and nose contraction assays, adult worms were placed on NGM plates containing various concentrations of dichlorvos. Plates containing dichlorvos was prepared by adding 100 mM stock solution of dichlorvos (prepared in double distilled water) to autoclaved NGM agar media at varying final concentrations (0, 5, 50, 100,150 and 200μM). Plates were poured and dried overnight and OP50 bacteria were spread on the dried plates and incubated overnight at 37°C to grow a thin lawn.

3.3.1 Paralysis

Worms (100 adults) were placed on NGM plates with varying concentrations of dichlorvos and incubated for 4h at 20°C. The number of paralyzed worms was counted at the end of every hour and the data expressed as percent animals paralyzed (Lesa et al., 2003).

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3.3.2 Nose contraction

Nose contraction response was determined by exposing 100 adult worms to varying concentrations of dichlorvos in NGM plates for 4h and at the end of every hour counting the number of worms with any discernible nose rounding under a dissecting microscope (at 40X magnification). The data was expressed as percent worms with contracted nose (Choy and Thomas, 1999).

3.4 Effect of dichlorvos on egg-laying rates

The egg laying rates of worms in the presence of dichlorvos was determined in NGM agar media containing dichlorvos in 24-well tissue culture plates. 10 age-synchronized worms (adults, 3.5d old) were included for each concentrations of dichlorvos (0, 5, 50, 100,150 and 200μM). The number of eggs laid per worm during 1 h of exposure to dichlorvos was counted. For each condition tested, the assay was repeated 12 times, thus using a total of 120 animals (Bany et al., 2003).

3.5 Effect on AChE activity and ACh content

To examine the effect of dichlorvos on the activity of AChE and ACh content in *C. elegans*, worms (15,000-20,000 / well) were exposed to dichlorvos (0, 5, 50, 100, 150 and 200 μM) in K-medium for 4h at 20 ± 1°C. At every 1h interval, aliquots of worms were drawn, washed three times with K-medium and subsequently processed for AChE and ACh assays as described below.

4.0 ASSAY METHODS

4.1 Acetylcholinesterase (AChE) (EC 3.1.1.7) (Ellman et al., 1961)

After the exposure period, the worms were washed with K-medium and processed as described earlier (*Chapter IB, Section 4.3*).

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4.2 Acetylcholine content (Augustinson, 1957)

After the exposure period, worms were pelleted and re-suspended in 1N formic acid/acetone (85:15) and then subjected to low-speed centrifugation (1000 rpm for 2 min). The pellets were suspended in 2M NaOH and aliquots were used to determine protein concentration as described in Section 4.3. The supernatant was withdrawn, lyophilized and used for acetylcholine determination. Briefly, the lyophilized content was dissolved in 2.0 ml of distilled water to which 2 ml of alkaline hydroxylamine hydrochloride and 1.0 ml of HCl (6N) was added. The contents were centrifuged and 1.0 ml of ferric chloride was added to the supernatant and the absorbance of the sample was measured at 540 nm in a spectrophotometer against a reagent blank. The ACh values were calculated from standard graph and expressed as μg ACh /mg protein.

4.3 Protein content (Lowry et al., 1951)

10 µl of supernatant was added to 490 µl of distilled water and incubated with 2.5ml alkaline copper sulfate solution for 10 min at room temperature 250 µl of Folin's reagent (1:1 diluted with water) was added and the color was read after 30min at 670nm. Bovine serum albumin (BSA) was used as the standard.

4.4 Statistical analysis

Each assay was conducted in triplicate, on three different days. Data were expressed as mean ± S.E. Statistical significance was assessed either by Student's t-test ($p<0.001$) or by ANOVA and post-hoc Duncan's Multiple Range Test (DMRT) ($p< 0.05$) using SPSS 10 for windows (SPSS, Chicago, IL, USA).

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5.0 RESULTS

5.1 Feeding inhibition

There was a gradual time-related decrease in bacterial density in the control worm suspension indicating normal feeding by these worms. Dichlorvos at all the tested concentrations induced cessation in feeding after 4h of exposure. While the extent of feeding inhibition after 4h in worms exposed to 5μM was 52%, worms exposed to 50 - 200 μM showed 77 – 79% inhibition in feeding **(Figure 2.1).** Feeding inhibition after 4h exposure to dichlorvos was confirmed by uptake of red acrylic paint particles by the treated worms. While untreated worms took up the pigment particles, which accumulated in the pharynx within 10min, dichlorvos treated worms showed no pigment particles in the pharynx **(Figure 2.2).**

5.2 Paralysis

Paralysis rates were measured at the end of every hour of exposure to dichlorvos. There was a time and concentration related increase in the number of paralyzed worms (6-23%) exposed to dichlorvos. The percent of worms that were paralysed at the end of 4h exposure ranged from 22-81%. Further, more than 50% of the worms were paralyzed at 150 and 200μM after 4h of exposure **(Figure 2.3).**

5.3 Nose contraction response

Nose contractions were monitored at the end of every hour of exposure to dichlorvos. Worms exposed to dichlorvos hyper contracted their nose muscles **(Figure 2.4)** and there was a time- and dose-dependent increase in the number of worms contracting their noses. The percent of worms exhibiting nose contraction at the end of 1h was 10-32%. The number of worms hyper contracting their noses increased between 2-3h. However, there was a decrease in the number of worms exhibiting nose contraction at all the concentrations of dichlorvos at the end of 4h exposure period **(Figure 2.5).**

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5.4 Effect on egg laying

Egg laying rates in the presence of dichlorvos was evaluated and the results are presented in **(Table 2.1).** The average number of eggs laid in the control group was 3 ±0.2 whereas, in the worms exposed to the highest concentration (200µM) it was one or no egg laid /worm. The percent decrease in egg laying at 5µM was 53% and at highest dose (200 µM) was 69%.

5.5 Effect of dichlorvos on AChE activity

Worms exposed to dichlorvos exhibited significant reduction in the AChE activity in a concentration and time dependent manner **(Table 2.2, Figure 2.6).** Dichlorvos at 5µM induced nearly 32-46% inhibition while at 200 µM it induced 65-82% inhibition between 1 – 4h of exposure.

5.6 Effect of dichlorvos on ACh content

Accumulation of ACh in worms exposed to sublethal concentrations of dichlorvos revealed an interesting trend. ACh accumulation showed a concentration-dependent increase (20 - 59%) after 1h, which increased further after 2h (73 - 86%). However, there was a decrease in ACh content after 3h which, further decreased at 4h to near control levels **(Table 2.3, Figure 2.7).**

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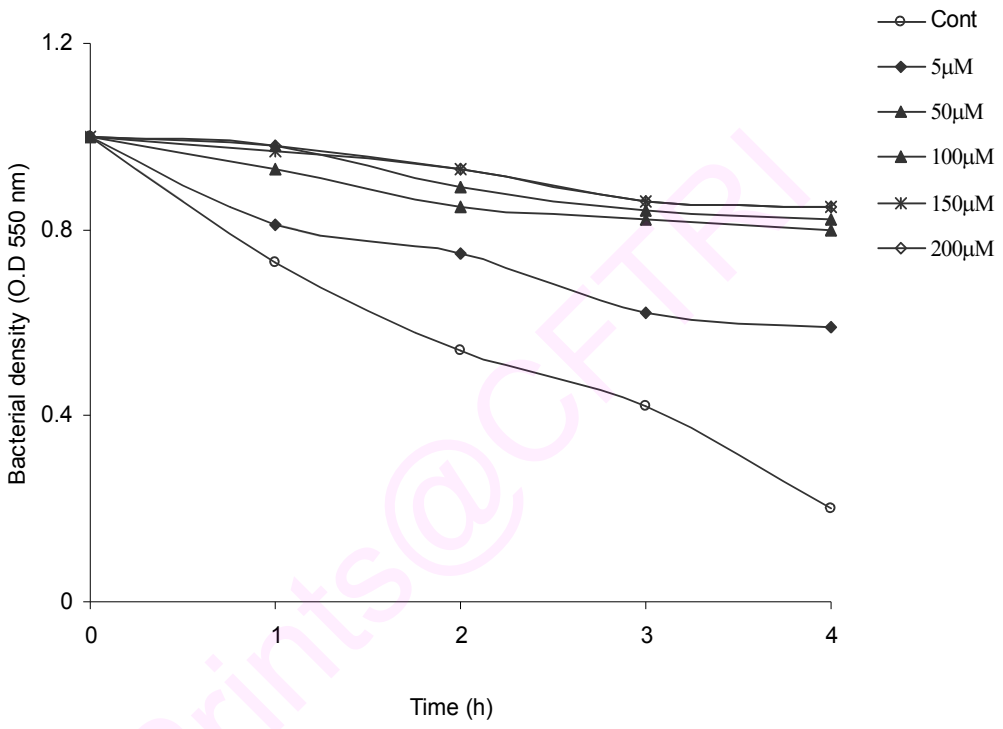

Figure 2.1 The effect of dichlorvos on feeding rate in *C. elegans* (N2). Each data point is the mean ± S.E.M of three replicates from three independent experiments.

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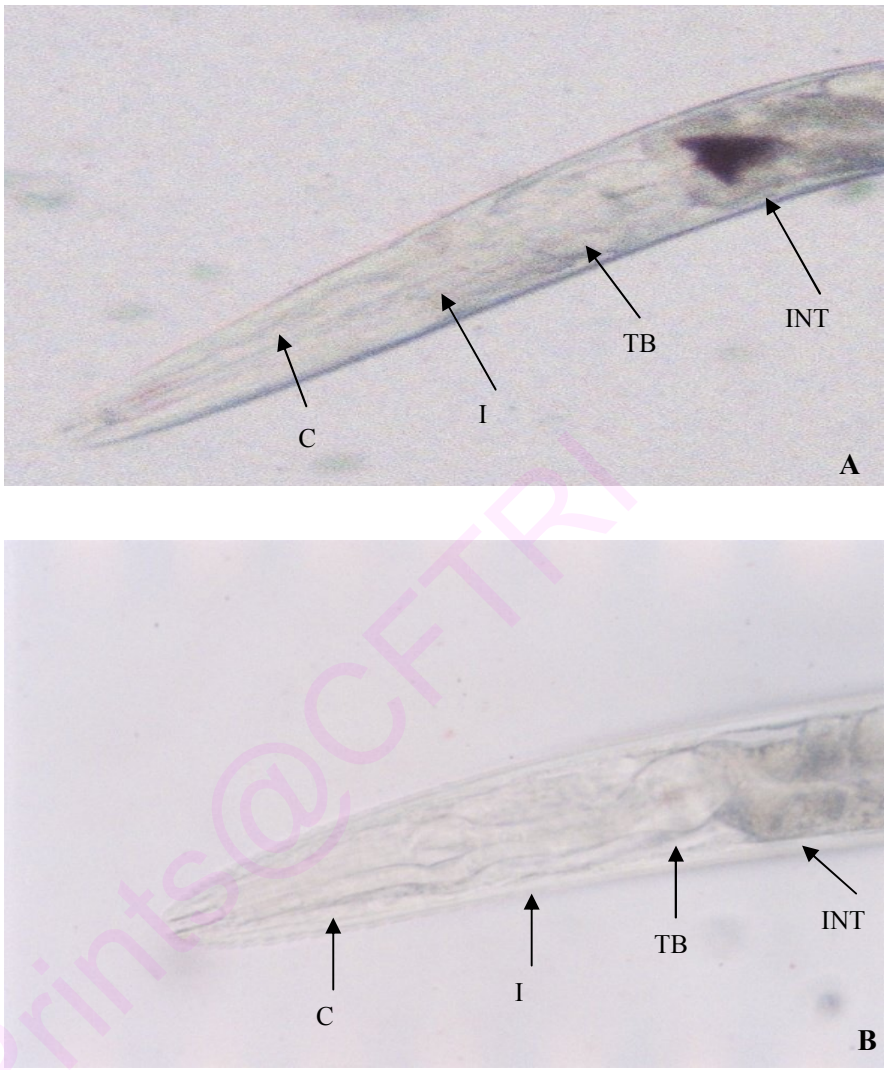

Figure. 2.2 Effect of dichlorvos on pharyngeal pumping in *C. elegans* (N2) after 4h of exposure.
A: Control worms showing particles of red acrylic paint below the pharynx;
B: Dichlorvos-treated (100 μM) worms showing absence of pigment particles below the pharynx.
(The anatomical regions of the pharynx are the corpus (C), isthmus (I) and terminal bulb (TB); below the terminal bulb is the intestine (INT)).

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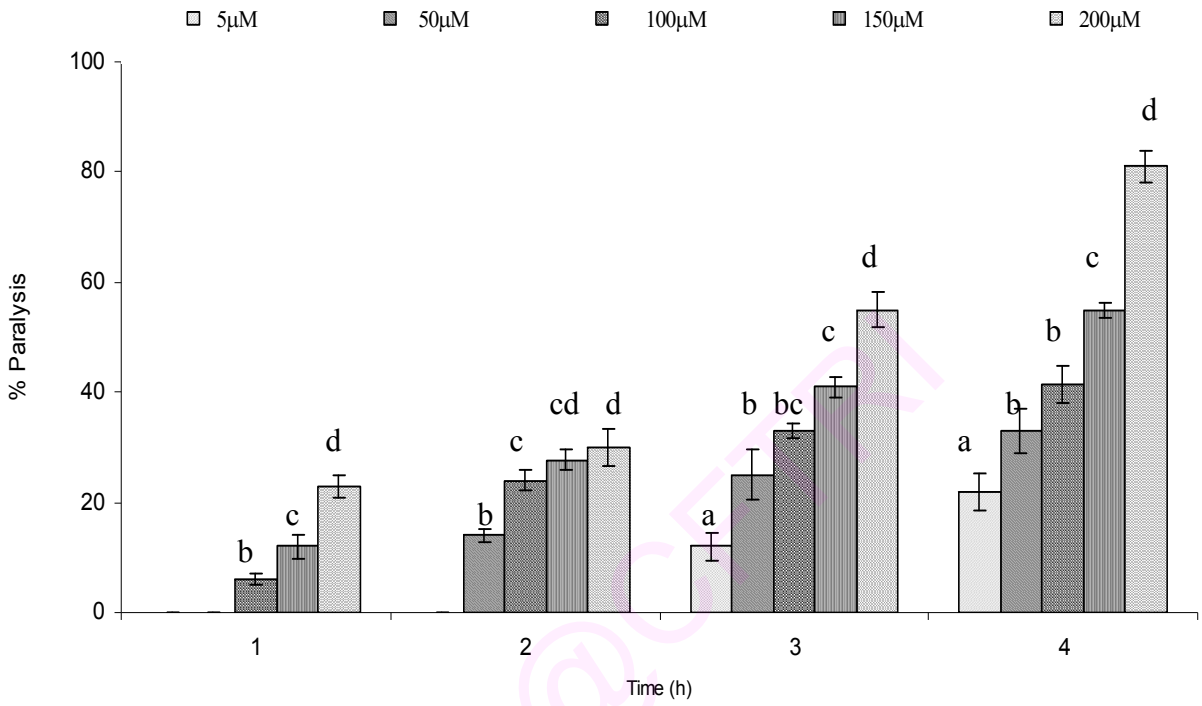

Figure 2.3 Induction of paralysis in *C. elegans* (N2) by sublethal concentration of dichlorvos.

Each data point is the mean ± S.E.M of three replicates from three independent experiments. Bars with different letters are significantly different (ANOVA, post hoc DMRT, P<0.05).

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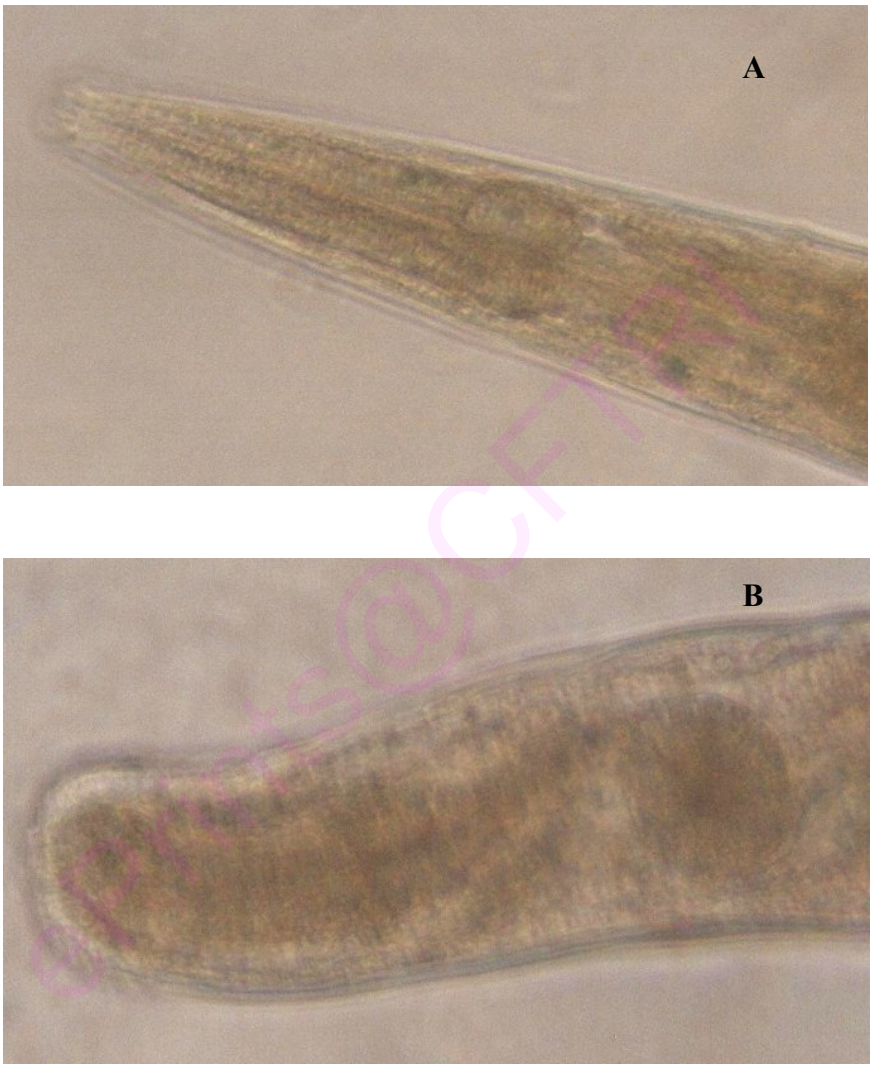

Figure.2.4 Nose contraction response in *C. elegans* (N2) after 4h of exposure to dichlorvos.
A: Control worm showing normal nose region
B: Dichlorvos-treated (100μM) worm showing hyper contracted nose.

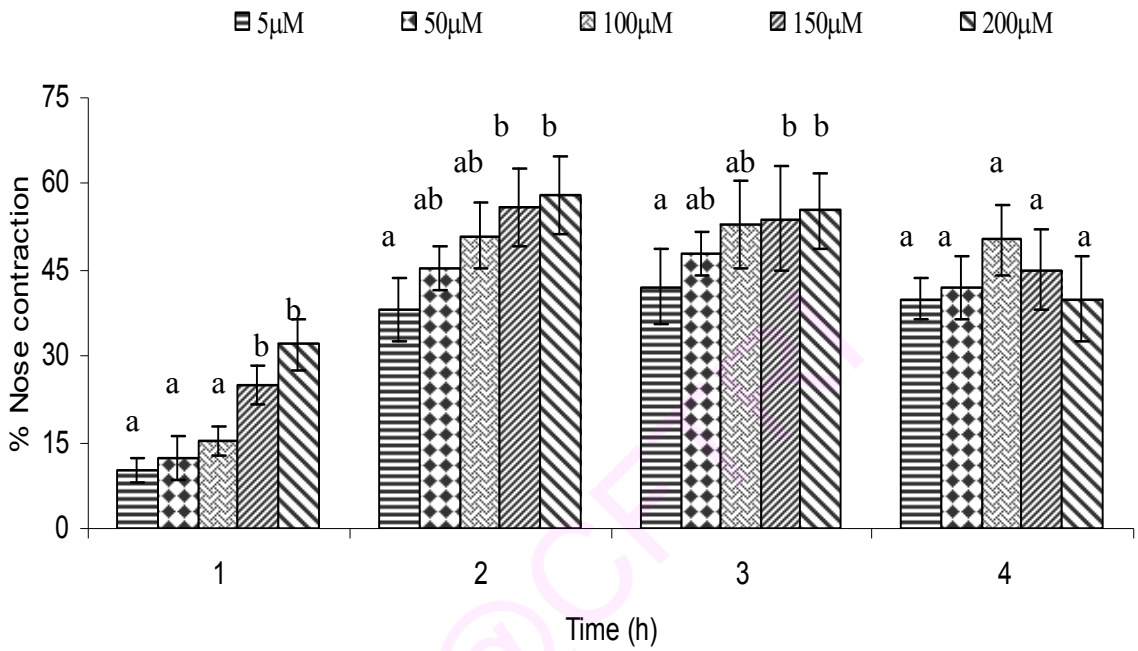

Figure 2.5 Nose contraction response in *C. elegans* (N2) exposed to dichlorvos: time and concentration response.

Each data point is the mean ± S.E.M of three replicates from three independent experiments. Bars with different letters are significantly different (ANOVA, post hoc DMRT, P<0.05).

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Table 2.1 Inhibition of egg laying in *C. elegans* (N2) on exposure to dichlorvos.

Dichlorvos (μM)	Mean Eggs / worm*	%Inhibition
0	3.0 [a] ±0.20	0
5	1.5 [b] ±0.25	53
50	1.4 [bc] ±0.14	58
100	1.3 [bc] ± 0.23	59
150	1.0 [bc] ±0.21	63
200	1.0 [c] ± 0.20	70

* Eggs laid by a single worm after 1h of exposure to dichlorvos (n= 120).
Means in the same column with different superscript differ significantly ($p < 0.05$)

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Table 2.2 Acetylcholinesterase activity in *C. elegans* exposed to dichlorvos.

	AChE (nmol substrate hydrolysed/min/mg protein)			
Dichlorvos (μM)	1h	2h	3h	4h
0	24.90 [a] ± 1.05	23.08 [a] ± 1.42	23.51 [a] ± 0.77	23.71[a] ± 1.90
5	16.74 [b] ± 1.62	14.77 [b] ± 1.02	13.03 [b] ± 1.05	12.88 [b] ± 1.17
50	13.97 [c] ± 2.28	10.43 [c] ± 1.06	9.83 [c] ± 1.60	7.64 [c] ± 2.30
100	10.26 [d] ± 2.50	7.74 [d] ± 0.60	6.85 [d] ± 0.20	6.33 [c] ± 1.66
150	9.09 [d] ± 1.91	7.67 [d] ± 0.65	6.70 [d] ± 0.26	5.85 [c] ± 1.02
200	8.44 [d] ± 1.75	6.52 [d] ± 0.50	5.56 [d] ± 1.90	4.34 [c] ± 1.30

Values are mean ± S.E of 3 observations with 3 replicates each.
Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)

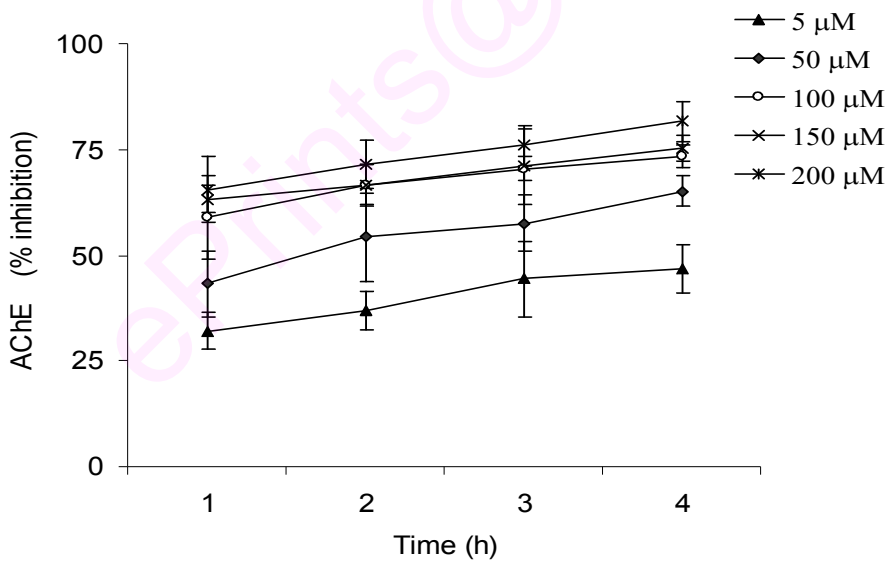

Figure 2.6 Inhibition of AChE in *C. elegans* (N2) exposed to sublethal concentrations of dichlorvos: Time and concentration response.
All values are mean ± S.E.M (n=9). Bars with different letters are significantly different (ANOVA, post hoc DMRT, P<0.05).

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Table 2.3 Accumulation of acetylcholine in *C. elegans* exposed to dichlorvos

	ACh (μg/mg protein)			
Dichlorvos (μM)	1h	2h	3h	4h
0	16.74 [a] ±2.44	18.58 [a] ± 2.5	19.51 [a] ±3.27	19.22 [a] ± 4.70
5	20.10 [a] ±4.38	32.10 [b] ± 1.49	32.18 [b] ± 2.94	20.60 [a] ± 1.32
50	20.54 [ab] ±2.51	32.53 [b] ± 2.25	31.45 [b] ± 3.09	21.72 [a] ± 2.70
100	22.50 [ab] ±2.27	32.47 [b] ± 1.73	32.45 [b] ± 2.18	22.10 [a] ± 3.83
150	24.70 [b] ±0.86	32.52 [b] ± 6.33	30.08 [b] ± 1.76	22.03 [a] ± 2.50
200	26.55 [b] ± 1.80	34.61 [b] ± 2.01	29.54 [b] ± 1.55	21.67 [a] ± 3.35

Values are mean ± S.E of 3 observations with 3 replicates each.
Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)

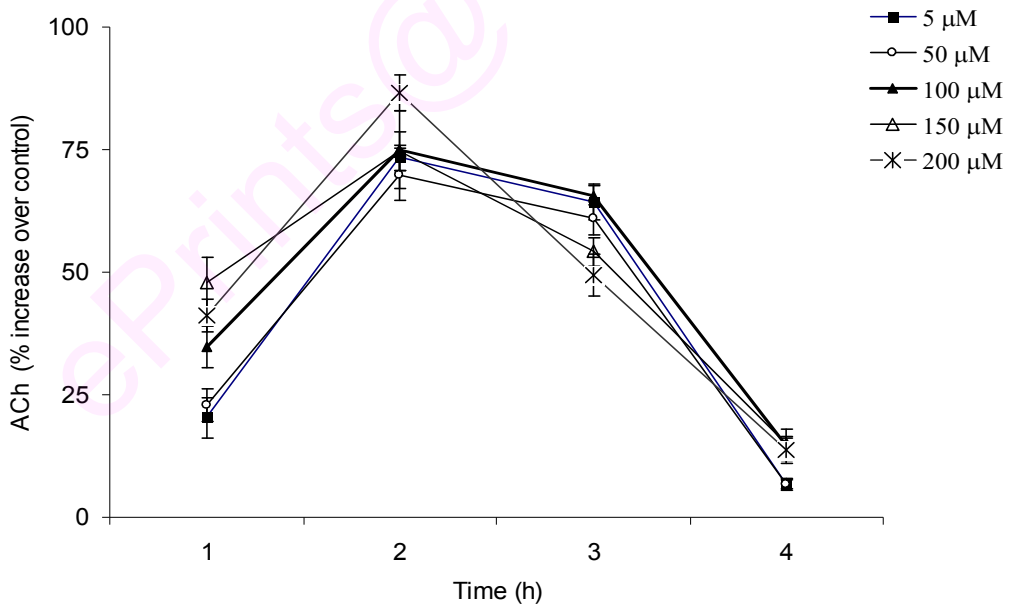

Figure 2.7 Acetylcholine accumulation in *C. elegans* (N2) exposed to sublethal concentrations of dichlorvos: Time and concentration response.
All values are mean ± S.E.M (n=9). Bars with different letters are significantly different (ANOVA, post hoc DMRT, $P<0.05$).

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6.0 DISCUSSION

In the present study, we measured the behavioral impairments in *C. elegans* as a result of increased acetylcholine signaling through inhibition of acetylcholinesterase by a known inhibitor, dichlorvos by measuring behavioral endpoints such as paralysis, nose contraction, feeding and egg laying.

OPI exert their toxicity in target and non-target species through inhibition of AChE in nerve and muscle tissues, which results in the persistence of acetylcholine in cholinergic synapses leading to repetitive stimulation of muscarinic and nicotinic receptors in target tissues. The action of acetylcholine, however, is terminated by the direct enzymatic hydrolysis of the neurotransmitter in the synaptic cleft by AChE (Miller et al., 1996). Acetylcholinesterase is an enzyme that modulates the amount of neurotransmitter substance at neuron junctions (O'Brien, 1967) and it is also concerned with the ionic content. AChE acts to clear acetylcholine released in synaptic clefts, thus ensuring that signaling is rapidly terminated. Acetylcholine (ACh) is an excitatory transmitter at nematode neuromuscular junctions (Rand, 2007). More than a third of the cells in the C*. elegans* nervous system are known to release acetylcholine. Cholinergic transmission is suggested to be involved directly or indirectly, in many *C. elegans* behaviors, including locomotion, egg laying, feeding and male mating (Rand, 2007).

In the present study, we have explored the possible correlations among behavioral alterations in *C. elegans* elicited by impaired acetylcholine levels due to inhibition of AChE by dichlorvos. Exposure to dichlorvos resulted in a significant inhibition in AChE and marked accumulation of ACh in the worms suggesting enhanced cholinergic transmission. Similar elevation of ACh content consequent to decrease in AChE activity has been reported in other vertebrate models such as fish (*Tilapia mossambica*) exposed to malathion for 48h (Sahib and Rao, 1990). Further, it is speculated that greater inhibition of AChE activity with a concomitant increase in ACh content in the tissues is an implication of

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greater inhibition in the integratory activity of the central nervous system and ACh accumulated in brain and other tissues (Reddy et al.,1992).

Earlier several workers have examined the effects of OPI on AChE levels in aquatic invertebrates (Karnak and Collins,1974; Flannagan, 1978). In the present study, we observed that dichlorvos induced AChE inhibition which increased with increasing exposure time at all the tested concentrations as have been reported in certain aquatic invertebrates (Detra and Collinsz,1991; Legiers et al.,1999). Further, accumulation of acetylcholine was evident at all concentrations. The increase in ACh content was maximum at all the tested concentrations at 2h after exposure where after there was a significant decrease in ACh content in the exposed worms. Inhibition of acetylcholine accumulation has also been reported on exposure to other OPI such as Soman and Parathion (Murumatsu and Kuriyama, 1985; Thompson and Thomas, 1985) as a result of inhibition of choline acetyl transferase (ChAT) which results in reduction in the synthesis of acetylcholine itself. Acetylcholine levels are also reportedly reduced by auto inhibition at higher levels of acetylcholine esterase inhibition (Moor, 1994) and also as a result of muscarinic auto receptor function, an important step in feedback inhibition of acetylcholine release (Weiler, 1989; Feuerstein et al.,1992). Similar mechanisms might be operating in *C. elegans* on prolonged exposure to dichlorvos.

Besides causing inhibition of acetylcholinesterase activity, toxicants can disrupt physiological function at cholinergic synapses by altering cholinergic receptor number. So it is likely that neurological dysfunction induced by toxicant exposure results in behavioral changes. In our study dichlorvos induced inhibition of AChE was associated with a consequent buildup of synaptic ACh and this excess ACh caused both hyper contraction and paralysis in the worms. The degree of paralysis was concentration dependent and percent worms paralyzed was highest after 4h of exposure and at the highest tested concentration. Similar behavioral impairments have been reported earlier in *C. elegans* following acute exposure to aldicarb, an inhibitor of

acetylcholinesterase wherein the percent of animals totally paralyzed after 4h was a function of aldicarb concentration (Nonet, et al.,1998). Cholinergic neuromuscular junction (NMJ) function in *C. elegans* when probed using the acetylcholinesterase inhibitor aldicarb revealed the enhanced effect of endogenously released ACh (Lesa et al., 2003). Marza et al., (2008) have recently confirmed that rate of paralysis by aldicarb was proportional to the levels of acetylcholine at the synapse.

We also observed a concentration and time-related increase in nose-contraction in worms exposed to dichlorvos. ACh is reported to be the primary excitatory neurotransmitter for the body muscle in *C. elegans* (Choy and Thomas, 1999). Fluoxetine, an antidepressant has been also been reported to induce nose contraction (Choy and Thomas, 1999) similarly as a result of increased release of ACh from anterior hypodermal cells. Hence, ACh accumulation in the anterior hypodermal cells in *C. elegans* may be playing a role in dichlorvos-induced nose-contraction. Since the nose-contraction response correlates with ACh accumulation, this endpoint might serve as an easily measurable behavioural endpoint for agents inducing ACh accumulation in *C. elegans.*

We observed a significant inhibition in feeding in worms exposed to the sublethal concentrations of dichlorvos for 4h. The rate of nematode feeding was quantified by measuring the rate of reduction of the optical density (at 550 nm) of the bacterial suspension. On addition of a toxicant, the rate of reduction of the optical density was markedly reduced, as a consequence of feeding inhibition (Jones and Candido, 1999). Effect on feeding could be further confirmed by observation under a microscope as a shut down of the pumping action of pharynx. Feeding behavior in *C. elegans* has also been shown to be affected upon heavy metal exposure. A decrease in feeding was observed when worms were exposed to Cd or Hg (Jones and Candido, 1999 ; Boyd et al., 2003).

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C. elegans is a filter feeder and it feeds by pumping and concentrating food using a neuromuscular organ, the pharynx (Avery and Shtonda, 2003; Shtonda and Avery, 2005). The pharynx is a muscular organ that pumps food into the pharyngeal lumen, grinds it up, which then moves it into the intestine. The pharynx is formed of muscle cells, neurons, epithelial cells, and gland cells (Albertson and Thomson, 1976). A pharyngeal-intestinal valve connects the pharynx to the intestine by the peristaltic pumping action. The pharynx is innervated by the MC neuron which is cholinergic and stimulates the pharynx muscle (via the EAT-2 receptor) to control pharyngeal pumping rate (McKay et al., 2004). The pharyngeal motor neurons M1, M2L, M2R, M4 and M5 are presumably cholinergic (Altun and Hall, 2002, 2006). Feeding requires a different neuronal circuitry including M3 (involved in pharyngeal relaxation), MC (control of pumping rate), M4 (control of isthmus peristalsis), NSM (stimulate feeding), RIP, and I neurons (Riddle et al., 1997). Wild type animals pump >250/min; pumping for animals ablated for MC is ~ 45/min (Avery and Horvitz, 1989; Raizen et al., 1995). Numbers of other ACh receptor types are expressed on pharyngeal cells and appear to mediate several aspects of pharynx function (Steger and Avery, 2004).

The idea of noxious stimuli eliciting stress responses in the pharynx has already been established (Stringham et al.,1994; Jones et al.,1996). Our recent study showed a good correlation between AChE inhibition, stress protein induction and inhibition in feeding in the transgenic *C. elegans* exposed to sublethal concentrations of dichlorvos (Jadhav and Rajini, 2009). In the present study, AChE activity and feeding rate were significantly decreased in following exposure to dichlorvos. Feeding effect could be observed by microscopy as a shut down of the pumping action of pharynx. The rate of nematode feeding could be quantified by measuring the rate of reduction of the optical density (at 550 nm) of the bacterial suspension. On addition of a toxicant, the rate of reduction of the optical density was markedly reduced, as a consequence of feeding inhibition (Miller et al.,1996). A physiological mechanism that inhibits

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feeding may be essential for survival when animals are exposed to severe chemical stress. Such a mechanism would serve both to limit the intake of toxins/toxicant. Thus feeding inhibition also has implications for toxicological studies in *C. elegans* since it imposes a limit on the concentration of toxicant that can be ingested. A correlation between the interference of the organophosphate, DDVP, on feeding behavior and brain acetylcholinesterase was demonstrated in the bream (*Abramis brama*) (Pavlov et al., 1992). In our study the inhibition of 46-81% AChE after 4 hr exposure period led to feeding inhibition of 78-80%.

A mechanism capable of rapidly detecting stressors and inhibiting feeding (and further intake of the stressor) would have obvious survival value for the nematode. A physiological mechanism that inhibits feeding may be essential for survival when animals are exposed to severe chemical or physical stress. Such a mechanism would serve both to limit the intake of toxins and to reduce the energy expended in food absorption in the gut, energy that might be better utilized in moving away from the stressor. Feeding inhibition also has implications for toxicological studies on *C. elegans* since it imposes a limit on the dose of stressors that can be ingested.

Egg laying in *C. elegans* serves as an excellent behavioral parameter, since it has been extensively characterized, can be effectively quantitated and more importantly has a simple anatomical basis. Eggs are laid when the two hermaphrodite-specific neurons (HSNs) stimulate the contraction of 16 egg-laying muscle cells to push eggs through the uterus and out the vulva (Desai et al.,1988**)**. The HSNs reportedly use at least three neurotransmitters: serotonin (Horvitz et al., 1982; Desai et al., 1988), acetylcholine (Duerr et al., 2001), and one or more neuropeptides (Schinkmann and Li, 1992; Kim and Li, 2004). Egg laying behavior thus involves the action of several neurotransmitters and ACh can affect egg laying through multiple mechanisms. The major effect of ACh appears to be an inhibition of egg laying (Bany et al., 2003). In the present study, exposure of worms to dichlorvos induced significant decrease in the rate

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of egg laying, a phenomenon consistent with recent reports of decreased egg laying observed in mutant animals (deficient for acetylcholinesterase) exposed to aldicarb, a well known AChE inhibitor (Bany et al., 2003).

The behavior of organism is complex and co-ordinated by various biochemical, physiological and neuronal functioning as integration that are necessary for survival, reproduction, feeding and normal behavior. It is suggested that the behavioral endpoints of movement and feeding may be more sensitive to neurotoxicants than the tests based on developmental endpoints. AChE inhibition is used as a biomarker to OPI exposure at the biochemical level and not directly linked with effects at the level of the whole organism. Our behavioral observations such as feeding, paralysis, egg laying and physiological nose contraction in *C. elegans* on sublethal exposure to dichlorvos at the organismal level may also be used as an indicator of exposure to dichlorvos in invertebrates.

7.0 SUMMARY

1. Dichlorvos at all the tested concentrations induced significant cessation in feeding in *C. elegans* after 4h of exposure. While the extent of feeding inhibition after 4h in worms exposed to 5μM was 52%, worms exposed to 50 μM - 200 μM showed 77 – 79% inhibition in feeding.

2. There was time and concentration related increase in the number of paralyzed worms (6-23%) exposed to dichlorvos.

3. Greater than 50% of the worms were paralyzed at 150 and 200μM after 4h of exposure.

4. Worms exposed to dichlorvos hyper contracted their nose muscles and there was a time- and dose-dependent increase in the number of worms contracting their noses.

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5. However, there was a decrease in the number of worms exhibiting nose contraction at all the concentrations of dichlorvos at the end of 4h exposure period.

6. The average number of eggs laid in the control group was 3 ±0.2 whereas in the worms exposed to the highest concentration (200μM) it was one or no egg laid /worm. The percent decrease in egg laying at 5μM was 53% and at highest dose (200 μM) was 69%.

7. Worms exposed to dichlorvos exhibited significant reduction in the AChE activity in a concentration and time dependent manner. At 5μM, it induced nearly 32-46% inhibition while at 200 μM, it induced 65-82% inhibition between 1 – 4h of exposure.

8. ACh accumulation showed a concentration-dependent increase (20 - 59%) after 1h, which increased further after 2h (73 - 86%). However, after 3h, there was a decrease in ACh content after 3h which further decreased at 4h to near control levels.

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SECTION II B

Impact of atropine and pralidoxime chloride on the toxic outcome of dichlorvos in *C. elegans*

1.0 INTRODUCTION

The primary effects of dichlorvos and other OPI on organisms are through the inhibition of the enzyme AChE, which is responsible for terminating the transmission of the nerve impulse. AChEs get irreversibly inhibited by phosphorylation of their active site serine (MacPhee-Quigley et al., 1985; Taylor et al., 1995). The inability of OP-inhibited AChE to hydrolyze acetylcholine is followed by an accumulation of the neurotransmitter in the synaptic gaps and results in generalized cholinergic symptoms including convulsive seizures, paralysis and lethality (Marrs, 2000). Standard treatment of OP poisoning includes the administration of an antimuscarinic agent (atropine) and an oxime as reactivator of inhibited AChE (Volans, 1996). While atropine relieves muscarinic signs and symptoms, oximes are supposed to shorten the duration of respiratory muscle paralysis by reactivation of cholinesterases (Johnson et al., 2000). The presently available oximes, obidoxime and pralidoxime, are considered to be rather ineffective in treatment of poisoning with certain nerve agents, e.g. soman and cyclosarin (Lundy et al., 1992; Sidell, 1996; Worek et al., 2004). The approval of numerous new oximes, which were synthesized and tested concerning their efficacy as antidotes (Bismuth et al., 1992), is hampered by difficulties in drug evaluation. The investigation of oxime efficacy *in vivo* is not possible in humans for ethical reasons. Therefore, testing of new substances was primarily performed in animal experiments in the last decades.

In our earlier studies, employing 100μM of dichlorvos, we observed that after 4h of exposure, the worms exhibited decreased feeding, increased incidence in paralysis, nose contraction and significantly inhibited AChE

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activity. In the present study, we have attempted to use *C. elegans* to evaluate the protective effect of atropine and oxime 2-PAM from dichlorvos-induced neurotoxicity and further effectiveness of atropine 2-PAM treatment as a reactivator of inhibited AChE. We speculate that interactive study of this nature is likely to yield pertinent data which may lead to a reduction in the number of animal experiments which are a prerequisite for assessment and approval of newer antidotes.

In the present set of investigations, we evaluated the protective effect of atropine and 2-PAM against dichlorvos-induced toxicity to *C. elegans* by using both physiological and biochemical end points viz: feeding, paralysis, nose contraction, curvature, AChE activity and acetylcholine content.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Acetylthiocholine iodide, BSA (fraction V), Atropine sulphate and 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB) were procured from Sigma Chemical Co., (St. Louis, MO, USA). Folin's reagent, Ferric chloride and Hydroxylamine hydrochloride were procured from Sisco Research Lab, (Mumbai, India) while acetylcholine iodide was procured from Hi Media (Mumbai, India). 2-pyridine aldoxime methchloride or Pralidoxime chloride (2-PAM) and Acrylic red paint were procured from local pharmacy/ market. All other chemicals used were of analytical grade.

Dichlorvos (DDVP; Technical grade; 97.6%) was a gift from Hyderabad Chemical Supplies Ltd., (Hyderabad, India)

2.2 Pesticide solution

Stock solution (1mM) of Dichlorvos (DDVP) was prepared in double distilled water and further diluted to obtain working solution of 100μM for the assays.

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2.3 Worm culture and maintenance

The wild-type *Caenorhabditis elegans* Bristol strain N2 was obtained from Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA) which is funded by the NIH National Center for Research Resources (NCRR). The worms were cultured and maintained as described in Chapter IA (*Section 2.3.1 – 2.3.3*).

3.0 EXPERIMENTAL PROCEDURE

Adult worms (3.5 d old) were used for these studies. The dichlorvos concentration (100µM) employed was selected based on the results obtained in our earlier study (Chapter IIA). This concentration, which induced 73% inhibition of acetylcholinesterase, 15% increase in acetylcholine accumulation and induced significant paralysis (in 41% of the worms). Employing this concentration of dichlorvos, two experiments were conducted.

Experiment 1:

Worms (20,000 ± 100) were exposed to dichlorvos (100µM) or Atropine (1, 5, 10 and 15mM) or dichlorvos (100µM) + atropine (1, 5, 10 and 15mM) for 4h. After 4h of exposure, the worms were washed and observed for the extent of feeding/ pharyngeal pumping, paralysis, body curvature, AChE inhibition and ACh accumulation.

Experiment 2:

Worms (20,000 ± 100) were exposed to dichlorvos (100µM) + atropine or atropine alone as described in experiment 1. After 4 h of exposure, the worms were washed and then exposed to 2-PAM at 1 and 10mM for 90 min. After the exposure period the worms were evaluated for all the parameters as listed in Experiment1.

3.1 Effect on pharyngeal pumping

After the exposure period, aliquot (20µl of 20 mg/ml solution) of acrylic polymer emulsion (Tristar, Mumbai, India) was added to the worm suspension.

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The worms were observed for 90 min at 30 min intervals for the pharyngeal localization of the dye. Number of worms showing dye particles in pharynx were counted and expressed as percent fed (Jones and Candido, 1999).

3.2 Induction of paralysis

Paralysis rates were determined by aliquoting 100 adult animals after exposure and counting the number of worms paralyzed. Data was expressed as percent animals paralyzed (Giovanni et al., 2003).

3.3 Induction of body curvature assay

Body curvature was determined by aliquoting 100 adult animals after exposure and counting the number of worms with curved or body bends. For each condition tested, the assay was repeated 3 times, and data obtained is expressed as percent worms curved.

3.4 Effect on Acetylcholinesterase activity and Acetylcholine content

Worms after the exposure period were processed for estimation of assays of acetylcholinesterase activity and acetylcholine content. The worms were washed three times with K-medium and subsequently processed as described earlier (*Chapter IB , Section 4.3* & *Chapter II A, Section 4.2*) for measuring AChE activity and ACh content.

4.0 ASSAY METHODS

4.1 Acetylcholinesterase (AChE) (EC 3.1.1.7) (Ellman et al., 1961)

AChE activity was determined in the worm homogenate as described earlier (*Chapter IB, Section 4.3).*

4.2 Acetylcholine estimation (Mushigeri and David, 2005)

ACh content in the worm homogenate was determined as described earlier (*Chapter II A, Section 4.2).*

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4.3 Protein estimation (Lowry et al., 1951)

Protein content of *C. elegans* homogenate was estimated as described earlier (*Chapter IB, Section 4.4).*

4.4 Statistical analysis

Each assay was conducted in triplicate, on three different days. Data were expressed as mean ± S.E. Statistical significance was assessed either by Student's t-test ($p<0.001$) or by ANOVA and post-hoc Duncan's Multiple Range Test (DMRT) ($p< 0.05$) using SPSS 10 for windows (SPSS, Chicago, IL, USA).

5.0 RESULTS

5.1 Feeding inhibition and recovery

Dichlorvos (100μM) induced almost total inhibition in feeding in the worms at the end of 4h as indicated by the absence of red acrylic paint particles in the pharyngeal region or intestine (**Figure 2.8A**). Atropine alone at all the tested concentrations (1 - 15mM) did not affect the feeding. Worms exposed to both dichlorvos + atropine showed inhibition in feeding as in worms treated with dichlorvos alone.

Worms washed after 4h of exposure to dichlorvos, or dichlorvos + atropine started feeding after 30 min and gradually increased feeding by 90min (**Figure 2.8B**). Maximum recovery in feeding was evident in worms exposed to dichlorvos alone or in worms exposed to dichlorvos + atropine (1 and 5mM) Worms treated with atropine alone for 4h and washed revealed normal feeding at all time points (**Figure 2.8B**).

Worms exposed for 4h to dichlorvos ± atropine and then washed and exposed to 2-PAM for 90 min revealed varying degrees of recovery in feeding (**Figure 2.8C**). Worms exposed to PAM at 1 and 10 mM after exposure to dichlorvos alone revealed concentration dependent marked reversal in feeding. Similarly, maximum reversal in feeding was also evident in worms

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exposed to 10mM PAM after exposure to dichlorvos and 5 and 10mM atropine. These results indicate the potential of 2-PAM to facilitate feeding in worms which was inhibited by dichlorvos ± atropine.

5.2 Paralysis

Extent of worms with paralysis was measured after 4h exposure to dichlorvos, atropine, dichlorvos + atropine. Dichlorvos (100μM) induced paralysis in 45% of the worms while atropine alone did not induce any paralysis. Worms exposed to dichlorvos + atropine showed a concentration related decrease in the extent of paralysis (**Figure 2.9**). Atropine at 10 and 15mM, completely relieved the paralysis induced by dichlorvos.

Worms washed after exposure to dichlorvos, atropine and dichlorvos + atropine recovered from paralysis within 10 min. Hence none of the worms further exposed to 2-PAM showed paralysis (data not shown).

5.3 Curvature

Number of worms revealing body bends (curvature) was monitored after exposure to dichlorvos, atropine and dichlorvos + atropine for 4h. Worms exhibited none to marginal curvature after exposure to atropine or dichlorvos respectively (**Figure 2.10, 2.11**). Interestingly worms exposed to dichlorvos + atropine revealed a concentration dependent (23 - 38%) increase in percent worms with curvature.

5.4 Effect on AChE activity

Tables 2.4a and 2.4b depict the AChE activity in worms exposed to the various treatments. Worms of the control group as well as those exposed to atropine (1-15mM) showed no inhibition of AChE (**Table 2.4a**), exposure to dichlorvos (100μM) for 4h induced 63% inhibition of AChE. Worms exposed to dichlorvos + atropine (1-15mM) also showed significant (61- 63%) inhibition in AChE activity. Apparently atropine offered no protection against AChE inhibition by dichlorvos.

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Worms washed after exposure to dichlorvos ± atropine, followed by treatment with 2-PAM (1 and 10mM) showed varying degrees of AChE inhibition (**Table 2.4b**).

Worms treated with dichlorvos ± atropine for 4h, washed and observed after 90min still revealed significant inhibition of AChE activity (56 - 64%). Worms exposed to 2-PAM after exposure to dichlorvos alone showed marked decrease (20 - 40%) in the extent of inhibition of AChE. Worms exposed to dichlorvos + atropine (1, 5 and 10mM) followed by 2-PAM (1 and 10mM) revealed significant recovery in AChE activity (**Table 2.4b**). Worms treated with dichlorvos + atropine (10 and 15mM) followed by treatment with 2-PAM (10mM) showed 100% recovery from AChE inhibition (0% inhibition). These results suggest that PAM offers protection against DDVP induced AChE inhibition and offers further protection in presence of atropine.

5.6 Effect on ACh accumulation

The extent of ACh accumulation in worms exposed to various treatments is depicted in **Table 2.5**. As clearly evident, atropine treatment elicited a marked increase (86 - 120%) in ACh accumulation in the worms. Worms exposed to dichlorvos revealed 29% increase in ACh content. Worms exposed to dichlorvos + atropine showed increased ACh content (47 - 64%) compared to those exposed to dichlorvos alone, but to lesser extent than those exposed to atropine alone (**Table 2.5**). However worms post-treated with 2-PAM (10mM) for 90 min after 4h exposure to dichlorvos + atropine (10mM) showed ACh content on par with those of control worms.

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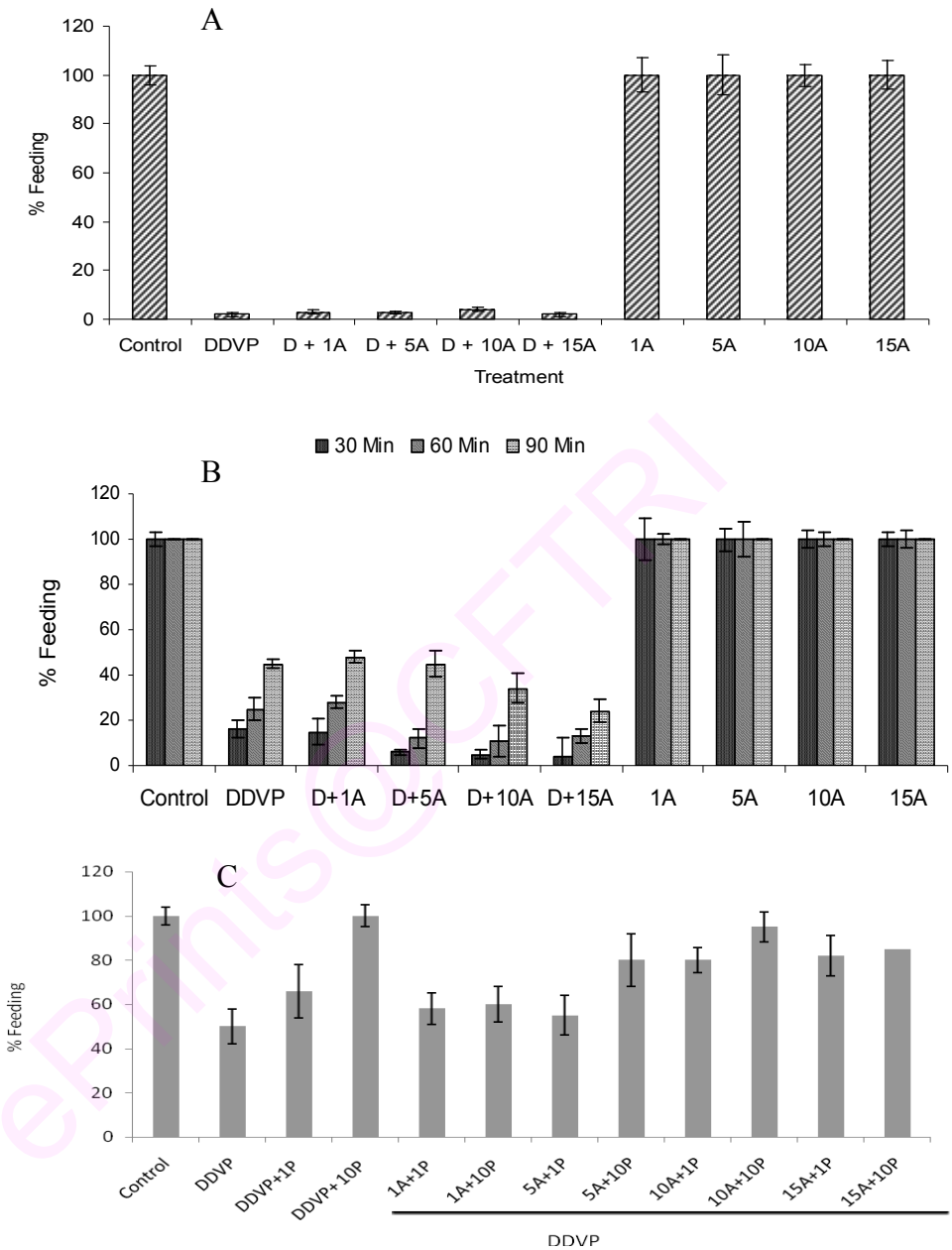

Figure 2.8 Feeding pattern in *C. elegans* in various treatment groups (Data indicates % worms showing acrylic paint particles in the pharynx).**(**DDVP or D-Dichlorvos(100µM); A-Atropine; P-2-PAM; 1, 5, 10, 15-in mM**)**

A: Worms evaluated after 4h of exposure

B: Worms washed after 4h of exposure and observed at various time intervals

C: Worms washed after 4h of exposure to D **±** A and exposed to 2-PAM and observed after 90min.

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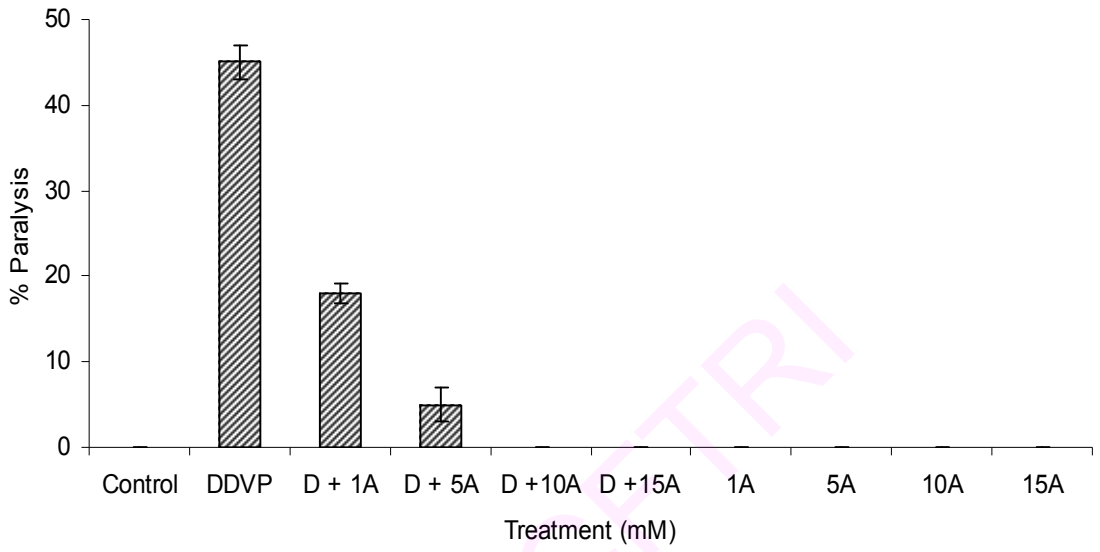

Figure 2.9 Paralysis induced in *C. elegans* in various treatment groups (Data shows % worms paralysed).
Each data point is the mean ± S.E.M of three replicates from three independent experiments.
(DDVP or D- Dichlorvos (100µM); A-Atropine; 1, 5, 10, 15-in mM)

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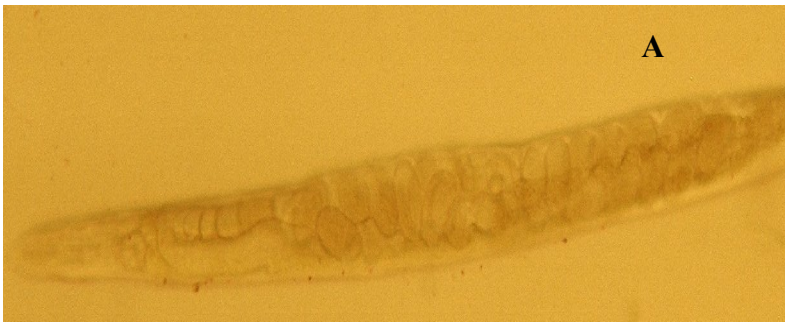

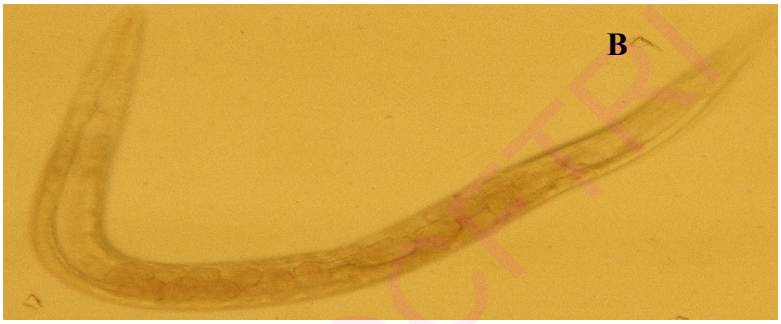

Figure.2.10 Curvature response in *C. elegans* after 4h of exposure

A. Control worm
B. Worm treated with DDVP (100µM) + Atropine(10 mM) .

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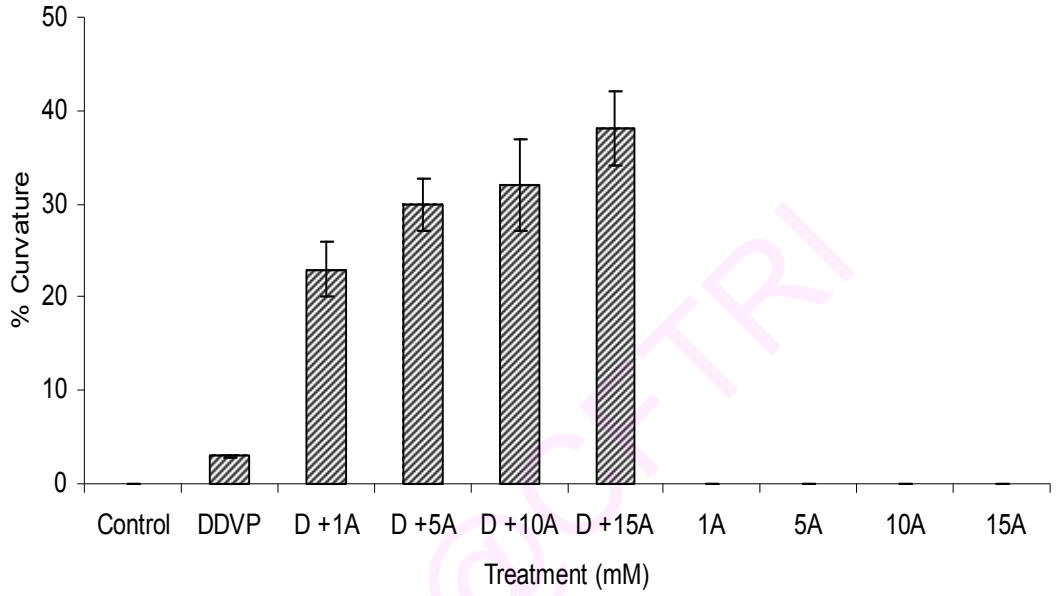

Fig.2.11 Extent of curvature induction in *C. elegans* in various treatment groups (Data shown % worms showing curved body).
Each data point is the mean ± S.E.M of three replicates from three independent experiments.
(DDVP or D- Dichlorvos (100µM); A-Atropine; 1, 5, 10, 15-in mM)

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Table 2.4a Acetylcholinesterase activity in *C. elegans* exposed to various treatments

Treatment	AChE (nmol substrate hydrolysed min/mg protein)	% inhibition
Control	21.02 [a] ± 2.34	0
DDVP	07.83 [ab] ± 0.60	63
A1	19.92 [cd] ± 0.56	5
A5	21.43 [d] ± 1.24	0
A10	21.98 [d] ± 2.13	0
A15	22.23 [d] ± 1.56	0
DDVP+1A	07.89 [bc] ± 0.67	63
DDVP+5A	08.23 [bc] ±0 .45	61
DDVP+10A	07.83 [bc] ± 0.50	63
DDVP+15A	07.99 [bc] ± 2.56	62

Values are mean ± S.E of 3 observations with 3 replicates each. Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)
(DDVP - Dichlorvos (100µM); A-Atropine; 1, 5, 10, 15-in mM)

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Table 2.4b Acetylcholinesterase activity in *C. elegans* exposed to various treatments.

Treatment	AChE (nmol substrate hydrolysed/min/mg protein)	% inhibition
Control	21^{a} ± 1.30	0
DDVP	7.53^{d} ± 0.56	64
DDVP +1A	8.13^{d} ± 1.20	61
DDVP +5A	8.58^{d} ± .84	59
DDVP +10A	9.27^{d} ± 1.45	56
DDVP +15A	8.40^{d} ± 2.13	60
DDVP +1P	10.98^{cd} ± 1.10	48
DDVP +10P	16.64^{bcd} ± 1.05	21
DDVP +A+P	13.26^{d} ± 0.69	37
DDVP +1A +10P	16.63^{bcd} ± 0.95	21
DDVP+5A +P	14.63^{cd} ± 0.85	30
DDVP+5A+10p	18.41^{abc} ±0.94	12
DDVP+10A +1P	13.30^{d} ± 1.13	37
DDVP+10A+10P	19.96^{ab} ± 0.80	0
DDVP +15A +1P	13.90^{d} ± 1.33	34
DDVP+15A+10P	20.92^{a} ± 1.20	0

Values are mean ± S.E of 3 observations with 3 replicates each.
Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)
(DDVP - Dichlorvos (100µM); A-Atropine; P- 2-PAM; 1, 5, 10, 15-in mM**)**

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Table 2.5 ACh content in worms of various treatment groups after 4 h of exposure

Treatment	ACh µg/mg protein	% increase
Control	16.25[a] ± 1.09	0
DDVP	20.89 [ab] ± 2.33	29
A1	30.3 [cd] ± 3.51	86
A5	33.4 [d] ± 2.52	106
A10	35.45 [d] ± 2.94	118
A15	35.78 [d] ± 3.92	120
DDVP+ A1	23.91 [bc] ± 2.45	47
DDVP+ A 5	23.93 [bc] ± 1.29	47
DDVP+ A10	26.11 [bc] ± 1.90	61
DDVP+ A 15	26.65 [bc] ± 2.29	64
DDVP+10A+10P	17.40 ± 1.56	7

Values are mean ± S.E of 3 observations with 3 replicates each.
Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)
(DDVP - Dichlorvos (100µM); A-Atropine;P- 2-PAM; 1, 5, 10, 15-in mM**)**

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7.0 DISCUSSION

The present study clearly demonstrated that a co-treatment of dichlorvos with atropine and further post-treatment with 2-PAM completely protected the *C. elegans* from dichlorvos-induced toxicity. While worms exposed to both dichlorvos + atropine showed inhibition in feeding as in worms treated with dichlorvos alone, these worms post-treated with 2-PAM revealed varying degrees of recovery in feeding. Feeding effect could be observed by microscopy as a shut down of the pumping action of pharynx (Jones and Candido, 1999) by red acrylic paint particles entering the pharynx. The inhibition of pharyngeal pumping was observed on exposure to dichlorvos and the worms co-treated with atropine as there were no acrylic paint particles in the pharynx. Atropine alone did not induce feeding inhibition, whereas in worms post treated with 2-PAM, feeding recovery was evident a dose dependent manner.

The *C. elegans* pharyngeal muscle contracts upon acetylcholine release from the MC motor neurons (McKay et al., 2004; Raizen et al., 1995). Acetylcholine is the critical neurotransmitter not only to effect fast pumping through nicotinic receptors but also to regulate pumping through muscarinic receptors (Raizen et al., 1995; Steger and Avery, 2004). The probable reason for the observed effect in the present study may be accumulation of acetylcholine and inhibition of AChE in worms treated with dichlorvos and co-treated with atropine, whereas on post-treatment with PAM there is recovery of inhibited AChE and levels of acetylcholine were equivalent to that of control, and hence the worms feed similar to the control.

The worms exposed to dichlorvos were paralyzed to a large extent and, similar observation was reported in studies with the nematicide piperazine which also acts by inhibiting AChE and thereby blocking the neurotransmitter acetylcholine in the worm, leading to paralysis (Castillo et al.,1964). Worms exposed to dichlorvos and co-treated with atropine, showed a dose-dependent

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decrease in the incidence of paralysis. Organophosphates and carbamates are inhibitors of AChE and induce paralysis (Nonet et al.,1993, Schade et al., 2005) in several model systems on exposure. Atropine relieves muscarinic receptors from continuous stimulation in response to accumulation of acetylcholine and muscarinic receptors are G protein-coupled acetylcholine receptors, whose roles in smooth muscle contractibility and neuronal plasticity are known (Bartus et al., 1982; Wess et al., 2003). Muscarinic receptors located on the smooth muscle cells were blocked by atropine, and thus the motor activity was reduced (Furuichi et al., 2001). We speculate that similar mechanism may be operating in *C. elegans* on co-treatment with atropine which could have relieved the muscarinic receptors from continuous stimulation thereby protecting worms from paralysis induced by dichlorvos.

Worms exposed to dichlorvos or dichlorvos + atropine exhibited significant inhibition in AChE activity. Worms washed after 4h of exposure (to dichlorvos or dichlorvos + atropine) and treated with 2-PAM interestingly showed recovery of AChE activity. AChE inhibited with OP insecticides, having mainly dimethyl and diethyl phosphate structure, can spontaneously reactivate with half- times for dimethyl phosphates of 1.3h *in vitro* and 2.1h *in vivo* (Reiner et al., 1979). The onset of action of pralidoxime occurs within 10 to 40 minutes (Minton and Murray, 1988; Simmons et al., 1990; Lotti, 1991). In our study we have obtained an reactivation time of 90 min after treating with 2-PAM, Among different OP insecticides, it has been reported that *in vivo* cholinesterase recovery is probably related to differences in absorption, biotransformation, and, most importantly, to spontaneous enzyme reactivation (Pope et al.,1991). The 2-PAM can control the nicotinic symptoms caused by the OP agents, and the atropine counteracts the muscarinic effects of the accumulating neurotransmitter acetylcholine by competing with the OP molecules for the acetylcholine receptor site.

Worms exposed to dichlorvos or dichlorvos + atropine exhibited significant accumulation in ACh. Similarly ACh levels in the presence of

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neostigmine were not affected by atropine, however in the presence of 100nM of neostigmine ACh levels with atropine were four fold higher than with neostigmine alone. The increase in concentrations of ACh is attributed to the blockade by atropine of the muscarinic auto receptors located on the cholinergic nerve terminals that inhibit ACh release by a negative feedback mechanism, as has been shown by both *in vivo* and *in vitro* studies (Morita et al.,1982; Kilbinger, 1984; Kostka et al.,1992). We, presume that a similar mechanism may be involved in the worms treated with atropine.

7.0 SUMMARY

1. Dichlorvos at 100μM induced almost total inhibition in feeding in the worms at the end of 4h as indicated by the absence of red acrylic paint particles in the pharyngeal region or intestine.
2. Atropine alone at all the tested concentrations (1 - 15mM) did not affect the feeding.
3. Worms exposed to both dichlorvos + atropine showed inhibition in feeding as in worms treated with dichlorvos alone.
4. Worms washed after 4h of exposure to dichlorvos, or dichlorvos + atropine started feeding after 30 min and gradually increased feeding by 90min.
5. Worms exposed for 4h to dichlorvos ± atropine and then washed and exposed to 2-PAM for 90 min revealed varying degrees of recovery in feeding.
6. Worms exposed to PAM at 1 and 10 mM after exposure to dichlorvos alone revealed concentration dependent marked reversal in feeding. Similarly, maximum reversal in feeding was also evident in worms exposed to 10mM PAM after exposure to dichlorvos and 5 and 10mM atropine.
7. Dichlorvos (100μM) induced paralysis in 45% of the worms while atropine alone did not induce any paralysis. Worms exposed to dichlorvos + atropine showed a concentration related decrease in the extent of paralysis.
8. Atropine at 10 and 15mM, completely relieved the paralysis induced by dichlorvos.

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9. Worms washed after exposure to dichlorvos, atropine and dichlorvos + atropine recovered from paralysis within 10 min. Hence none of the worms further exposed to 2-PAM showed paralysis.
10. Worms exhibited none to marginal curvature after exposure to atropine or dichlorvos respectively.
11. Interestingly worms exposed to dichlorvos + atropine revealed a concentration dependent (23 - 38%) increase in percent worms with curvature.
12. Worms of the control group as well as those exposed to atropine (1-15mM) showed no inhibition of AChE.
13. Exposure to dichlorvos (100μM) for 4h induced 63% inhibition of AChE. Worms exposed to dichlorvos + atropine (1-15mM) also showed significant (61- 63%) inhibition in AChE activity.
14. Worms washed after exposure to dichlorvos ± atropine, followed by treatment with 2-PAM (1 and 10mM) showed varying degrees of AChE inhibition.
15. Worms treated with dichlorvos ± atropine for 4h, washed and observed after 90min still revealed significant inhibition of AChE activity (56 - 64%).
16. Worms treated with dichlorvos + atropine (10 and 15mM) followed by treatment with 2-PAM (10mM) showed 100% recovery from AChE inhibition .
17. Atropine treatment elicited a marked increase (86 - 120%) in ACh accumulation in the worms.
18. Worms exposed to dichlorvos revealed 29% increase in ACh content.
19. Worms exposed to dichlorvos + atropine showed increased ACh content (47 - 64%) compared to those exposed to dichlorvos alone, but to lesser extent than those exposed to atropine alone.
20. Worms post-treated with 2-PAM (10mM) for 90 min after 4h exposure to dichlorvos + atropine (10mM) showed ACh content *on par* with those of control worms.

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SECTION II C

Involvement of oxidative stress in the toxicity of dichlorvos in *C. elegans*

1.0 INTRODUCTION

Organophosphorus insecticides (OPI) are indiscriminately used due to their high toxicity to insects and low environmental persistence. Humans get exposed to these pesticides as non-target organisms either during agricultural practices or as a result of residues in foods. OPI have potential to produce several different forms of neurotoxicity (Abou-Donia, MB 2003). The acute intoxication resulting from AChE inhibition is well understood, which when severe can have longer lasting consequences (Walker and Nidiry, 2002). But oxidative damage in relation to neurotoxicity of OPI is not well understood.

Oxidative stress caused by excessive reactive oxygen species production has been shown to lead to cellular dysfunction culminating in cell death. Cell death in neuronal tissues has been implicated in numerous neurodegenerative diseases (Price, 1999). ROS are part of normal oxidative metabolism, but when produced in excess, they cause tissue injury including lipid peroxidation, DNA damage, and enzyme inactivation (Halliwell and Gutteridge, 1999). In addition, oxidative stress is also a process related to xenobiotic exposure and different levels of environmental contamination (Halliwell and Gutteridge, 1999) and leading to oxidative damage by changing the balance between oxidants and antioxidants (Banerjee et al., 1988; Halliwell and Gutteridge, 1999; Torres et al., 2004). Involvement of oxidative stress following acute exposure to OPI has been reported recently (Banerjee et al., 2001) and it has been demonstrated unequivocally that lipid peroxidation is one of the molecular mechanisms involved in OPI-induced cytotoxicity (Ranjbar et al., 2002; Akhgari et al., 2003; Abdollahi et al., 2004).

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Dichlorvos an OPI, widely used in India and elsewhere for pest control has been shown to induce oxidative stress in fish (Hai et al., 1995), Drosophila (Gupta et al., 2005) and rats (Julka et al., 1992). Our earlier studies (Chapter II A and B) have demonstrated the diverse toxic actions of dichlorvos in *C. elegans* even when exposed at sublethal concentrations. Therefore, to understand the potential of dichlorvos to induce oxidative stress and its effect on brood size and life span in *C. elegans* were evaluated.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Acetylthiocholine iodide, BSA (fraction V), 2, 7-Dichlorofluorescin diacetate (DCFH-DA), 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB), 5-fluoro-2'-deoxyuridine (FudR), β-mercaptoethanol, *p*-nitrophenyl acetate (PNPA) and Quercetin were procured from Sigma Chemical Co., (St. Louis, MO, USA). 1-chloro-2,4-dinitrobenzene (CDNB), Ethylenediaminetetracetic acid (EDTA), Folin's reagent, Hydrogen peroxide (H_2O_2), Nicotine amide adenine dinucleotide phosphate reduced (NADPH), Reduced glutathione (GSH), Trichloroacetic acid (TCA) and Oxidised glutathione (GSSG) were procured from Sisco Research Lab, (Mumbai, India). All other chemicals used were of analytical grade.

Dichlorvos (DDVP; Technical grade; 97.6%) was a gift from Hyderabad Chemical Supplies Ltd., (Hyderabad, India)

2.2 Pesticide solution

Stock solution (1mM) of Dichlorvos (DDVP) was prepared in double distilled water and diluted further to obtain working solutions for the assays.

2.3 Worm culture and maintenance (Brenner, 1974)

The wild-type *Caenorhabditis elegans* Bristol strain N2 was obtained from Caenorhabditis Genetic Center (CGC, Minneapolis, MN, USA) which is funded by the NIH National Center for Research Resources (NCRR). The worms were cultured and maintained as described in Chapter IA (*Section 2.3.1 – 2.3.3*).

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3.0 EXPERIMENTAL PROCEDURE

3.1 Effect of dichlorvos on brood size

L4 staged worms were exposed to Dichlorvos (0, 5, 50,100,150 and 200 µM) for 4h at 20°±1°C. After exposure, the worms were washed with K-medium and a single worm was picked and transferred to 12-well tissue culture plates containing 1ml K-medium, which contained OP50 at a dilution of 1O.D at 550nm. The plate was incubated at 20°C for 72h. After 72h, the worms were pelleted and the progeny was counted under the dissecting microscope (Middendorf and Dusenbery, 1993).

3. 2 Effect of dichlorvos on life span

L4 staged worms were exposed to Dichlorvos (0, 5, 50,100,150 and 200 µM) for 4h at 20°±1°C. After exposure, worms were washed thrice with K-medium and 20±1 L4 worms (designated as Day 0 of life span estimate) were placed in a well of 15 mm flat bottom 24 well plates, each well containing 500 µl of K-medium (OD at 550nm adjusted to ≈ 01 with OP50) and FudR at a final concentration of 50 µM. Worms were raised and maintained at 20°C. The survivability was scored every day by gentle touching with platinum wire. The worms, which failed to move in response to touch, were considered as dead (Keaney et al., 2004).

3.3 Effect of dichlorvos on oxidative stress parameters

3.3.1 Generation of reactive oxygen species (ROS)

C. elegans (1500 worms/ml) were pre-incubated with DCFH-DA (1mM) for 1h 30min in a multi-well plate. Worms were washed thrice and exposed to various concentrations of dichlorvos (5, 50,100,150 and 200µM) for 4h in 12 well tissue culture plate. After exposure, worms were washed thrice with K-medium and homogenized in micro centrifuge tubes. Homogenates were spun at 2000 rpm for 5 min. The DCF formed was measured in the supernatant as described below.

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3.3.2 Effect on antioxidant system

To examine the effect of dichlorvos on the antioxidant system in *C. elegans*, adult worms (gravid) were exposed to varying concentrations of dichlorvos (0, 5, 50, 100, 150 and 200 μM) for 4h at 20 ± 1°C. After exposure, worms were washed thrice with K-medium and subsequently processed for antioxidant assays as described below.

3.4 Effect of dichlorvos on acetylcholinesterase and carboxylesterase activities

To examine the effect of dichlorvos on the esterase activities in *C. elegans*, adult (gravid) worms were exposed to varying concentrations (0, 5, 50, 100, 150 and 200 μM) of dichlorvos for 4h at 20 ± 1°C. After exposure, worms were washed thrice with K-medium and homogenized with 50mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 10,000 rpm for 10min and the supernatant was used for the assay of AChE and carboxylesterase activities after estimation of protein content.

4.0 ASSAY METHODS

4.1 Reactive oxygen species (ROS) (Keston and Brandt, 1965)

100μl of the supernatant obtained from exposed worms (Section 3.3.1) was added to 1,900μl of phosphate buffer (100mM, pH 7.4) and the intensity of fluorescence was measured at 530nm following excitation at 485nm. The amount of DCF resulting from the ROS mediated oxidation of DCFH, produced by hydrolytic cleavage of DCFH-DA by cellular esterases was calculated using the DCF standard graph. Results were expressed as pmoles of DCF/mg protein.

4.2 Antioxidant enzymes

4.2.1 Catalase (CAT) (EC 1.11.1.6) (Beers and Sizer, 1952)

Supernatant derived from worms after exposure (Section 3.3.2) equivalent to 250 μg protein along with 25μl of H_2O_2 (3%) was added to 3ml phosphate buffer (50mM, pH 7.4) and the decrease in absorbance due to H_2O_2 degradation

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was monitored at 240nm for 5min. The enzyme activity was calculated based on molar extinction coefficient at $43.6M^{-1}cm^{-1}$ and the results were expressed as µmoles of H_2O_2 consumed / min/mg protein.

4.2.2 Superoxide Dismutase (SOD) (EC 1.15.1.1) (Kostyuk and Potapovich., 1989)

SOD activity was measured by a method based on inhibition of auto-oxidation of quercetin by the enzyme at pH 10. Auto-oxidation of quercetin was followed by measuring decrease in absorbance at 406 nm. The reaction mixture contained: 0.016 M phosphate buffer, 0.8 mM N, N, N′, N′ tetramethyl ethylene diamine and 0.08 mM EDTA in a total volume of 3.0 ml. The reaction was started by addition of 0.1 ml of quercetin solution (1.5 mg/10 ml). For the assay of SOD, suitable aliquot derived from worms after exposure (Section 3.3.2) of supernatant was incorporated in the assay mixture in a final volume of 3.0 ml and inhibition of quercetin auto-oxidation was monitored. The results were expressed as units/mg protein. One unit of enzyme is defined as the amount of enzyme that inhibits quercetin auto-oxidation by 50%.

4.2.3 Glutathione S-transferase (GST) (EC 2.5.1.18) (Jakoby and Habig, 1980)

0.5ml of potassium phosphate buffer (0.2M, pH 7.2) were added 0.1ml of GSH (10mM), 0.01ml of 1-chloro-2,4-dinitro benzene (CDNB, 0.1M) and 390µl of distilled water and mixed. Supernatant derived from worms after exposure (Section 3.3.2) equivalent to 100 µg protein was then added to the above mixture and the formation of the adduct of CDNB (S-2,4-dinitrophenyl glutathione) was monitored by measuring net increase in absorbance at 340nm. The enzyme activity was calculated based on absorption coefficient of $9mM^{-1}cm^{-1}$. Results were expressed as µmoles of adduct formed/min/mg protein.

4.2.4 Glutathione peroxidase (GPX) (EC 1.11.1.9) (Flohe and Gunzler, 1984)

Supernatant derived from worms after exposure (Section 3.3.2) equivalent to 100 µg protein was incubated with 0.575ml phosphate buffer (0.2M, pH 7.0 containing 0.5mM EDTA), 100 µl of glutathione reductase (2.5U/ml), 100µl

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of GSH (10mM) and 100 μl of NADPH (1% in sodium bicarbonate solution) at 37ºC for 10 min. Finally, 100μl of 12mM -butylhydroperoxide was added to the above mixture and the oxidation of NADPH over 5min was recorded at 340nm. Results were expressed as nmoles of NADPH oxidized/min/mg protein.

4.2.5 Glutathione reductase (GR) (EC1.6.4.2) (Carlberg and Mannervik, 1975)

The reaction was started by adding supernatant derived from worms after exposure (Section 3.3.2) equivalent to100μg of protein to the reaction mixture (final volume 1ml) containing 0.2 M sodium phosphate buffer (pH 7.0), 2 mM EDTA, 1mM oxidized glutathione (GSSG) and 0.2 mM NADPH. The enzyme activity was measured indirectly by monitoring the oxidation of NADPH following decrease in OD/min for a minimum of 3 min at 340 nm. One unit enzyme activity was defined as nmoles of NADPH consumed/min/mg protein based on Molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

4.3 Non enzymatic antioxidants

4.3.1 Reduced Glutathione (GSH) (Benke et al., 1974)

Worms after exposure (Section 3.3.2) were washed thrice with K-medium and then homogenized in 5% TCA containing 1mM EDTA solution in micro centrifuge tubes using a micro pestle followed by centrifugation at 3000 rpm. 200μl of the supernatant was added to 4.75ml of sodium phosphate buffer (0.1 M, pH 8.0) with 1mM EDTA to which 50μl of DTNB (in phosphate buffer, 0.1M, pH 7.0) was added and mixed. The absorbance of the solution was read at 412nm. Results were expressed as μg GSH/mg protein.

4.4 Acetylcholinesterase and Carboxylesterase

4.4.1 Acetylcholinesterase (AChE) (EC 3.1.1.7) (Ellman et al., 1961)

AChE activity was determined in the worms after exposure (Section 3.4) as described earlier (*Chapter IB, Section 4.3).*

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4.4.2 Carboxylesterase (CaE) (EC 3.1.1.1) (Sidhu and Blair, 1975)

Homogenate of worms (15µl) after exposure (Section 3.4) was added to 3ml of Tris-HCI buffer (0.05 M pH- 7.4) and preincubated for 10 min at 30ºC in a water bath. The reaction was initiated by adding 50 µl of the substrate (20 mM PNPA stock solution in acetone) at a final concentration of 0.3mM. The reaction mixture was incubated for 30 minutes at 30°C. The absorbance of the reaction product, p-nitrophenol was measured at 405 nm against a reagent blank containing PNPA and buffer in a UV-Vis spectrophotometer (Shimadzu). Enzyme activity (CaE-PNPA) was calculated using the extinction co-efficient for p-nitrophenol (£405 = 1.83 X 10^4 M^{-l} cm^{-l}).

4.5 Protein estimation (Lowry et al., 1951)

Protein content of *C. elegans* homogenates was estimated as described earlier (*Chapter IB, Section 4.4).*

4.6 Statistical analysis

All the data results are presented as mean ± standard error of three separate experiments performed in triplicate on three different days, using the computer program Excel software (1999). The data were analyzed employing analysis of variance (ANOVA) using SPSS software 10 (Chicago, IL, USA) Duncan's multiple regression test (DMRT) for multiple comparisons were performed to determine the significant differences among the groups. *P* Values <0.05 were considered significant.

5.0 RESULTS

5.1 Effect on brood size

Brood size was evaluated in L4 stage worms exposed to sublethal concentrations of dichlorvos for 4h. While the number of progeny in untreated worms was 208 ± 10 / worm, exposure to dichlorvos significantly decreased the brood size by 16 - 46% at the tested concentrations. The decrease in brood size was distinctly concentration-related (**Figure 2.12**).

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5.2 Effect on life span

The impact of 4h exposure to dichlorvos at various concentrations on life span was assessed. The maximum survival in the control worms was 25d. A concentration-related decrease in survivability was evident in the worms exposed to dichlorvos after 5d (**Figure 2.13**). The percent survival was markedly reduced at the highest concentrations (150 and 200 μM) on day 20 onwards with no worm surviving at these concentrations beyond 21d.

5.3 Generation of reactive oxygen species (ROS)

Marginal increase in ROS generation was evident in worms exposed to dichlorvos for 4h (**Table 2.6**). The increase was however statistically not significant.

5.4 Effect on antioxidant enzymes

There was a significant decrease in catalase activity in worms exposed to varying concentrations of dichlorvos (5-200 μM) for 4h, which ranged from 34 – 50%. The response was not concentration-dependent (**Figure 2.14**).

A significant increase (38 – 56%) in superoxide dismutase activity was evident in worms exposed to higher concentrations (100-200 μM) of dichlorvos for 4h. Worms exposed to the lower concentration (5 and 50 μM) however showed SOD activity at par with that of control worms (**Figure 2.15).**

The activity of GST was not significantly altered in worms exposed to dichlorvos for 4h (**Figure 2.16).**

Glutathione peroxidase activity was decreased to varying extent (25-36%) in worms exposed to different concentrations of dichlorvos. The response was significant but not concentration-dependent (**Figure 2.17).**

Similarly, a marked reduction in glutathione reductase activity was evident in worms exposed to dichlorvos (**Figure 2.18**). While the decrease was only marginal at 5μM, the response was marked (42 - 57% decrease) at the higher concentrations.

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5.5 Effect on non-enzymatic antioxidants: reduced Glutathione (GSH)

A marginal (14-24%) but statistically significant decrease in reduced glutathione (GSH) was evident in worms exposed to dichlorvos for 4h. The response was however not concentration-related (**Table 2.7)**.

5.6 Effect on AChE activity

Table 2.8 depicts the AChE activity among control and dichlorvos treated worms. Worms exposed to dichlorvos for 4h exhibited significant inhibition in enzyme activity. Dichlorvos at the lowest concentration (5μM) induced 45% inhibition of AChE activity while it induced 85% inhibition at the higher concentration (200μM).

5.7 Effect on carboxylesterase activity

Table 2.9 shows the carboxylesterase activity among control and dichlorvos treated worms. Worms exposed to dichlorvos for 4h exhibited significant inhibition in the CaE activity. Dichlorvos at the lowest concentration (5μM) induced 17% inhibition of the enzyme activity while it induced 58% inhibition of the enzyme activity at the higher concentration (200μM).

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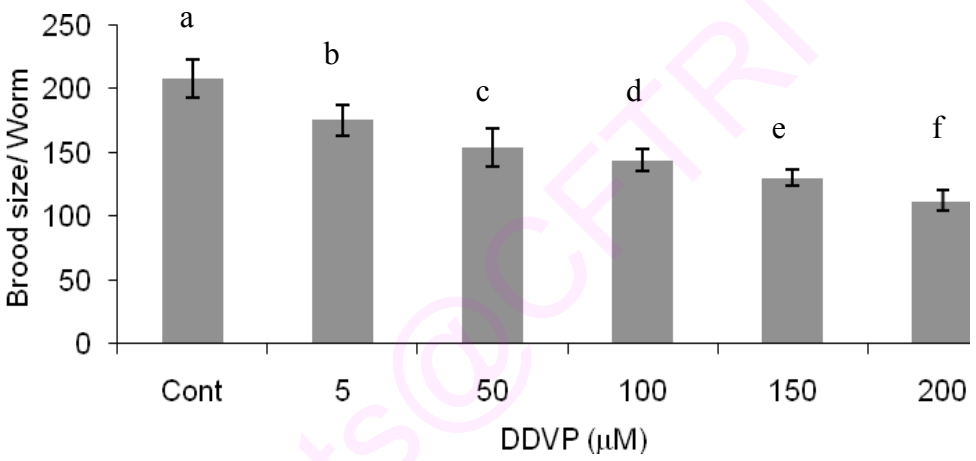

Figure 2.12 Effect of dichlorvos (DDVP) on brood size in *C. elegans*: outcome of 4h of exposure of L4 stage worms.
Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)

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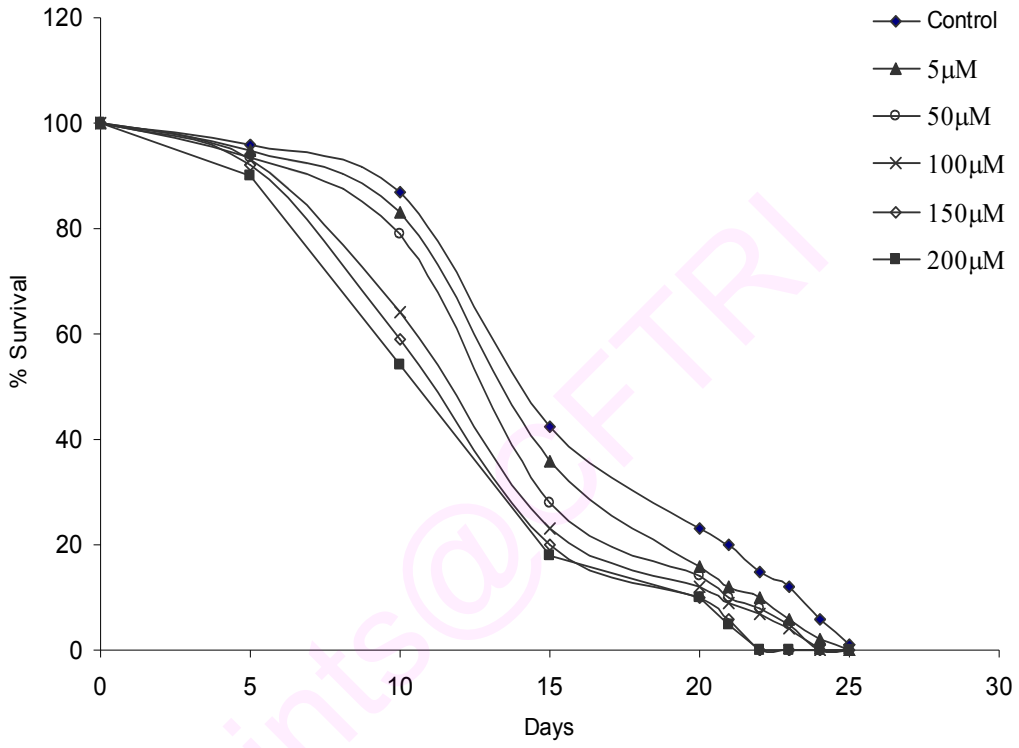

Figure 2.13 Impact of dichlorvos exposure for 4h on life span in *C. elegans*.
Values are mean ± S.E of 3 observations with 3 replicates each.

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Table 2.6 ROS generation in *C. elegans* exposed to dichlorvos for 4h

Dichlorvos (μM)	ROS (nmol DCF/ mg protein)
0	1.04 [a] ± 0.12
5	1.17 [a] ± 0.10
50	1.25 [a] ± 0.18
100	1.37 [a] ± 0.13
150	1.46 [a] ± 0.35
200	1.56 [a] ± 0.25

Values are mean ± S.E of 3 observations with 3 replicates each. Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)

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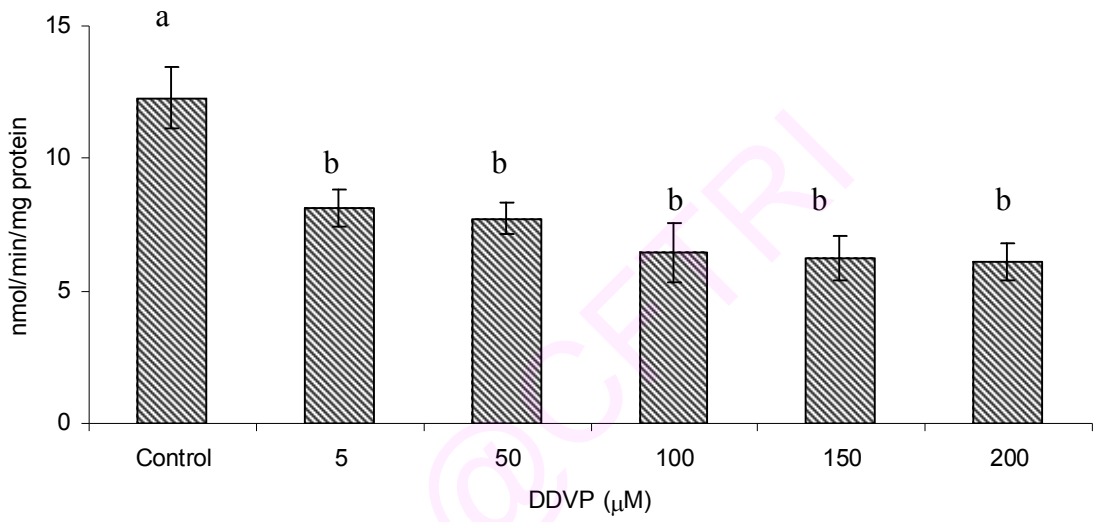

Figure 2.14 Catalase activity in *C. elegans* exposed to dichlorvos (DDVP) for 4h.

Values are mean ± S.E of 3 observations with 3 replicates each. Data analyzed by post-hoc test (Duncan). Means in the same column with different superscript differ significantly ($p < 0.05$)

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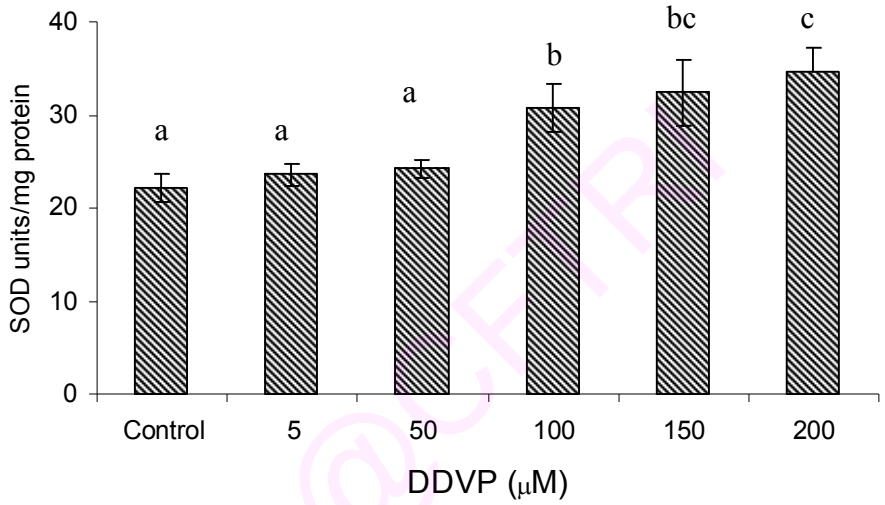

Figure 2.15 SOD activity in *C. elegans* exposed to dichlorvos (DDVP) for 4h.

Each data point is the mean ± S.E.M of three replicates from three independent experiments.

Data analyzed by post-hoc test (Duncan). Means in the same column with different superscript differ significantly ($p < 0.05$)

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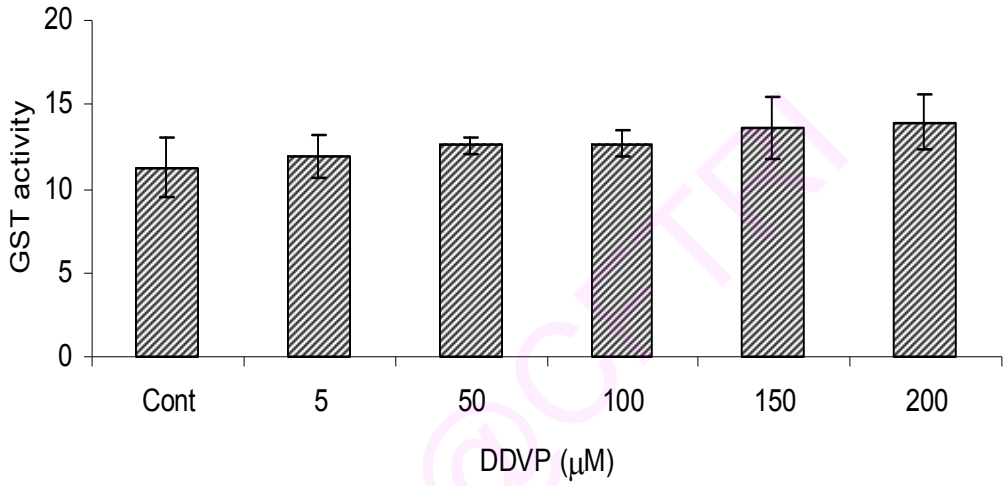

Figure 2.16 GST activity in *C. elegans* exposed to dichlorvos (DDVP) for 4h.
Each data point is the mean ± S.E.M of three replicates from three independent experiments.

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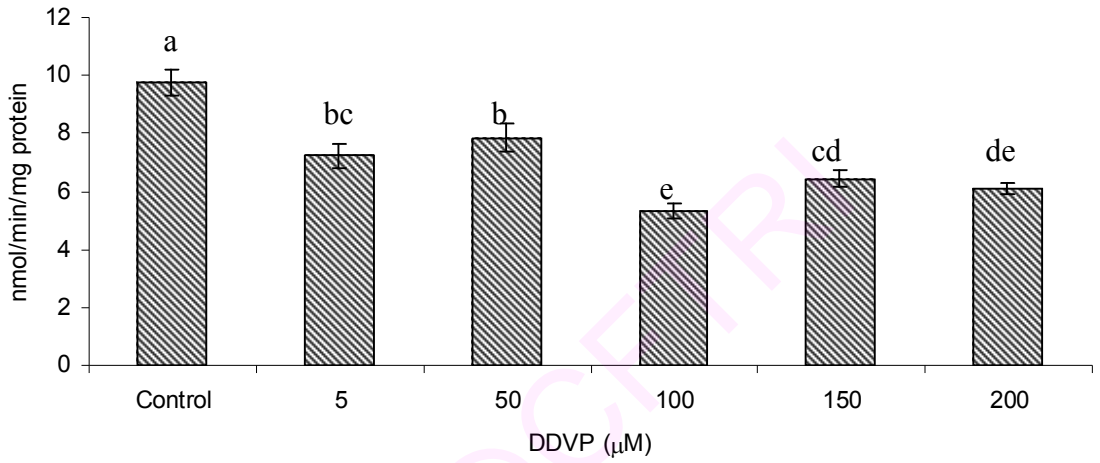

Figure 2.17 GPX activity in *C. elegans* exposed to dichlorvos (DDVP) for 4h.

Each data point is the mean ± S.E.M of three replicates from three independent experiments.

Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)

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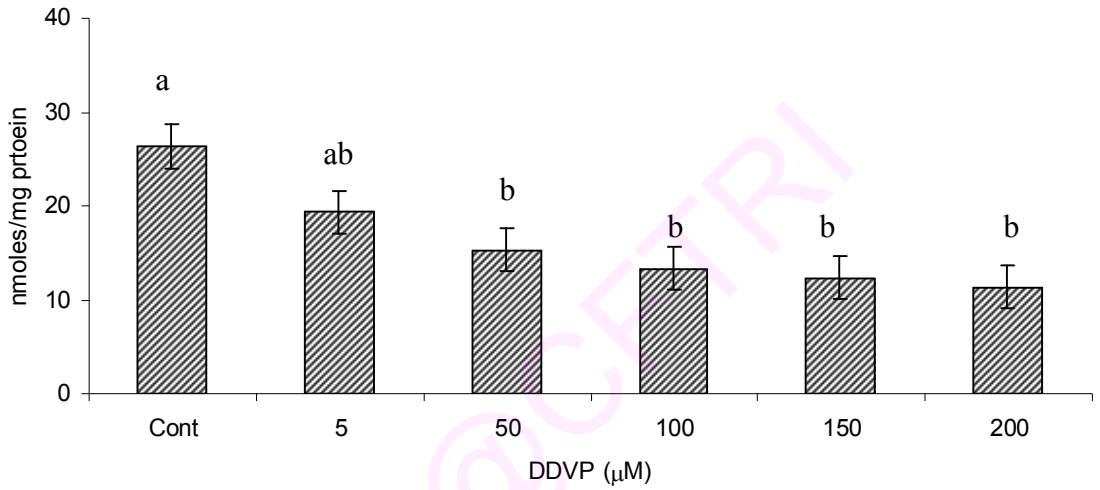

Figure 2.18 GR activity in *C. elegans* exposed to dichlorvos (DDVP) for 4h.

Each data point is the mean ± S.E.M of three replicates from three independent experiments.

Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)

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Table 2.7 Reduced glutathione (GSH) content in *C. elegans* exposed to dichlorvos (DDVP) for 4h

DDVP (μM)	GSH µg / mg protein
0	178.2[a] ± 7.50
5	135.9 [d] ± 8.00
50	140.4 [cd] ± 14.5
100	153.6 [b] ± 8.50
150	144.9 [c] ± 9.50
200	137.1 [d] ± 14.5

Values are mean ± S.E of 3 observations with 3 replicates each.
Data analyzed by post-hoc test (Duncan);
Means in the same column with different superscript differ significantly ($p < 0.05$)

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Table 2.8 Acetylcholinesterase (AChE) activity in *C. elegans* exposed to dichlorvos (DDVP) for 4h

Dichlorvos (μM)	AChE (nmol substrate hydrolysed / min/ mg protein)
0	$21.71^{a} \pm 0.89$
5	$11.89^{b} \pm 0.89$
50	$8.95^{c} \pm 1.58$
100	$5.18^{d} \pm 1.03$
150	$4.60^{de} \pm 1.03$
200	$3.33^{e} \pm 0.90$

Values are mean ± S.E of 3 observations with 3 replicates each. Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)

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Table 2.9 Carboxylesterase (CaE) activity in *C. elegans* exposed to dichlorvos (DDVP) for 4h.

Dichlorvos (μM)	CaE (nmol substrate hydrolysed/ min/ mg protein)
0	4.20 [a] ± 0.93
5	3.51 [a] ± 0.39
50	2.50 [a] ± 0.36
100	2.25 [a] ± 0.38
150	1.95 [a] ± 0.37
200	1.75 [a] ± 0.33

Values are mean ± S.E of 3 observations with 3 replicates each. Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)

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6.0 DISCUSSION

The results of the present study clearly demonstrated that 4h exposure of *C. elegans* to sublethal concentrations of dichlorvos resulted in increased levels of ROS, superoxide dismutase and glutathione-*S*-transferase but decreased levels of catalase, glutathione peroxidase, glutathione reductase and glutathione. Our results further demonstrated decrease in brood size and life span as a consequence of exposure to dichlorvos for 4h.

Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion and hydroxyl radical are produced in a number of cellular reactions and by enzymes such as lipoxygenases, peroxidases and dehydrogenases (Halliwell and Gutteridge, 1999). ROS are part of normal oxidative metabolism, but when produced in excess, lead to oxidative stress (OS) and they cause tissue injury including lipid peroxidation, DNA damage, and enzyme inactivation (Halliwell and Gutteridge, 1999). More recently, it has been postulated that OPI induce oxidative stress in different tissues through the formation of reactive oxygen species (ROS) (Banerjee et al., 1999; Ahmed et al., 2000; Akhgari et al., 2003; Abdollahi et al., 2004). In addition, OS also results due to xenobiotic exposure and different levels of environmental contamination (Halliwell and Gutteridge, 1999). In such cases, peroxidation of membrane lipids seems to be an unavoidable process in tissue injury, and may impair antioxidant defenses, leading to oxidative damage by changing the balance between oxidants and antioxidants (Banerjee et al., 1988; Halliwell and Gutteridge, 1999; Torres et al., 2004). Consequently, members of antioxidant enzyme machinery such as superoxide dismutase (SOD) and catalase (CAT) may contribute to the cellular defense system during oxidative stress conditions. ROS have been implicated in important pathologies such as cardiovascular, pulmonary and autoimmune diseases, inherited metabolic disorders, cancer, and aging (Halliwell and Gutteridge, 1999). Inactivation of free radicals occurs by antioxidant defenses and imbalance in the system can have deleterious health consequences. OPI have been shown to induce oxidative

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stress in experimental studies (Abdollahi et al., 2004) as well as acute poisoning cases in humans (Banerjee et al. 1999; Seth et al., 2001).

Oxidative stress induced by a test compound is evident by a significant elevation in ROS and alterations in antioxidant enzyme activities and content of GSH. A significant increase in ROS generation is an indicator of the prooxidant action of the compound in question. In the present study, we found only a marginal increase in ROS generation in worms exposed to dichlorvos. However, a negative correlation between ROS generation vs. GSH content is indicative of utilization of GSH as a non-enzymatic antioxidant in scavenging free radicals. Depletion of GSH, the most abundant cellular non-protein thiol, is associated with oxidative stress and cytotoxicity of pro-oxidant xenobiotic. Glutathione is presumed to be an important endogenous defense against the peroxidative destruction of cellular membranes. GSH can act either to detoxify activated oxygen species such as H_2O_2 or to reduce lipid peroxides themselves (Freeman et al., 1982). Tissue GSH concentration reflects the potential for detoxification and the levels may decrease due to an increased use of glutathione to detoxify. Indeed, a depletion in GSH induces an increase in the sensitivity of the organisms to xenobiotics or overall, with generating processes of radicals (Jones et al., 1995). Interestingly, we observed a marginal decrease in GSH content in worms exposed to dichlorvos. This observation strengthens the fact dichlorvos possesses the potential to induce OS in *C. elegans.*

Exposure of *C. elegans* to sublethal concentrations of dichlorvos also resulted in increased activities of superoxide dismutase and glutathione-*S*-transferase but decreased activities of catalase, glutathione peroxidase and glutathione reductase. The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are the main enzymatic defenses and act in concert with panoply of non-enzymatic antioxidants (Halliwell and Gutteridge, 1999). Under normal physiological conditions, a delicate balance exists between the rate of formation of H_2O_2 via dismutation of O_2 •− by SOD activity and the rate of removal of H_2O_2 by CAT and GPx. Therefore, any

impairment in this pathway will affect the activities of other enzymes in the cascade (Sinet and Garber, 1981). For example, reduction in the activity of SOD will result in an increased level of O_2 •−, while decrease in the activities of CAT and GPx will lead to accumulation of H_2O_2 in the cell, which leads to peroxidation of membrane lipids via Fenton-type reaction.

In *C. elegans*, antioxidant protection involves anti oxidant enzymes such as SOD, Catalase, Glutathione peroxidase and non-enzymatic anti-oxidant glutathione (Callahan et al., 1998). All nematodes have been reported to up regulate expression of SOD when subjected to oxidative stress (Tawe et al.,1998). SOD and to a lesser extent, catalase have been shown to be crucial for defense against oxygen toxicity in *C. elegans*. These two enzymes detoxify the reactive compounds superoxide and hydrogen peroxide. SOD converts superoxide into water and hydrogen peroxide, and catalase produces water and oxygen from hydrogen peroxide.

GSTs are also important as antioxidants in many organisms including nematodes. The enzyme in mammals and insects is inducible by exposure to potential substrates. *C. elegans* Gst -1 mutant has been shown by differential display to be induced by paraquat, which induces oxidative stress in adults and larvae (Tawe et al., 1998). Three enzyme systems (GST, esterases and monoxygenases) are involved in the detoxification of organophosphate insecticide class. These enzymes act by rapidly metabolizing the insecticide to non-toxic products or by rapidly binding and very slowly turning over the insecticide (Guengerich, 1963; Hemingway et al., 1998). In the present study, an increase in GST activity was observed. This can be understood in view of the fact that OPI consumes GSH through GST catalyzed reaction as a major way of detoxification and these chemicals are expected to induce the activity of GST as a potent protection mechanism of the organism.

Glutathione peroxidases are enzymes that catalyze glutathione dependent reduction of both hydrogen peroxide and fatty acid peroxides. The GR is not always recognized as an antioxidant enzyme. It can nevertheless be included in

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this category because it makes it possible to reduce the oxidized glutathione (GSSG) according to a NADPH-dependent process, and it is thus at the base of the regeneration of reduced GSH necessary to the operation of the GPxs and of many other enzymes of the cell. Balance between GSSG and GSH is capital in the maintenance of cellular homeostasis (Winston and Giulio, 1991). In the present study, we observed decrease in the activities of catalase, glutathione peroxidase and glutathione reductase in worms exposed to dichlorvos. Similar decrease in activities of GPx and catalase has also been recently reported in rat erythrocytes after exposure to sublethal concentration of methidathion (Altuntas et al., 2002).

Exposure of worms to sublethal concentrations of dichlorvos also resulted in significant inhibition in the activities of both AChE and carboxylesterase. This confirms the exposure of the worms to effective concentrations of dichlorvos since AChE and CaEs are considered to be important targets for organophosphorus compounds and inhibition of CaE by OPI has also been well documented in several tissues / organisms (Maxwell, 1992; Karanth and Shivanandappa, 1995).

In the present study, exposure to dichlorvos significantly decreased the brood size by 16 – 46% at the tested concentrations. Nematodes can change their reproductive speed, life cycle and other properties while exposed to stress surroundings (Swain et al., 2004). Hence, fecundity and reproduction rates are suggested as good end points to evaluate the generational effects of steroids and synthetic hormones on *C. elegans* (Tominaga et al., 2003). Reproduction and various life-cycle parameters have been used routinely as toxicity endpoints for *C. elegans* in several studies (Middendorf and Dusenbery, 1993; Donkin et al., 1995; Tominaga et al., 2002, 2003; Boyd et al., 2003; Anderson et al., 2001; Jonker et al., 2004a,b). Brood size was reported to be decreased to 15% in *C. elegans* on exposure to alkyl phenols and further decreased in second generation (Tominaga et al., 2002). Decreased brood size can be the result of lowered fecundity, increased generation time and an increased generation time may be the consequence of retarded post embryonic development, delayed

gonad maturation, abnormalities in gamete, or inhibition of embryogenesis. Hence the results obtained in the present study are indicative of the impact of dichlorvos on the above processes in *C. elegans*.

Under optimal laboratory conditions, the average life span of *C. elegans* is 2-3 wk. Studies have shown that environmental factors influence aging and lifespan of *C. elegans* (Gems and Riddle, 2000; Garigan et al., 2002; Herndon et al., 2002). Both acrylamide and cadmium have been reported to shorten life span of *C. elegans* (Hasegawa et al., 2004; Swain et al., 2004). In the presence of copper or cadmium or SDS, the life span of *C. elegans* was found to be significantly decreased in concentration dependent manner (Harda et al., 2007). In the present study, a concentration-related decrease in survivability was evident in the worms exposed to dichlorvos after 5 d.

Several studies have been revealed closed relationship between stress tolerance and longevity in *C. elegans* (Lightgow and Walker, 2002). In addition, aspects of aging have been found to be similar between nematodes and mammals, including humans. For instance, sarcopenia, the loss of muscle mass, is a major feature of aging in humans and *C. elegans* and contributes to aging-related behavioral declines in both (Herndon et al., 2002; Glenn et al ., 2004). Second, calorie restriction, the only known intervention that successfully extends lifespan in mammals, can prolong *C. elegans* lifespan (Lakowski and Hekimi, 1998). Finally, oxidative stress appears to be a major factor limiting lifespan in both *C. elegans* and humans (Larsen, 1993; Finkel and Holbrook, 2000). These findings show that studies of aging in *C. elegans* provide useful insights for identifying genes and compounds that can prolong or decrease lifespan in humans. A mutant of *C. elegans* with half the normal SOD activity was hypersensitive towards oxygen and paraquat and had a shortened life span (Darr and Fridovich, 1995). A long-lived strain of *C. elegans* contained elevated SOD and catalase (Larsen, 1993; Vanflerten, 1993). Hence the decrease in life span in worms exposed to dichlorvos could be linked to the OS induced.

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In conclusion, our experiments demonstrate that dichlorvos conferred an increase in oxidative stress markers and a reduction in brood size and life span in *C. elegans* indicating the possible role of oxidative stress in the observed OPI-mediated effects.

7.0 SUMMARY

1. While the number of progeny in untreated worms was 208 ± 10 / worm, exposure to dichlorvos markedly decreased the brood size by 16 - 46% at the tested concentrations.
2. The maximum survival in the control worms was 25d. A concentration-related decrease in survivability was evident in the worms exposed to dichlorvos after 5d.
3. The percent survival was markedly reduced at the highest concentrations of dichlorvos (150 and 200 μM) on day 20 onwards with no worm surviving at these concentrations beyond 21d.
4. Marginal increase in reactive oxygen species generation was evident in worms exposed to dichlorvos.
5. There was a significant decrease in catalase activity in worms exposed to varying concentrations of dichlorvos (5-200 μM) for 4h, which ranged from 34 – 50% and the response was not concentration-dependent.
6. A significant increase (38 – 56%) in superoxide dismutase activity was evident in worms exposed to higher concentrations (100-200 μM) of dichlorvos while the activity of GST was not significantly altered.
7. Glutathione peroxidase activity was decreased to varying extent (25-36%) in worms exposed to different concentrations of dichlorvos.
8. A marked reduction in glutathione reductase activity was evident in these worms.
9. A marginal (14-24%) but statistically significant decrease in reduced glutathione (GSH) was also evident.

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10. Dichlorvos at the lowest concentration (5μM) induced 45% inhibition of AChE activity while it induced 85% inhibition at the higher concentration (200μM).
11. Dichlorvos at the lowest concentration (5μM) induced 17% inhibition of CaE activity while it induced 58% inhibition of the enzyme activity at the higher concentration (200μM).

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CHAPTER III

Impact of low doses of dichlorvos in *C. elegans*: Biochemical investigations

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CHAPTER III

Impact of low doses of dichlorvos in *C. elegans*: Biochemical investigations

PREFACE

Our previous studies clearly demonstrated the potential of sublethal concentrations of dichlorvos to affect the neurological and physiological functions in *C. elegans* after a short exposure. Results of our studies also indicated i) the involvement of oxidative stress in the toxicity of dichlorvos and ii) the impact of dichlorvos on egg laying and life span.

In this series of investigations, we planned to understand the impact of low doses of dichlorvos on *C. elegans* when exposed for a longer period and through development.

This chapter is presented under **two** sections:

Section A describes studies on impact of low doses of dichlorvos on development of *C. elegans.*

Section B includes studies describing the effect of very low doses of dichlorvos on markers of ageing and neurological enzymes in *C. elegans*.

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SECTION III A

Effect of dichlorvos on the growth/ development in *C. elegans*

1.0 INTRODUCTION

The primary mechanism of acute toxic action of organophosphorus insecticides is the inhibition of acetylcholinesterase (AChE), which is responsible for degradation of the neurotransmitter acetylcholine. AChE inhibition results in acute cholinergic over stimulation at nicotine and muscarinic synapses of the peripheral, autonomic, and central nervous system (Jamal, 1997; Lotti, 2001). Further, the developmental neurotoxicity of organophosphate pesticides represents a biological riddle that has important ramifications for human exposures (Colborn, 2006; Costa, 2006). Besides AChE inhibition, non-AChE targets have also been proposed as alternatives that contribute to the acute lethal action and side effects of short- or long-term exposure (Casida and Quistad, 2004; Pope, 1999). Recent studies have shown the involvement of other systems as GABAergic, cytokine, or serine hydrolases, in the neurotoxic effects of OPI (Ray and Richards, 2001; Carvajal et al., 2005).

In our earlier studies, we observed that after 4h to 100 μM dichlorvos exposure, the worms exhibited - inhibited AChE activity (64-76%); increased incidence in paralysis and nose contraction and elevated oxidative stress associated with decreased feeding, egg laying, brood size and life span. Hence it was of interest to us to investigate the impact of low doses of dichlorvos on growth and development in *C. elegans.* Neuronal membranes are enriched in long-chain polyunsaturated fatty acids (LC-PUFAs) and alterations in LC-PUFA metabolism cause debilitating neuronal pathologies. *C. elegans* synthesizes many of the LC-PUFAs found in humans (Wallis et al., 2002) and represents a

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good model organism to systematically study the neuronal roles of these molecules.

In this study, in order to investigate the effects of dichlorvos on developmental neurotoxicity in *C. elegans*, initially an acute toxicity test was performed to estimate median lethal concentration (LC_{50}) at various time intervals (4, 24, 36, 48 and 72 h). A full life cycle assay was applied to assess the effect of dichlorvos on the development in *C. elegans* and also the associated biochemical and physiological functions upon sublethal exposure. Further, we also determined the effect of dichlorvos on the activity of AChE enzyme, dopamine content and fatty acid profile.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Acetylthiocholine iodide, BSA (fraction V) and 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB) and Dopamine chloride were procured from Sigma Chemical Co., (St. Louis, MO, USA). F.A.M.E. mix (C_4 - C_{24}) was purchased from Supelco (Bellefonte, USA). Dichlorvos (Technical grade; 97.6%) was gift from Hyderabad Chemical Supplies Ltd., (Hyderabad, India). All other chemicals used were of analytical grade.

2.2 Pesticide solution

Stock solution (1mM) of Dichlorvos (DDVP) was prepared in double distilled water and diluted further to obtain a working solution of 100µM.

2.3 Worm culture and maintenance (Brenner, 1974)

The wild-type *Caenorhabditis elegans* Bristol strain N2 was obtained from Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA) which is funded by the NIH National Center for Research Resources (NCRR). The worms were cultured and maintained as described in Chapter IA (*Section 2.3.1 – 2.3.3*).

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3.0 EXPERIMENTAL PROCEDURE

3.1 Toxicity test: Lethality assay

Each test consisted of five concentrations of dichlorvos and a control in which 10±1 L4 stage worms were transferred onto 24-well tissue culture plates containing 1 ml of test solution in each of the wells. The worms were exposed for varying intervals—24, 36, 48 and 72 h at 20ºC. The number of survivors was counted at the end of the treatment period. They were scored dead if they did not respond to a touch stimulus. Data were calculated from three independent experiments. The LC_{50} values, representing 50% lethality at a given exposure period were calculated using regression analysis.

3.2 Effect on growth, fatty acid profile, dopamine and AChE activity

NGM agar plates containing 100µM of dichlorvos were prepared as described earlier (*Chapter IIA, Section 3.3*). OP-50 was spread over the NGM agar and incubated overnight at 37ºC. Gravid adult worms were bleached to obtain eggs which were then transferred to the *E. coli* lawn of NGM plates with or without dichlorvos and incubated at 20ºC for 24, 36, 48 and 72h. At the end of each exposure period, the worms were washed off from the plates and then washed three times with K-medium. In order to obtain worms free from bacteria, they were suspended in K-medium for 2 to 3 h with constant shaking, till the OP-50 was digested from the worm intestine, which was confirmed by observing under dissecting microscope. The worms were then used for assessing growth, fatty acid profile, dopamine content and acetylcholinesterase activity at 24, 36, 48 and 72h of exposure as described under assay methods.

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4.0 ASSAY METHODS

4.1 Measurement of worm length

Worm length was measured using ocular micrometer (one scale=1/10mm (100µ) and stage micrometer (1Scale division =1/10mm) under a microscope. The ocular micrometer was calibrated by using the stage micrometer under 10X lens where each division corresponded to 18µm.

4.2 Fatty acid profile (Watts and Browse, 2002)

After the exposure period, the worms were homogenized with 100µl of 1.15% KCl and incubated overnight at 4°C with a solvent mixture of chloroform: methanol (1: 2). The contents were centrifuged at 2000rpm for 30min. Pellet was discarded and to the supernatant, n-hexane was added and shaken. The contents were centrifuged at low speed and pellet was discarded, supernatant was evaporated under nitrogen gas and transmethylated by adding 01ml of 2.5% H_2SO_4 in methanol and contents were boiled for 1h at 80°C in capped glass tubes. 0.2 ml of hexane and 1.5 ml of H_2O was added to the extract and supernatant was separated by shaking and centrifuging the tubes at low speed. Supernatant was evaporated under nitrogen gas. The content was dissolved in 50µl of dichloromethane and was used for GC and GC-MS analysis.

GC analysis was carried out using GC-8000 Series (Fisons Instruments, C.E. Instruments, and Italy) and OV-1 column of 30m X 0.25mm and 0.25µm thickness, with the programme temp. 60ºC (3) -10 - 260 (10). The same condition of temperature programme was used in GC-MS (Perkin Elmer Instruments, Turbo Mass Gold, Auto System XL, Gas Chromatograph, Perkin Elmer International, Switzerland) with Elite-1 column of 30m X 0.25mm and 0.25µm thickness. Mass range was detected between 40 - 400 amu and the ionization potential was 70 electron volts.

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Standard methyl esters (F.A.M.E mix) were injected for the methyl ester reference purposes. The fatty acid profile was identified by the fragmentation pattern using the cleavage of ester group first ($O\text{-}CH_3$) (31 M.W.) and then subsequent cleaving CH_2 group. Wherever there was a methylation in the fatty acid the corresponding fragmentation patterns were identified, in most of the cases M^+ fragments were easily detectable.

4.3 Dopamine content (Gruntenk et al., 2005)

After the exposure period, the worms were washed thrice with K-medium and then homogenized with 200µl Phosphate buffer (100mM, pH 8). The homogenate was centrifuged at 10,000 rpm for 10min. 20 µl of supernatant was injected to HPLC (Shimadzu, Model no: LC-10A, Asia specific PTS Ltd, Singapore) using mobile phase water: methanol (70:30), containing 0.2% of Trifluoroacetic acid. HPLC conditions were optimized by using Dopamine standard, dissolved in 1mg/2ml of 100mM Phosphate buffer (pH 8) using isocratic C-18 column, UV-detector at 280nm and flow rate of 1ml/ min for 10min. HPLC data was analyzed by comparing with the standard dopamine.

4.4 Acetylcholinesterase (AChE) (EC 3.1.1.7) (Ellman et al., 1961)

After the exposure period, the worms were washed with K-medium and processed as described earlier (*Chapter IB, Section 4.3*).

4.5 Protein estimation (Lowry et al., 1951)

Protein content of *C.elegans* homogenate was estimated as described earlier (*Section 4.3, Chapter IIA*).

4.6 Statistical analysis

All the data results are presented as mean ± standard error of three separate experiments performed in triplicate on three different days, using the computer program Excel software (1999). Student's t-test was performed to the results obtained in the two groups.

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5.0 RESULTS

5.1 Lethality: LC_{50}

The LC_{50} values of dichlorvos for the various exposure periods are presented in (**Table 3.1**). LC_{50} at 4, 24, 36, 48 and 72h were 16.00, 2.99, 1.66, 1.30 and 0.73mM respectively.

5.2 Effect on growth

The growth of the worms after exposure of eggs to dichlorvos in agar for various time intervals was monitored by measuring their body length. The data is depicted in **Figure 3.1.** Dichlorvos exposure induced drastic reduction in body length at all exposure periods. The decrease in length ranged from 50 - 67% between 24 - 72 h of exposure. While the control worm gained length from 0.7 to 1.3mm between 24 - 72h, worms exposed to dichlorvos measured 0.3 - 0.5mm between 24 - 72 of exposure. At 48 h, while the control worms measured 1.1 mm, worms exposed to dichlorvos (100µM) measure 0.48mm (56% decrease in length) (**Figure 3.1, 3.2.**)

5.3 Effect on fatty acid profile

The fatty acid profile was determined in control worms and worms exposed to dichlorvos 100uM from egg stage through development at 24, 36, 48 and 72 h. The fatty acids were not detectable in both control and treated worms at 24 and 36 h, probably due to the column employed. Hence this data is not shown. Significant differences in fatty acid composition was evident between control and dichlorvos exposed worms at 48 (**Figure 3.3, 3.4**) and 72 h (**Figure 3.5, 3.6**). The results of the qualitative analysis of the fatty acid methyl esters derived from lipids of the worms showed that although the control and dichlorvos treated worms contained several identical fatty acid components (**Figure 3.6 A, B)**, the relative quantities of the individual acids varied (**Table 3.2**). At both 48 and 72h the fatty acids in control worms were mainly composed of fatty acids containing 16 – 18 carbon atoms, while dichlorvos

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treated worms at 48 h showed less of fatty acids containing 16-18 carbon atoms, while at 72 h, the worms showed more of fatty acids with 16-18 carbon atoms.

5.4 Dopamine content

The dopamine content measured after 72 h of exposure (from egg stage) showed a dramatic increase (97%) in dichlorvos treated worms compared to that in the control worms (49.1μg dopamine/ mg protein in control vs 96.7 μg dopamine/ mg protein in dichlorvos treated worms) **(Figure 3.7).**

5.5 Acetylcholinesterase activity

Data on the activity of AChE measured in control and dichlorvos treated worms after various time intervals is presented in **Table 3.3**. AChE activity in control worms showed a marginal increase with age. Dichlorvos exposure elicited 40- 58% inhibition in AChE activity at 24, 36, 48 and 72 h.

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Table 3.1 Lethal concentration (LC_{50}) of dichlorvos in *C. elegans* after various exposure intervals.

Time of exposure (h)	LC_{50} (mM)*
4	16.00 ± 0.36
24	2.99 ± 0.04
36	1.66 ± 0.05
48	1.30 ± 0.02
72	0.73 ± 0.02

* Values are mean ± S.E of three replicates each from three independent experiments

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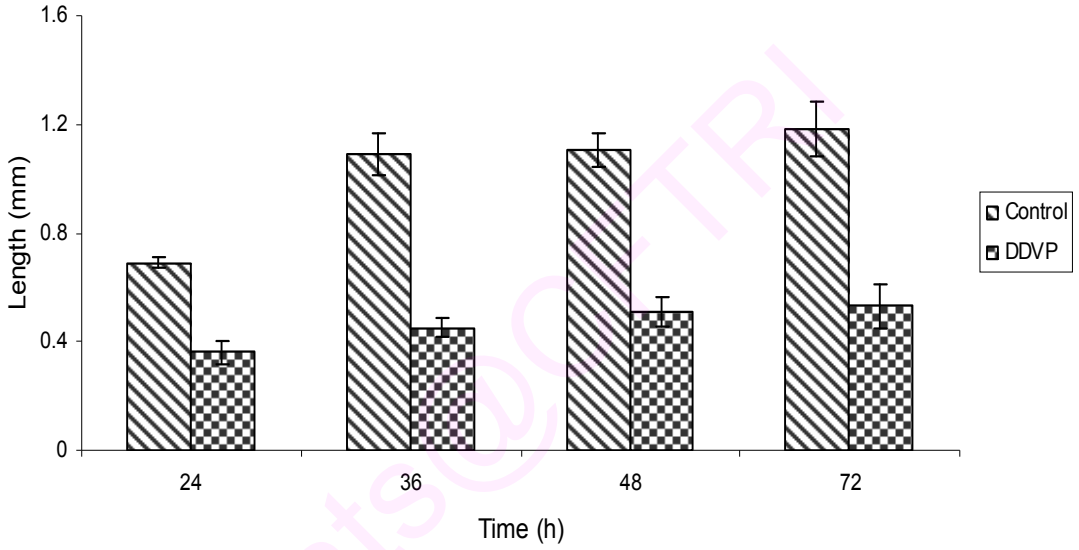

Figure 3.1 Length of *C. elegans* on exposure to 100µM of dichlorvos for 24, 36, 48 and 72h from egg stage.
(Length: average of 100 worms at each time point)

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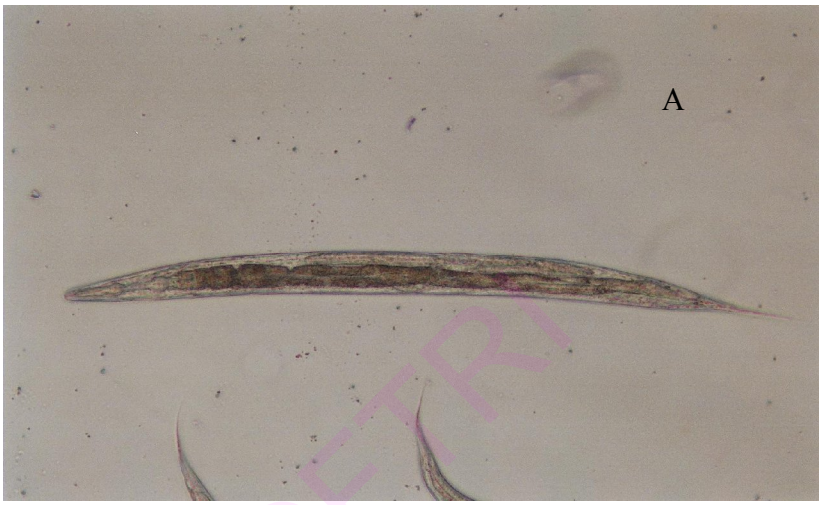

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Figure 3.2 Worm length on exposure to dichlorvos for 48h

A: Control worm (10X)
B: Dichlorvos (100 μM) (10X)

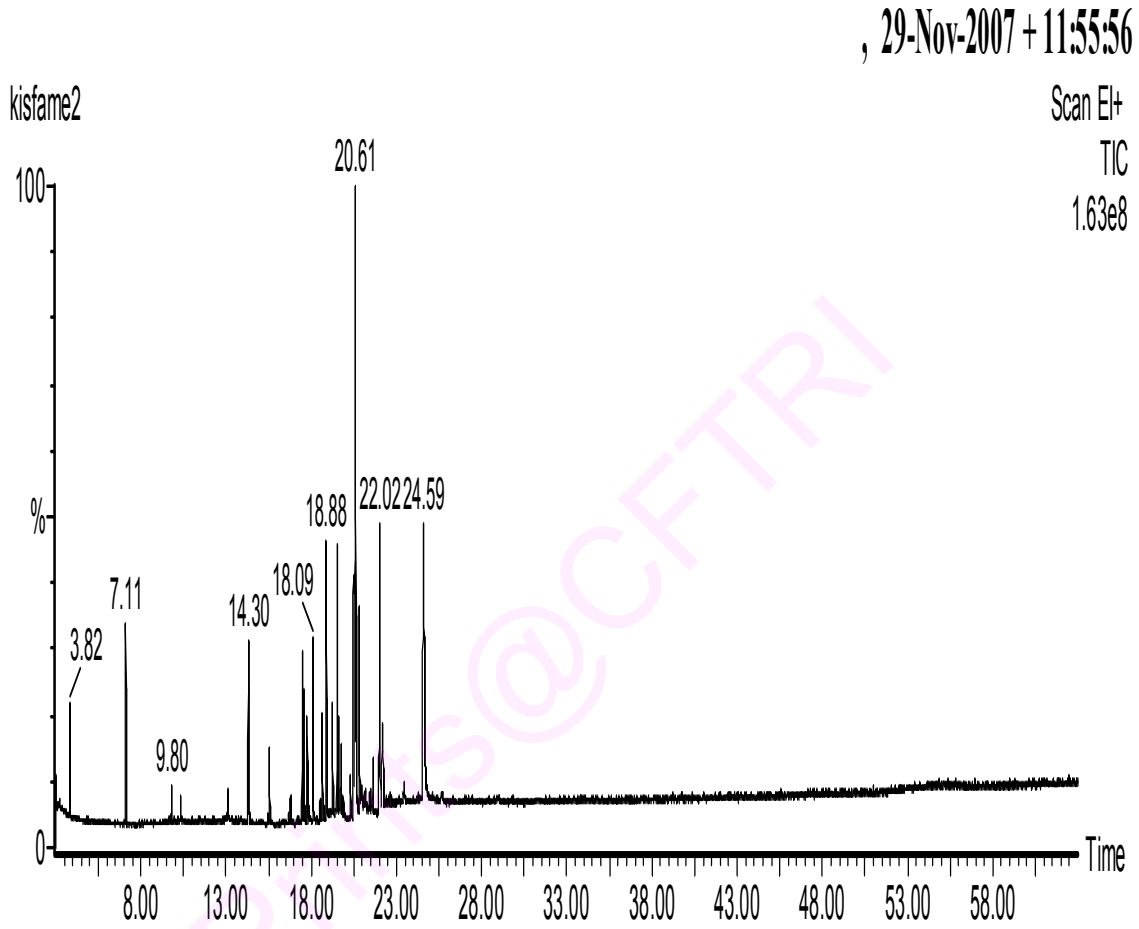

Figure 3.3 Fatty acid composition in control worms at 48h

GC profile: X- axis: Retention time
Y- axis: % Intensity of signals

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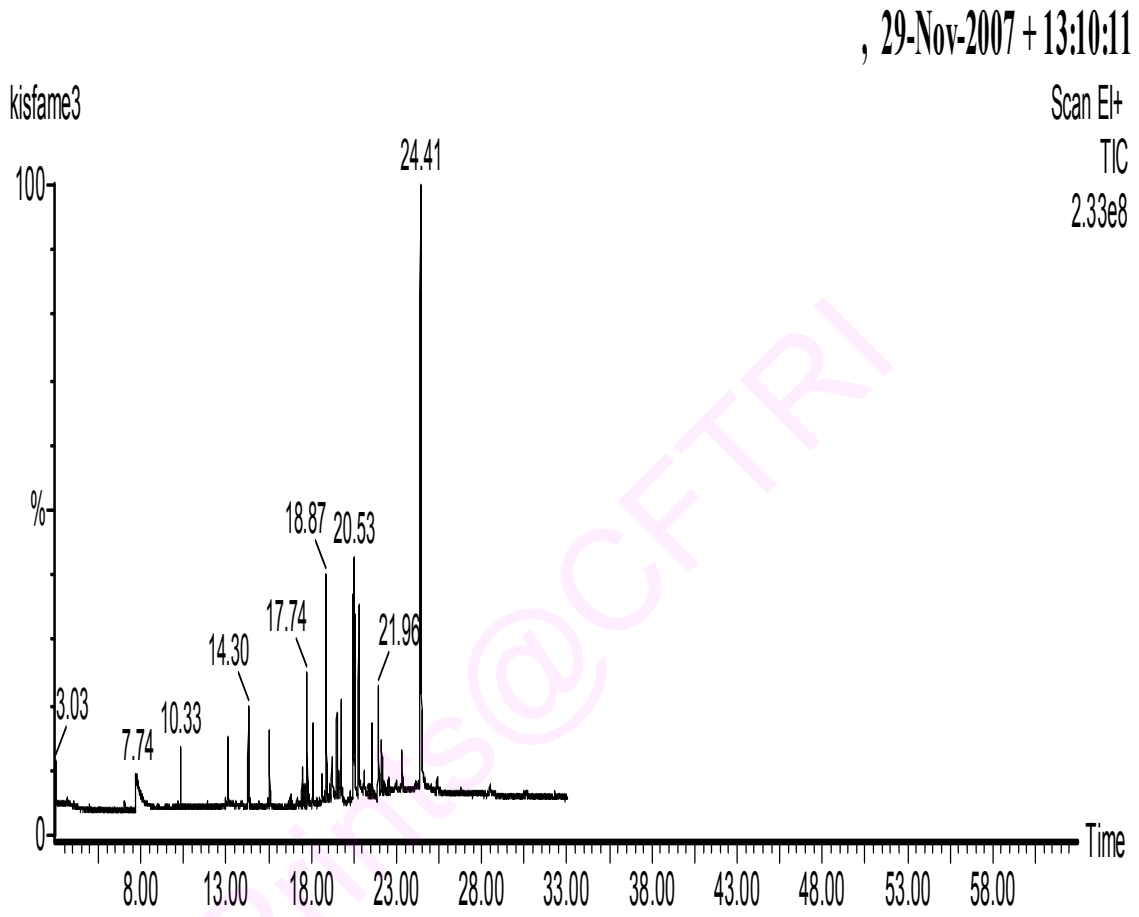

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Figure 3.4 Fatty acid composition in worms exposed to dichlorvos (100µM) at 48h

GC profile: X- axis: Retention time
Y- axis: % Intensity of signals

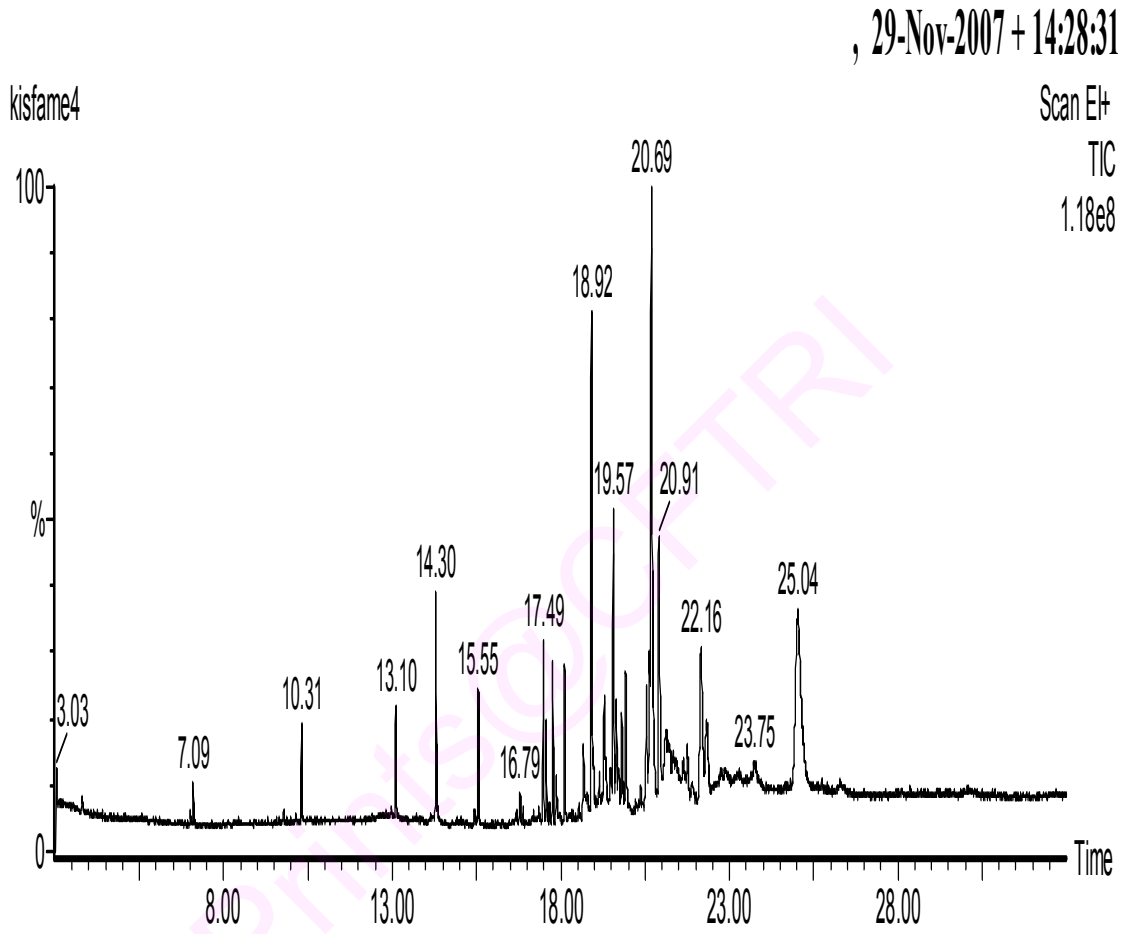

Figure 3.5 Fatty acid composition in control worms at 72h

GC profile: X- axis: Retention time
Y- axis: % Intensity of signals

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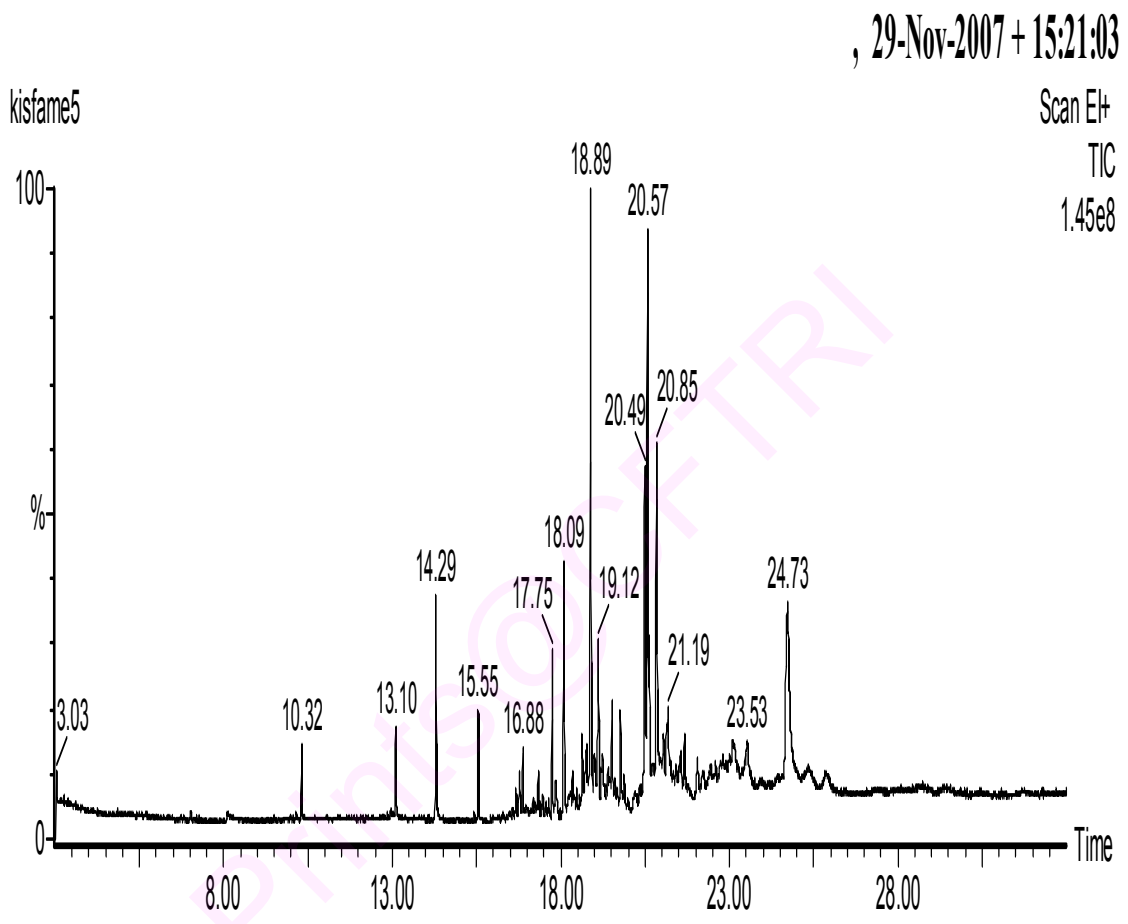

Figure 3.6 Fatty acid composition in worms exposed to dichlorvos (100µM) at 72h

GC profile: X- axis: Retention time
Y- axis: % Intensity of signals

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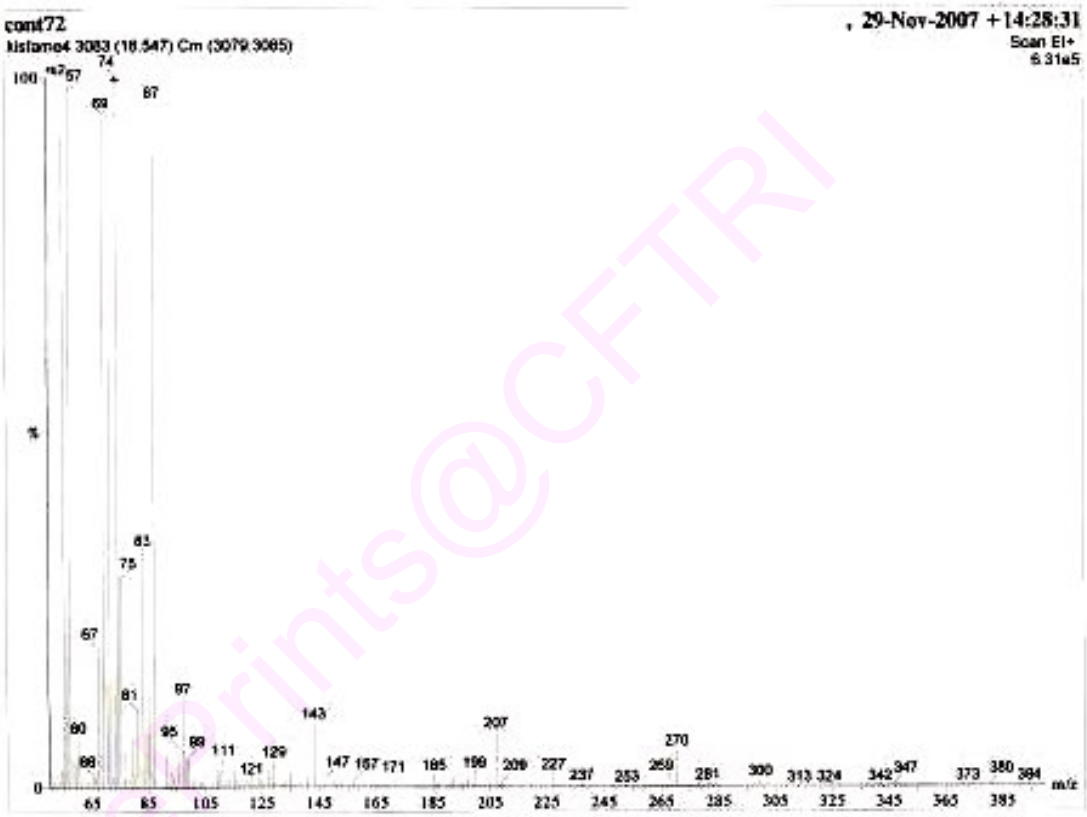

Figure 3.6A Mass spectra of Methyl-3-Methyl Pentadecanoate (3.15:0)

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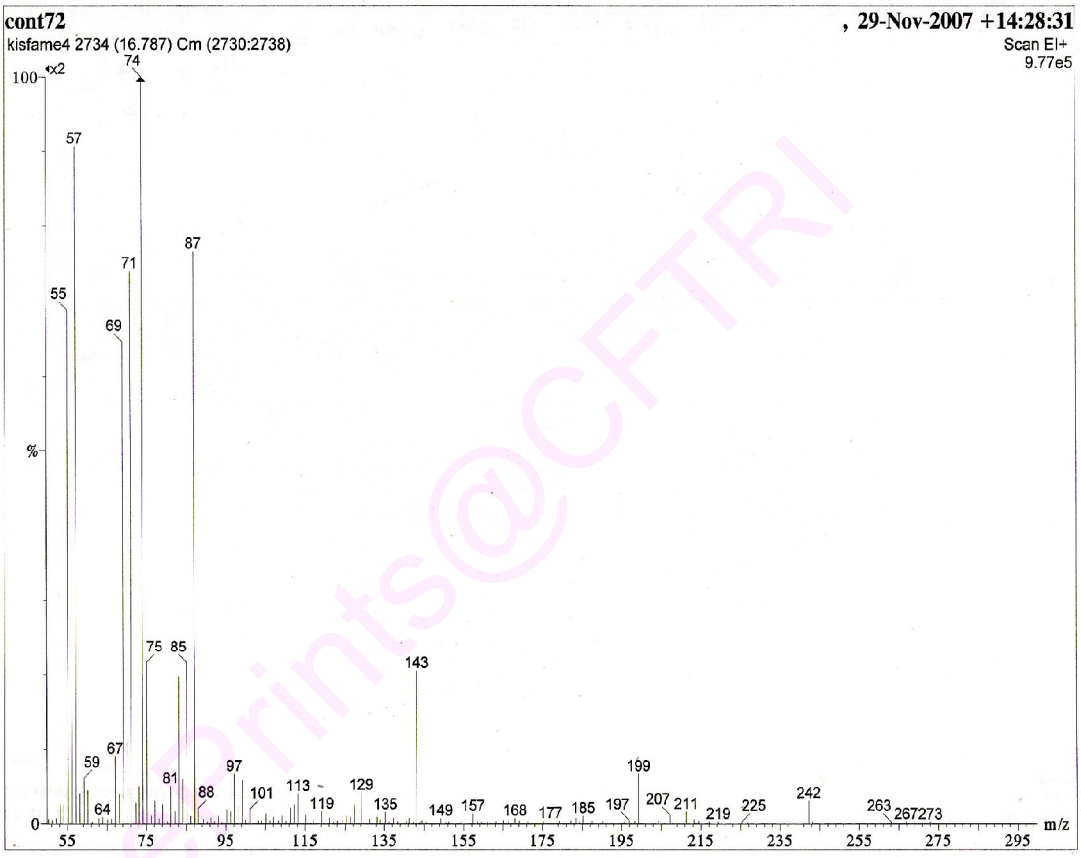

Figure 3.6B Mass spectra of Methyl Myristate (14:0)

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Table 3.2 Fatty acid profile of *C. elegans* at 48 and 72h

No.	Fatty acid	48h		72h	
		Cont	DDVP	Cont	DDVP
1	Methyl – tridecanoate (13:0)			+	
2	Methyl myristate (14:0)	+		+	+
3	Methyl–13-Methyl-tetradecanoate (iso) (13.14:0)	+		+	+
4	Methyl – pentadecanoate (15:0)	+	+		
5	Methyl –3- Methyl- pentadecanoate(3. 15:0)	+		+	
7	Methyl –14- methyl hexadecanoate (14-16:0)		+		
8	Methyl palmitate (16:0)	+		+	+
9	Methyl –9 - hexadecenoic acid (9 – 16:1)	+			
10	Methyl–15-methyl hexadecanoate (iso) (15-16:0)	+			
11	Methyl–10– Methyl- hexadecanoate (10- 16:0)	+		+	
12	Methyl 6,9 – octadecadienoate (6.9., 16:0)			+	+
13	Methyl –16 – methyl heptadeconate (iso) (16- 19:0)			+	+
14	Methyl–11-hexadecenoate(11–16:1)			+	
15	Methyl heptadecanoate (17:0)	+	+	+	
16	Methyl–8–heptadecenoate(8 – 17:1)	+		+	
17	Methyl heptadecenoate (17:0)				+
18	Methyl–11-hexadecenoate (11 –18:1)	+			
19	Methyl trans – 2 – octadecenoate (2 –18:1)	+		+	+
20	Methyl – octadecanoate (18:0)	+			+
21	Methyl -11- methyl – octadecanoate (11- 18:0)	+			
22	Methyl 9-octadecenoate (9 – 18:1)			+	
23	Methyl –6,9,12 – octadecatrienoate (18: 3(n-6)			+	
24	Methyl–9,12–octadecadienoate (18:2 (n-9)		+		
25	Methyl–13–octadecenoate(13–18:1)				+
26	Methyl 11– nonadecenoate(11 –19:1)	+			
27	Methyl – nonadecanoate (19:0)	+	+		

Cont: untreated worms.
DDVP: worms exposed to dichlorvos (100μM).
+ : indicates presence.

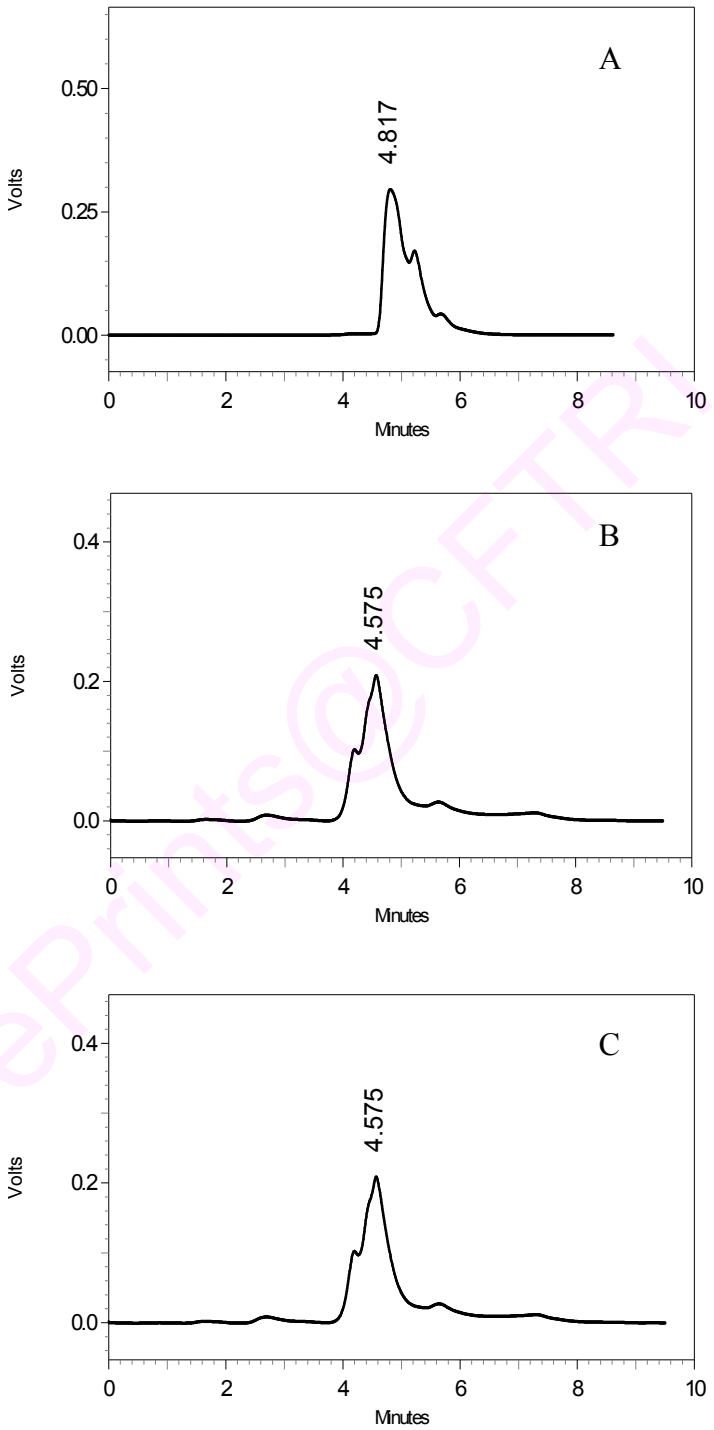

Figure 3.7 HPLC-Dopamine determinations at 72 h of worm exposure to dichlorvos.
A: Standard dopamine; B: Control worms
C: Worms exposed to dichlorvos (100 μM).

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Table 3.3 AChE activity in *C. elegans* exposed to dichlorvos (100µM)

	AChE (nmol substrate hydrolysed min/mg protein)		
Time (h)	Control	DDVP	%Inhibition
24	13.36 ± 2.00	7.97 [a] ± 0.92	40
36	14.65 ± 1.67	8.61 [a] ± 1.44	41
48	15.33 ± 2.02	8.01 [a] ± 1.24	48
72	16.36 ± 1.70	6.95 [a] ± 0.74	58

Values are mean ± S.E of 3 observations with 3 replicates each.
Data analyzed by Student's *t* test; [a] $p < 0.05$

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6.0 DISCUSSION

In the present study, worms exposed to dichlorvos during development (from egg stage) exhibited a marked decrease in body length as well as delay in development. Associated alterations were also evident in fatty acid composition among worms exposed to dichlorvos. Development-related inhibition in AChE activity and dopamine content were also evident in these worms.

Developmental neurotoxicity of OPI represents a separable set of mechanisms that operate at lower exposures (Slotkin, 2004, 2005; Colborn 2006). Of additional concern is the possibility that developmental exposure to neurotoxic pesticides may accelerate age-related decline in function (Rice and Barone, 2000). Furthermore it has been suggested that sub-clinical effects may lead to lifetime morbidity (Reigart and Roberts, 2001). *C. elegans* provides an excellent model system for obtaining an integrated picture of cellular, developmental, and molecular aspects of toxicity (Liao and Freedman, 2002). Hence in the present study, we exposed the eggs of *C. elegans* to varying periods to dichlorvos to obtain an insight into its impact on development and associated biochemical indices.

The most commonly used indicator for progress through postembryonic development is the length attained by individuals in a synchronously growing population. Each stage of *C. elegans* has its characteristic length range, although this range may vary as a function of culture conditions. Growth of the worm occurs during the intervals between molts, with a cessation of growth during molts. Normally an adult worm measures between 1 – 1.3 mm in length. In the present study, growth of the worms emerging from the eggs exposed to 100μM dichlorvos for varying periods was drastically reduced. The worms appeared to be much shorter (50 - 67%) in length compared to the untreated worms. The use of length attained by a synchronously growing population as an indicator of the toxic effects of environmental agents offers several advantages.

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Of the possible indicators, length attained represents the parameter most suitable for routine detection of sublethal toxic effects. In this study the decrease in length of the worms is again a clear indicator of the sublethal effect of dichlorvos on growth and development in these worms. Similar decrease in growth and development has been reported in *C. elegans* exposed to various concentrations of metals (Popham and Webster, 1979; Anderson et al., 2001; Harda et al., 2007).

In the present study, concomitant with the decreased body length, the worms developed from eggs exposed to dichlorvos revealed a significant difference in their fatty acid profile. While at 48 and 72h, the control worms showed mainly fatty acids containing 16 - 18 carbon atoms, dichlorvos treated worms at 48h showed less of fatty acids with 16-18 carbon atoms, while at 72h showed relatively more of fatty acids with 16 -18 carbon atoms.

Several groups have biochemically determined the composition of *C. elegans* fat content. This is accomplished by extraction of total lipids from whole animals, fractionation to phospholipids and neutral lipid moieties, and further analysis by column and thin-layer chromatography as well as gas-chromatography/mass spectrometry (Kniazeva et al., 2003). Triacylglyceride fat stores make up approximately 40–55% of total lipids depending on diet and growth stage (Ashrafi, 2006). Phospholipids pools are composed of approximately 55% ethanolamine glycerophospholipid, 32% choline glycerophospholipid, 8% sphingomyelin. Cardiolipin, inositol glycerophospholipids and lyso-cholineglycerophospholipids account for the remaining 5%. Relative abundance of these phospholipid constituents changes with growth temperature (Tanaka et al., 1996). Similar to mammals, *C. elegans* contains a wide range of saturated, monounsaturated and polyunsaturated fatty acids (PUFAs) including arachidonic (20:4n-6) and eicosapentaenoic acid (20:5n-3) as well as monomethyl branched chain fatty acids (Kniazeva et al., 2004).

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C.elegans combines the full range of desaturase and PUFA elongase activities that are found separately in plants and animals, abrogating the requirement for dietary supply of linoleic acid (18:2n6) and linolenic acid (18:3n3), which are essential fatty acids in mammalian diets (Wallis et al., 2002; Brock et al., 2006). Inactivation of *C. elegans* desaturase and elongase family members, encoded by *fat* and *elo* genes, respectively, causes imbalances in fatty acid composition and is associated with metabolic, physiological and behavioral phenotypes. These include altered total fat levels, growth retardation, slowed movement, reduction in body size, germ cell maintenance and reproductive defects, aberrations in rhythmic behavior, defects in sensory signaling, defects in neurotransmission and reduced adult lifespan (Kahn-Kirby et al., 2004; Kniazeva et al., 2004; Brock et al., 2006). Our results clearly point towards the fact that dichlorvos-induced growth/developmental arrest may in part be due its effect on the fatty acids in these worms.

In the present study, dichlorvos treated worms revealed 40-58% inhibition in AChE activity and interestingly a 97% increase in dopamine content at 72h of exposure. While much progress has been made in the understanding of dopamine signaling and its role in behavioral plasticity in mammalian organisms, the molecular basis for dopamine-induced plasticity in *C. elegans* is not well understood. Dopamine is made in eight neurons in the hermaphrodite and in an additional six neurons located in the tail of the male. Each of these fourteen neurons are thought to be mechanosensory, and ablation of these cells or mutations that block synthesis or release of dopamine are reported to cause defects in the animal's ability to sense or respond to changes in its environment (Sawin et al., 2000; Sanyal et al., 2004). Dopamine signaling has thus established roles in the modulation of locomotion behavior and in learning.

Dopamine signaling allows *C. elegans* to respond to changes in its environment by modulating locomotion behavior (Sawin et al., 2000). Dopamine-dependent effects on *C. elegans* behavior have been gleaned

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primarily from the analysis of: 1) wild-type animals exposed to exogenous dopamine; 2) wild-type animals in which the dopaminergic neurons have been ablated; and 3) mutants for *cat-2*, which encodes a tyrosine hydroxylase responsible for catalyzing the rate-limiting step in the synthesis of dopamine (Weinshenker and Thomas, 1995; Sawin et al., 2000). Exogenous dopamine has been reported to inhibit motility in *C. elegans* (Schafer and Kenyon, 1995) and it has been observed that 20mM dopamine is capable of completely immobilizing worm populations when preincubated on NGM plates containing dopamine (Sanyal, 2004). However, until to date, there has been no published data on the dopamine content in worms exposed to OPI. Our results for the first time try to correlate between the growth retardation and decrease in motility in worms exposed to the OPI, dichlorvos.

7.0 SUMMARY

1. The LC_{50} values of dichlorvos for 4, 24, 36, 48 and 72h were 16.00, 2.99, 1.66, 1.30 and 0.73mM respectively.
2. The growth of the worms after exposure of eggs to dichlorvos in agar was drastically reduced.
3. The decrease in body length ranged from 50 - 67% between 24 - 72 h of exposure.
4. While the control worm gained length from 0.7 to 1.3mm between 24 - 72h, worms exposed to dichlorvos measured 0.3 - 0.5mm between 24 - 72 of exposure.
5. Significant differences in fatty acid composition were evident between control and dichlorvos exposed worms at 48 and 72 h.

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6. The results of the qualitative analysis of the fatty acid methyl esters derived from lipids of the worms showed that although the control and dichlorvos treated worms continued several identical fatty acid components, the relative quantities of the individual acids varied.
7. The dopamine content measured after 72 h of exposure (from egg stage) showed a 97% increase in dichlorvos treated worms compared to that in the control worms (49.1μg dopamine/ mg protein in control vs. 96.7μg dopamine/ mg protein in dichlorvos treated worms).
8. AChE activity in control worms showed a marginal increase with age.
9. Dichlorvos exposure elicited 40- 58% inhibition in AChE activity at 24, 36, 48 and 72 h.

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SECTION III B

Influence of dichlorvos exposure on aging and neurological enzymes in *C. elegans*

1.0 INTRODUCTION

Acetylcholinesterase (AChE) activity has traditionally been employed as a biomarker of organophosphorus insecticide (OPI) exposure. However, AChE activity may not be the most sensitive endpoint for these pesticides since OPI can cause adverse physiological effects at concentrations that may or may not affect AChE activity. Carboxylesterases are a related family of enzymes that have higher affinity than AChE for some of the OPI and have been reported to be more sensitive indicators of exposure to these pesticides (Wheelock et al., 2005).

Our earlier studies (Chapter II) demonstrated the potential of dichlorvos to induce oxidative stress and neurophysiological alterations in *C. elegans* at sublethal concentrations when exposed for 4h and also reduce the life span and brood size. We have also demonstrated (Chapter III A) that dichlorvos at 100 µM has the potential to impair growth / development in these worms. The purpose of the present investigation was to study the impact of dichlorvos on the content of 'age pigment' – lipofuscin – in worms when exposed at extremely low concentrations during their life.

Lipofuscin (age pigment) is considered a hallmark of aging since its amount increases with age in most cells (Terman and Brunk, 1998). The rate of lipofuscin accumulation reportedly correlates negatively with longevity (i.e., positively correlates with the rate of aging) (Brunk and Terman, 2002). Several pathological conditions, such as lysosomal storage diseases, radiation injury, tumors are associated with the accumulation of lipofuscin (Brunk and Terman, 2002). Lipofuscin levels have been reported to increase with aging in many organisms, including *C. elegans* (Hosokawa et al., 1994). It has been

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unequivocally demonstrated that oxidative stress promotes lipofuscin formation. Further, the deposition of lipofuscin in large amounts is likely to make cells considerably more vulnerable to oxidative stress, decrease adaptability and favor the pathologies associated with aging (Beal, 1996).

Hence it was of interest to investigate the influence of dichlorvos to affect lipofuscin / aging in the worms especially at concentrations that do not affect the normal physiology and functioning. It was also of interest to follow up the activity of AChE and carboxylesterase (CaE) in the worms as they aged in order to study the impact of dichlorvos on the enzyme activities at different ages. Here, we report that low dose exposure to dichlorvos increases lipofuscin an ageing marker, inhibits AChE and affects CaE, the detoxifying enzyme at the doses where worms feed normally and no physiological or behavioral alterations were observed.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Acetylthiocholine iodide, BSA (fraction V), 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB), 5-fluoro-2'-deoxyuridine (FudR) and *p*-nitrophenyl acetate (PNPA) were procured from Sigma Chemical Co., (St. Louis, MO, USA). Folin's reagent was procured from Sisco Research Lab, (Mumbai, India). Dichlorvos (DDVP; Technical grade; 97.6%) was gift from Hyderabad Chemical Supplies Ltd., (Hyderabad, India). All other chemicals used were of analytical grade.

2.2 Pesticide solution

Stock solution (5mM) of Dichlorvos (DDVP) was prepared in double distilled water and diluted further to obtain working solutions for the assays.

2.3 Worm culture and maintenance (Brenner, 1974)

The wild-type *Caenorhabditis elegans* Bristol strain N2 was obtained from Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA) which is funded

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by the NIH National Center for Research Resources (NCRR). The worms were cultured and maintained as described in Chapter IA (*Section 2.3.1 – 2.3.3*).

3.0 EXPERIMENTAL PROCEDURE

3.1 Feeding

Aliquots (1ml) of K-medium with OP50 suspended at a concentration of 1.0 OD (at 550 nm) were dispensed into the wells of 12 well plates. Dichlorvos was added to the wells at different concentrations (0, 5, 100, 200, 250 and 500nM). The assay was performed as described earlier (*Chapter IB, Section 3.3*) by dispensing 500±10 worms/ well (Jones and Candido, 1999).

3.2 Pharyngeal pumping

Worms were exposed to dichlorvos at 0, 5, 100, 200, 250 and 500nM for 12 and 24h. After the exposure period, aliquot of acrylic polymer emulsion (Tristar, Mumbai, India; 20 µl of 20 mg/ml solution) was added to the worm suspension. The worms were observed after 10 min for the pharyngeal localization of the dye (Jones and Candido, 1999).

3.3 Worm exposure for acetylcholinesterase, carboxylesterase and lipofuscin

Age synchronized worms (2d old) were harvested and exposed to dichlorvos (0, 5, 100 and 200nM) and grown at low population densities (approx.2000 worms/ml) in K-medium and a relatively constant *E. coli* OP50 concentration of 1.0 O.D at 550nm. FudR was added to the medium at a final concentration of 50µM to prevent progeny production. Worms were transferred every day by settling and pelleting and then suspended in fresh medium with dichlorvos concentrations and with 50µM FudR along with OP50 (1 O.D at 550nm). The worms were maintained at 20 ± 1°C and assayed for acetylcholinesterase and carboxylesterase activities at : 0h, 24h, 5d, 10d, 15d and 20d) and lipofuscin was quantified on: 5, 10, 15 and 20 d of exposure.

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3.4 Fluorescence microscopy

On 15 d after exposure, worms were immobilized by adding a drop of sodium azide (0.1%) and placing them on slide with cover slip and then observed under phase contrast microscope with a fluorescence module for the localization of lipofuscin under GFP filter and photographed under 10X microscope (Hosokawa et al., 1994).

4.0 ASSAY METHODS

4.1 Acetylcholinesterase and carboxylesterase activities

After the exposure period, worms were washed thrice with K-medium and homogenized with 50mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 10,000 rpm for 10min and the supernatant was used for the assay after estimation of protein content.

4.1.1 Acetylcholinesterase (AChE) (EC 3.1.1.7) (Ellman et al., 1961)

After the exposure period, the worms were washed with K-medium and processed as described earlier (*Chapter IB, Section 4.3*).

4.1.2 Carboxylesterase (CaE) (EC 3.1.1.1) (Sidhu and Blair, 1975)

After the exposure period, the worms were washed with K-medium and processed as described earlier (*Chapter IIC, Section 4.4.2*).

4.2 Measurement of fluorescent material- lipofuscin (Hosokawa et al., 1994)

After exposure, worms were homogenized in methanol (1 ml of methanol with 0.5ml of washed nematodes) for 2 min on ice. The extract was mixed by vortexing and then centrifuged at 16,000rpm for 10 min at 4°C. The fluorescence of the supernatant was measured in a Fluorometer (Shimadzu, Model No: RF-5301PC, Asia specific PTS. Ltd., Singapore) with excitation at 360nm and emission at 415 nm.

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4.3 Protein estimation (Lowry et al., 1951)

Protein content of *C. elegans* homogenate was estimated as described earlier (*Section 4.3, Chapter IIA*).

4.4 Statistical analysis

Data is presented as mean ± standard error of three separate experiments performed in triplicate on three different days, using the computer program Excel software (1999). The data were analyzed employing Two way analysis of variance (ANOVA) using SPSS software 10 (Chicago, IL, USA) Duncan's multiple regression test (DMRT) for multiple comparisons was performed to determine the significant differences among the groups. *P* Values <0.05 were considered significant.

5.0 RESULTS

5.1 Effect on feeding

Worms were exposed for 12 and 24 h to various concentrations of dichlorvos (0, 5, 100, 200, 250 and 500nM) initially to select the dose which had no effect level on feeding. There was a gradual and time-related decrease in bacterial density in the control worm suspension and dichlorvos exposure at 5,100 and 200nM, indicating normal feeding by these worms. This was further confirmed by uptake of red acrylic paint particles which accumulated in the pharynx within 10 min. Dichlorvos concentration at 250 and 500nM induced cessation in feeding at 24h of exposure (**Figure 3.8**), which was also confirmed by absence of pigment particles in the pharynx clearly indicating cessation in feeding at these concentrations of dichlorvos.

Based on the feeding pattern we selected 5, 100 and 200nM of dichlorvos for further studies.

5.2 Acetylcholinesterase activity

In untreated worms, the AChE activity increased up to 5d and then decreased after 10 d. A similar trend was also evident in worms exposed to

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dichlorvos concentration (5, 100nM). However in worms exposed to 200nM dichlorvos, the AChE activity decreased after 5d. As evident from **Table 3.4** and **Figure 3.9**, varying degrees of inhibition in the enzyme activity was evident from 1d in worms exposed to dichlorvos. The inhibition due to exposure to dichlorvos ranged between 12 – 40% (1d) 18 – 16% (5d), 29 – 73% (10d). Maximum inhibition of AChE activity was evident on day 10.

5.3 Carboxylesterase activity

As evident from **Table 3.5** and **Figure 3.10,** an increase in carboxylesterase activity was evident in untreated worms up to day 5, beyond which the enzyme activity decreased drastically. Interestingly, in worms exposed to dichlorvos, the enzyme activity was significantly higher than control worms on day1 (**Figure 3.10**). On day 5 – 15, the CaE activity in worms exposed to 5 and 100nM was *on par* with that in control worms. The enzyme activity in worms exposed to 200nM was lower than that in control worms at all the time intervals. Interestingly, the enzyme activity in worms exposed to 5nM dichlorvos was higher than control beyond day 5.

5.4 Effect on lipofuscin content

Age-dependent increase in lipofuscin content was observed in the control worms. Interestingly, remarkable concentration and age dependent increase in lipofuscin accumulation was observed on exposure to dichlorvos. Dichlorvos at 5,100 and 200nM after 5d of exposure caused 1.3, 4.6 and 5.3 fold increase in lipofuscin levels compared to controls. Similarly lipofuscin accumulation on day 20 at 5,100 and 200nM was 0.6, 1.1 and 1.2 fold higher compared to controls. While lipofuscin accumulation in control worms was restricted to intestine only, exposure to dichlorvos resulted in significant fluorescence throughout body of the worm **(Table 3.6, Figure 3.11 and 3.12).**

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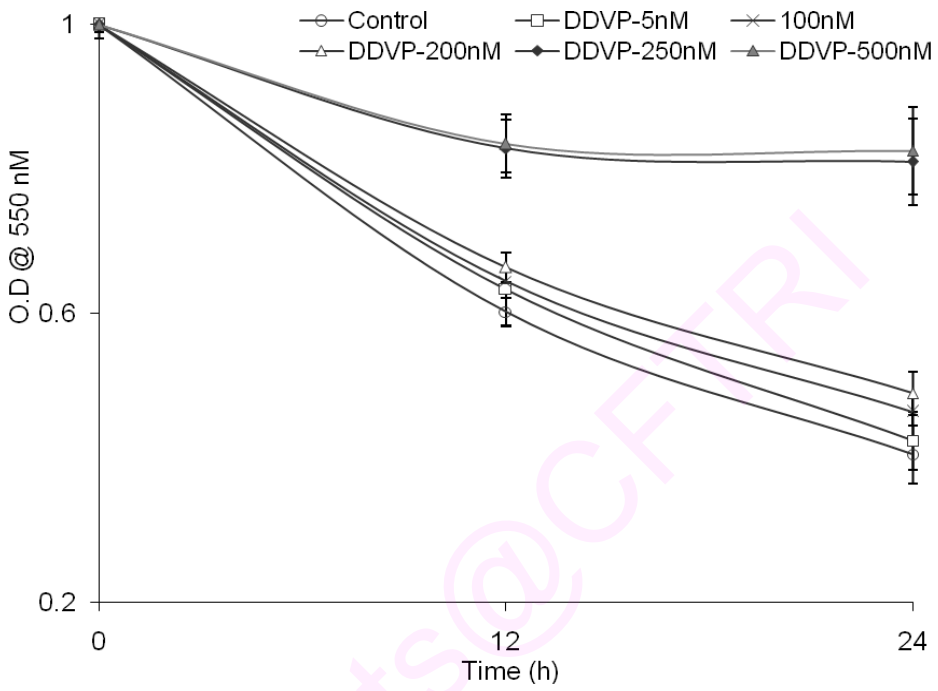

Figure 3.8 Effect of low concentrations of dichlorvos on feeding in *C. elegans*

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Table 3.4 Activity of AChE in *C. elegans* on exposure to dichlorvos with age.

	AChE (nmol/min/mg protein)					
	Day					
Dichlorvos (nM)	0	1	5	10	15	20
0	10.22[a] ± 1.34	20.4[b] ± 1.24	20.35[c] ± 1.32	17.8[c] ± 1.89	8.81[c] ±1.58	5.44[c] ±1.23
5	9.73[a] ± 1.45	17.92[ab] ± 1.23	16.6 ± 0.56	12.63[b] ± 0.89	8.51[c] ±1.23	4.96[bc] ± 0.96
100	9.86[a] ± 2.05	17.22[ab] ± 1.23	14.54[b] ± 1.28	5.337[a] ± 0.68	4.71[b] ±1.23	3.49[ab] ± 0.82
200	10.0[a] ± 1.20	12.31[a] ± 1.23	7.95[a] ± 1.56	4.83[a] ± 1.09	3.75[a] ±0.54	3.29[a] ±0.35

Values are mean ± S.E of 3 observations with 3 replicates each. Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)

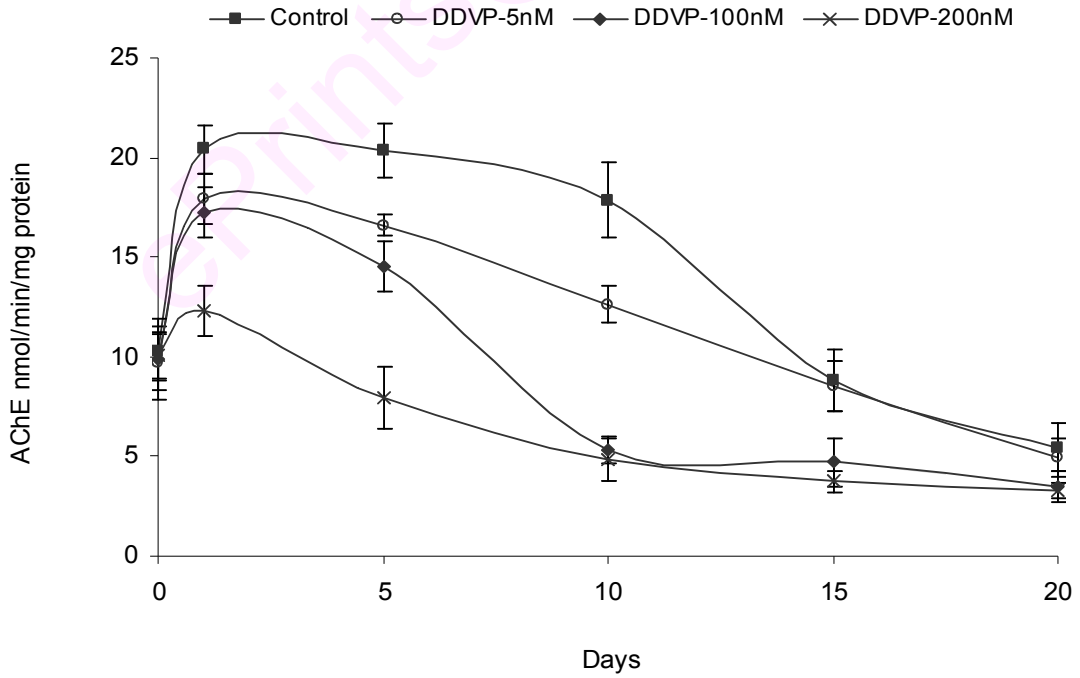

Figure 3.9 Age-related activity of AChE in *C. elegans.*
Each data point is the mean ± S.E.M of three replicates from three independent experiments.

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Table 3.5 Activity of Carboxylesterase in *C. elegans* on exposure to dichlorvos with age

	Carboxylesterase (nmol/min/mg protein)					
	Day					
Dichlorvos (nM)	0	1	5	10	15	20
0	10.43[a] ±1.23	32.46[a] ± 2.03	64.98[b] ± 2.56	31.22[b] ± 3.62	23.53[b] ± 2.78	14.96[a] ± 3.45
5	12.00[a] ± 3.58	61.55[b] ± 10.97	67.72[b] ± 4.56	31.95[b] ± 2.22	28.78[c] ± 2.78	24.00[b] ± 1.56
100	12.50[a] ± 3.56	63.56[b] ± 4.89	63.83[b] ± 3.66	12.56[a] ± 3.45	17.85[a] ± 4.78	13.93[a] ± 1.30
200	12.60[a] ± 2.67	47.78[ab] ± 3.54	36.53[a] ± 4.87	13.27[a] ± 1.91	15.75 [a] ± 2.34	13.33[a] ± 2.93

Values are mean ± S.E of 3 observations with 3 replicates each.
Data analyzed by post-hoc test (Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$)

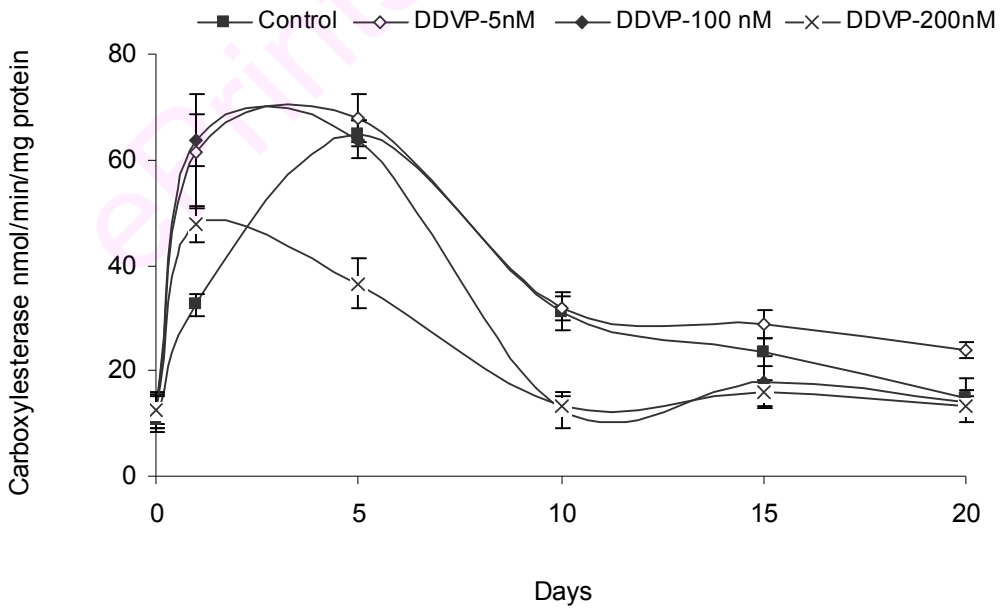

Figure 3.10 Age-related activity of carboxylesterase in *C. elegans*
Each data point is the mean ± S.E.M of three replicates from three independent experiments

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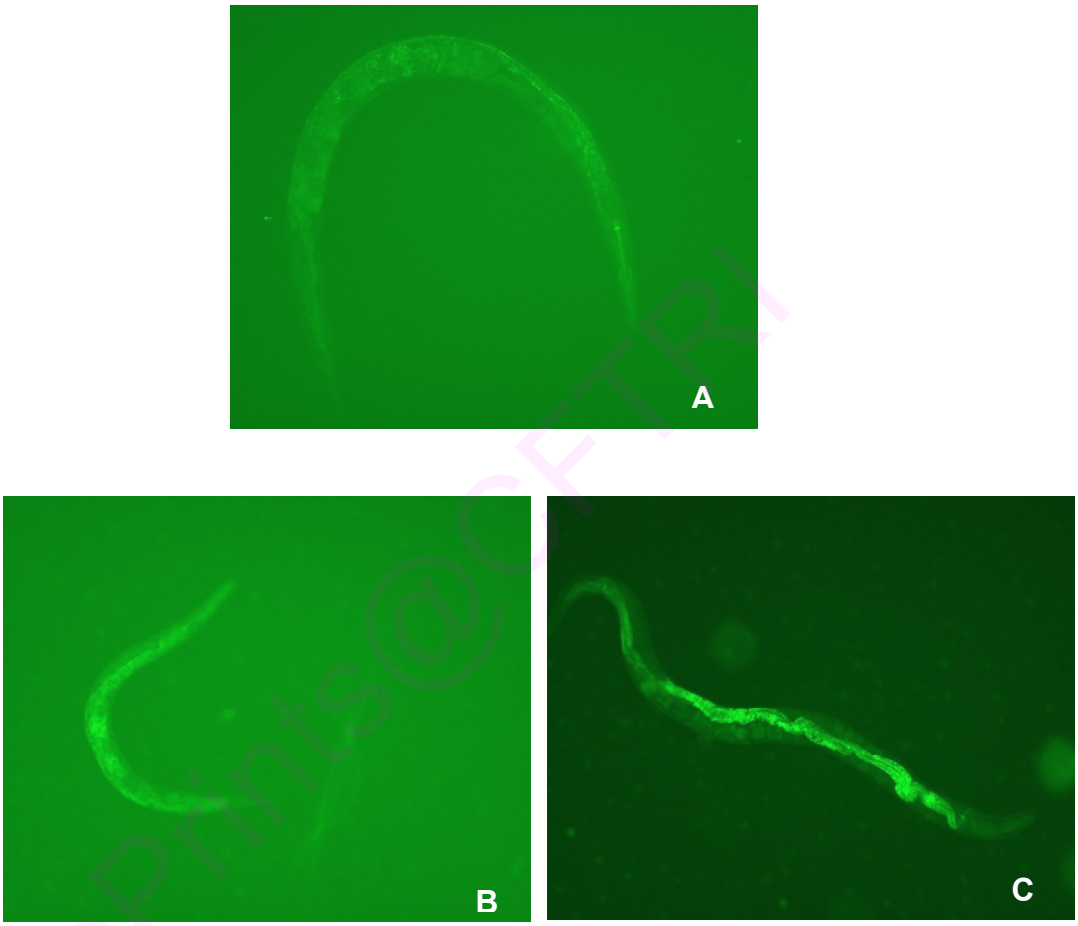

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Fig.3.11 Localization of lipofuscin in *C. elegans* exposed to dichlorvos

A: Control worm
B: Worm exposed to dichlorvos (5nM)
C: Worm exposed to dichlorvos (200nM)

Table 3.6 Lipofuscin accumulation in *C. elegans* on exposure to dichlorvos with age.

Dichlorvos (nM)	Lipofuscin (Relative fluorescence/mg protein)			
	Day			
	5	10	15	20
0	1.89[a] ± 0.45	6.29[a] ± 0.98	8.38[a] ± 1.25	9.10[a] ± 1.56
5	4.49[b] ± 0.58	10.49[b] ± 1.23	13.37[b] ± 1.56	14.71[b] ± 1.35
100	10.74[b] ± 1.20	14.94[b] ± 1.76	17.65[b] ± 1.89	18.68[b] ± 1.43
200	12.02[c] ± 0.67	16.33[c] ± 1.67	18.83[c] ± 1.89	19.69[c] ± 1.45

Values are mean ± S.E of 3 observations with 3 replicates each. Data analyzed by post-hoc test(Duncan); Means in the same column with different superscript differ significantly ($p < 0.05$).

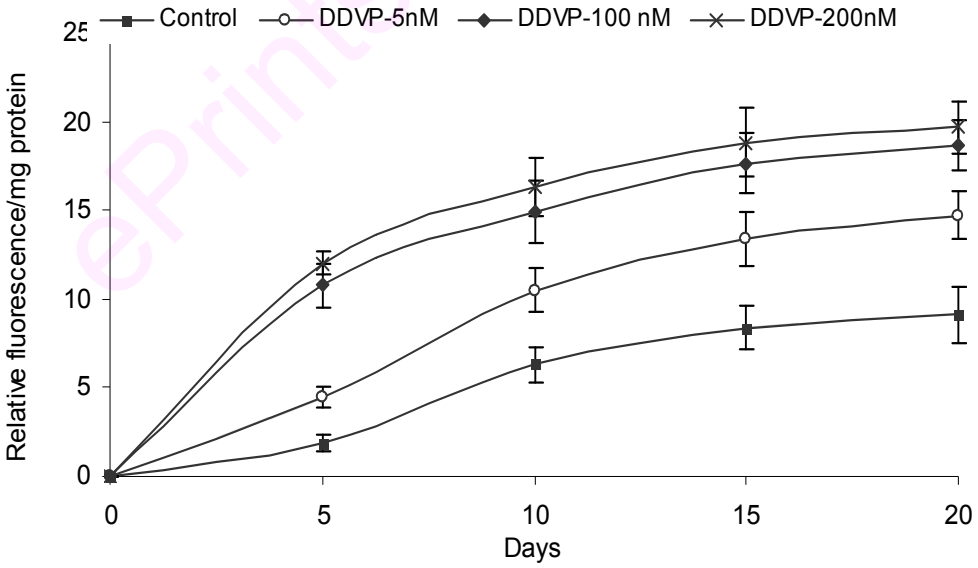

Figure 3.12 Lipofuscin accumulation with age in *C. elegans*
Each data point is the mean ± S.E.M of three replicates from three independent experiments.

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6.0 DISCUSSION

In our exposure conditions, we found that feeding was an important parameter to consider for OPI exposure in *C. elegans*. Since there is no literature available on low dose, long-term exposure in invertebrate system, we designed a study based on feeding responses on exposure. We found that dichlorvos induces significant inhibition in feeding beyond 250nM while there were no effects on physiology of worms below this concentration. Based on this criterion, in the present study, we selected dichlorvos concentrations below 250 nM and the exposure period of 20d.

The measurement of age-related changes is a necessary starting point for studies that can define causal relationships during aging. While measurements of age-related changes alone do not define these causal relationships, these measurements are an essential element of experiments that have the potential to define causal relationships. These experiments include the analysis of genetic, environmental, dietary and pharmacological factors that influence aging and longitudinal studies that define relationships between different age-related changes (Huang et al., 2004).

Our results demonstrated that in general the AChE activity increased in worms up to 5d and then decreased after 10d. Maximum inhibition in AChE activity on exposure to dichlorvos was evident at 10d. However, CaE activity increased in untreated worms up to 5d beyond which the activity decreased. Age-dependent increase in lipofuscin content was evident among control worms and interestingly, there was a concentration and age-dependent increase in lipofuscin content was discernible in worms exposed to dichlorvos.

CaEs are thought to act as a detoxifying enzyme by decreasing the overall concentration of the organophosphate at the target enzyme site, ChE (Maxwell, 1992). Many OPI are reported to cause inhibition of liver CaE at lower dietary levels than those required to cause brain ChE inhibition (Su et al.,1971). CaE alters the toxicity profile of many nerve agents (Maxwell, 1992) and some

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anticholinesterase pesticides (Chambers et al., 1990; Gupta and Dettbarn, 1993).

It has also been reported that newborn and young humans also have lower metabolic capacity for some key xenobiotic metabolism pathways compared to adults (Johnson, 2003; Mueller et al., 1983), which probably accounts for the age-related phenomena observed in animals. Mammalian carboxylesterases (both A and B esterases) play important roles in deactivating organophosphates (Moser et al., 1998; Karanth and Pope, 2000; Padilla et al., 2000). In rats, both A and B esterases increase occurs during the first few weeks postpartum, with a subsequent upward trend to at least 8–9 weeks of age (Brodeur and DuBois, 1967; Benke and Murphy, 1975). In addition to carboxylesterases, other pathways of organophosphate deactivation exhibit age related increases in activity as the animals mature. Karanth and Pope (2000) reported that the level of CaEs was significantly different between neonatal (7 d), juvenile (21 d), and adult (3 months) rat tissues (lung, plasma, and liver) and showed age-dependent increases of CaE activity in all evaluated tissues. Similarly, age-related increase in brain AChE activity has been demonstrated in both rats and mice (Atterberry et al., 1997; Timchalk et al., 2006).

The chronological accumulation of age pigment lipofuscin is characteristic of most animals, including humans and the increased accumulation of these substances has been considered the most constant marker of advancing age in animals. These fluorescent compounds include lipofuscin, a heterogeneous mix of oxidized and cross-linked molecules (proteins, lipids and carbohydrates) thought to accumulate in lysosomes (Yin, 1996). It has been suggested that highly cross-linked lipofuscin compounds accumulate in lysosomes where they fail to be efficiently degraded and there by impair normal protein turnover to diminish cell function (Brunk and Terman, 2002). Among humans and small animals, where the increase in lipofuscin accumulation has been extensively studied in heart muscle and nerve cells, it is estimated that 90% of the volume of certain nerve cells in old animals is taken

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up by age pigment, a fact that indicates that the function of the cell organelles may be impaired.

C. elegans hermaphrodites are demonstrated to display age-related accumulation of fluorescent material characterized by an excitation maximum of about 340nm and an emission maximum of about 430nm. The fact that significant changes in nematode age pigment accumulation can be demonstrated within weeks provides a clear rationale for the use of nematodes to study this phenomenon. In our study, we quantified the lipofuscin as a function of age and on exposure to dichlorvos and we observed an increase in lipofuscin with age and concentration of dichlorvos. Studies with mammalian age pigments under *in vitro* conditions of extreme oxidative stress have suggested that free radicals promote lipofuscin and advanced glycation end-product formation (Yin et al., 1995; Yin, 1996; Brunk and Terman, 2002). That age pigments might reflect physiological aging state has been suggested based on other work which correlated metabolic rate with lipofuscin levels (Sohal, 1981; Sohal and Brunk, 1989).

Studies have also examined age pigment accumulation in senescing *C. elegans* (Klass, 1977; Davis et al., 1982; Hosokawa et al., 1994; Braeckman et al., 2002a,b). Age pigment components are known to accumulate at a low rate during adult days (5–10d) in *C. elegans*. That is period as reproductive midlife, but after day 10 (advanced post-reproductive age), the rate of age pigment accumulation increases significantly (day 10 to day 15) which is just after reproduction by self-fertilization normally ceases, suggesting a midlife post-reproductive shift to a distinct physiological state (Gerstbrein et al., 2005). Similarly, we also observed an increased accumulation of age pigment after day 10 in control and significant increase in dichlorvos-exposed worms. This suggests that a metabolic transition, reflected by increased age pigment accumulation, occurs in aging adults.

An age dependent accumulation of lipofuscin in the intestinal cells of *C. elegans* is an established marker of ageing in this organism (Garigan et al.,

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2002; Gerstbrein et al., 2005). In *C. elegans* lipofuscin is thought to accumulate in secondary lysosomes, which appear as granules in intestinal cells (Clokey and Jacobson, 1986; Garigan et al., 2002). However, the material is not restricted to the intestine and detergent cleaned cuticle preparations display an age-related increase in fluorescence that may represent changes in collagen (Davis et al.,1982). In the present study, we observed the presence of these granules in the intestine and throughout the body of treated worms, whereas in control worms on day 15, the pigment was visible only in the intestine.

Toxicity data on mature animals may be insufficient for extrapolation to immature animals since the mechanism responsible for age related differences in toxicity has not been well characterized (Atterberry et al., 1997). The greater neonatal/juvenile animal sensitivity to OPs has primarily been attributed to the lack of complete metabolic competence during development (Murphy, 1982; Padilla et al., 2004). Age-related differences may be associated with quantitative differences in the target tissues and the biological responses. Studies have demonstrated that juvenile animals are more vulnerable than adults to the acute toxic effects of some OPI (Pope and Liu, 1997; Moser and Padilla, 1998; Moser et al., 1998). In contrast, a lack of juvenile animal sensitivity has also been reported for some other OPI, like methamidophos (Moser, 1999), suggesting chemical-specific differences. Karanth and Pope (2000) reported that the level of CaEs was significantly different between neonatal (7 days), juvenile (21 days), and adult (3 months) Sprague-Dawley rats' tissues (lung, plasma, and liver) and showed age dependent increases of CaE activity in all evaluated tissues.

Methyl parathion is reported to cause greater inhibition of brain cholinesterase in neonates compared to adults irrespective of the dosing (Pope and Chakraborti, 1992), over 7 d (Liu et al., 1999) or over 14 d (Pope and Liu, 1997). Methamidophos was equally inhibitory to brain and blood cholinesterase in neonates and adults following acute dosing (Moser, 1999), and all cholinesterase endpoints were more sensitive in younger animals compared to

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adults for methyl parathion. However, younger animals were more sensitive to chlorpyrifos. Similarly, our data on both carboxylesterase and acetylcholinesterase showed greater inhibition in young and adult worms when compared to ageing worms.

In conclusion, our results clearly demonstrate that AChE activity is maximally inhibited by very low concentrations of dichlorvos after 10 d of exposure when the CaE activity is relatively lower. Further, dichlorvos also increased the lipofuscin content in the worms and thereby appeared to accelerate aging in these worms when exposed to very low concentrations.

7.0 SUMMARY

1. Dichlorvos concentration at 250 and 500nM induced cessation in feeding at 24h of exposure while the worms showed normal feeding at 5,100 and 200nM. We selected 5, 100 and 200nM of dichlorvos for further studies.
2. In untreated worms, the AChE activity increased up to 5d and then decreased after 10 d. A similar trend was also evident in worms exposed to dichlorvos concentration (5, 100nM).
3. However in worms exposed to 200nM dichlorvos, the AChE activity decreased after 5d.
4. The inhibition due to exposure to dichlorvos ranged between 12 – 40% (1d) 18 – 16% (5d), 29 – 73% (10d) and maximum inhibition of AChE activity was evident on day 10.
5. An increase in carboxylesterase activity was evident in untreated worms up to day 5, beyond which the enzyme activity decreased drastically.
6. Interestingly, in worms exposed to dichlorvos, the enzyme activity was significantly higher than control worms on day1.
7. The enzyme activity in worms exposed to 200nM at all the time intervals was lower than that in control worms.

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8. Age-dependent increase in lipofuscin content observed in the control worms.
9. Interestingly, remarkable concentration and age dependent increase in lipofuscin accumulation was observed on exposure to dichlorvos.
10. While lipofuscin accumulation in control worms was restricted to intestine only. Exposure to dichlorvos resulted in significant fluorescence throughout body of the worm.

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CHAPTER IV

Responses of selected enzymes to dichlorvos: A comparative *in vitro* study in rat brain and *C. elegans* homogenates

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CHAPTER IV

Responses of selected enzymes to dichlorvos: A comparative *in vitro* study in rat brain and / or *C. elegans* homogenates

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PREFACE

Our preceding studies have clearly established the neurotoxicity and mode of action of dichlorvos in *C. elegans*. In order to validate this organism as a model for neurotoxicity studies, it was proposed to compare the sensitivity of selected enzymes involved in OPI toxicity both in rat brain and / or *C. elegans* homogenate system *in vitro*.

This chapter has been presented under **two** sections:

Section A includes studies on AChE of rat brain homogenate and *C. elegans* homogenate *in vitro*.

Section B describes the kinetic studies on carboxylesterase of *C. elegans* homogenate *in vitro*.

SECTION IV A

Comparative responses of AChE of rat brain and *C. elegans* to inhibition by dichlorvos, *in vitro*

1.0 INTRODUCTION

Cholinesterase enzymes are widely distributed across the animal kingdom. Acetylcholinesterase (AChE) is perhaps the only cholinesterase which has received maximum attention of researchers world over, owing to its important role in regulation of neurotransmission and toxicity of environmental pollutants. With regard to neurotransmission, AChE plays a crucial regulatory role in cholinergic systems by terminating the nerve impulse in a process involving hydrolysis of the neurotransmitter, acetylcholine (ACh) (Rand, 2007). AChE is also a functional target of OPI, which act as pseudo-substrates to inhibit the enzyme by phosphorylating the serine –OH group of the active site (Marcell et al., 1998). The acute toxicity of OPI has been explained on the basis of cholinergic stress resulting from inhibition of AChE.

Acetylcholine (ACh) is the primary excitatory neurotransmitter involved in *C. elegans* motor function, as in higher animals (Rand and Nonet, 1997). Also conserved is the synaptic degradation of ACh by acetylcholinesterase (AChE) enzymes. While vertebrates have a single AChE gene whose product can undergo alternative splicing post-transcriptionally to yield different isoforms, *C. elegans* has four separate genes yielding four AChE enzymes (Combes et al., 2001). Three class of AChE have been distinguished (A, B and C) in *C. elegans* on the basis of their K_m values for ACh, their selectivity for related substrates, and their sensitivities to cholinesterase inhibitors. More than one form of activity within given class can be identified using sucrose density dimension and ion exchange chromatography (Johnson and Russell, 1983; Kolson and Russell, 1985). Detergent is required for solubilization of some of these forms. None appears to be collagen tailed, as with some vertebrate AChEs.

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Monitoring of AChE activity in wildlife populations has been proposed as a general method for detecting the environmental contamination from OPI (Gagnaire et al., 2007). In laboratory studies, detection of residual AChE has extensively been employed as an index of OPI toxicity following *in vivo* exposures. AChE inhibitory potential of dichlorvos (Jadhav and Rajini, 2009) and other OPI (Rajini et al., 2008) in *C. elegans* following *in vivo* exposure have been recently reported. However, these studies are not sufficient to obtain information regarding mechanistic details of AChE inhibition. *In vitro* studies designed to investigate kinetic parameters of an enzyme provide invaluable information about substrate specificities, mechanistic insights, and nature of inhibition and relative sensitivities of enzymes for inhibition.

Organ and species variations sensitivity is a hallmark of OPI toxicity. One example for such variations is the relative toxicities of parathion and chlorpyrifos to rats and catfish. While, in rats, toxicity of parathion is much greater than chlorpyrifos (as reflected by LD_{50} values), their toxicities are reversed in the case of catfish with chlorpyrifos having higher LC_{50} value than parathion (Carr and Chambers., 1996). Main and Iverson (1966) have found differences in the sensitivities of various tissue enzymes to OP exposure. These differences may partly be explained on the basis of variability in the kinetic parameters of AChE from different organs or species. This view receives support from the studies of Anderson et al., (1972, 1977) suggesting that differential sensitivities of AChE from various organs is a consequence of differences in relative affinity of various OPI to the active site of the enzyme. Earlier studies (Singh.,1985) not only demonstrated variations in the sensitivity of brain and RBC AChE to acephate and methamidophos, but also differential sensitivity of different AChEs (brain and RBC) from the same organism to individual OPI (acephate or methamidophos). More importantly, the work attributes differences in AChE inhibitory potential of two OPI to differences in their phosphorylation rates. Thus, *in vitro* kinetic and inhibition studies serve as an extremely useful tool for

understanding the nature of inhibition and sensitivity of the enzyme to an inhibitor.

The present investigation deals with studies that were designed to understand basic kinetic parameters of *C. elegans* AChE with regard to substrate hydrolysis and inhibition by dichlorvos *in vitro*. Efforts were also made to compare the same with basic kinetic parameters of rat brain AChE with regard to substrate hydrolysis and inhibition by dichlorvos *in vitro* for the purpose of validation.

2.0 MATERIALS AND METHODS

2.1 Chemicals

Acetylthiocholine iodide, BSA (fraction V) and 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB) were procured from Sigma Chemical Co., (St. Louis, MO, USA). Dichlorvos (DDVP; Technical grade; 97.6%) was gift from Hyderabad Chemical Supplies Ltd., (Hyderabad, India). All other reagents used were of analytical grade.

2.2 Pesticide solution

Stock solution (5mM) of Dichlorvos (DDVP) was prepared in double distilled water and further diluted to obtain working solutions for *in vitro* assays.

2.3 Worm culture and maintenance (Brenner, 1974)

The wild-type *Caenorhabditis elegans* Bristol strain N2 was obtained from Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA) which is funded by the NIH National Center for Research Resources (NCRR). The worms were cultured and maintained as described in Chapter IA (*Section 2.3.1 – 2.3.3*). Adult worms (3.5 d old) were used for the studies.

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2.4 Animals and care

Male Wistar rats (CFT strain, 8 week old, 300-350 ± 5 g) obtained from the Central Food Technological Research Institute animal house were used for the studies. They were housed in polypropylene cages (two per cage) at room temperature (25 ± 2º C) with relative humidity of 50- 60% and on a 12h light - darkness cycle. They had free access to commercial pellet diet (Saidurga Feeds and Foods Pvt. Ltd., Bangalore, India) and water *ad libitum*. All procedures with animals were conducted strictly in accordance with approved guidelines by the 'Institute Animal Ethical Committee' regulated by the committee for the purpose of control and supervision of experiments on animals (CPCSEA) Ministry Social justice and Empowerment, Government of India. During the experiments, maximum care was taken to minimize animal suffering.

2.5 Preparation of rat brain homogenate

Rats were sacrificed under mild either anesthesia, brains were excised, washed and placed on ice. 10% homogenate were prepared (50 mM Tris HCl, pH 7.4) and the supernatant was used for *in vitro* assay.

2.6 Preparation of *C. elegans* homogenate

Worms (gravid stage/ 3.5d old) were harvested and homogenized in 50mM Tris HCl, pH 7.4. Homogenates were centrifuged at 10,000 rpm for 10 min and supernatant was stored at –20°C until the assay.

3.0 EXPERIMENTAL PROCEDURE

3.1 Substrate saturation

Acetylcholinesterase activities were determined based on the method of Ellman et al. (1961) with modifications suggested by Zhu and Clark (1994) using various concentrations of the substrate, ATCI, ranging between 0.10 - 10mM. Various kinetic parameters viz: Michaelis constant (K_m), maximum

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velocity (V_{max}), and catalytic efficiency (V_{max}/K_m) were determined for AChE of rat brain and *C. elegans* from the respective Lineweaver-Burk (LB) plot.

3.2 Determination of IC_{50}

In vitro inhibition of AChE by dichlorvos was monitored by incubation of both homogenate systems *in vitro* with concentrations of dichlorvos ranging from 5 to 200nM. Following incubation of both homogenate systems for 30min at room temperature, AChE activity was assayed as described above (Section 3.3) and IC_{50} was determined by linear regression analysis of percent inhibition versus dichlorvos concentration.

3.3 Kinetic properties of acetylcholinesterase by dichlorvos

Enzyme from the two sources were incubated with IC_{25}, IC_{50}, and IC_{75} concentrations of dichlorvos for 30 min at room temperature and residual AChE activity was measured in presence of increasing concentration of ATCI (0.10 to10 mM). The K_m and V_{max} was calculated from LB plot.

3.4 Protein estimation (Lowry et al., 1951)

Protein content was estimated in 10 µl of rat brain or *C. elegans* homogenate, as described in the earlier (C*hapter IIA, Section 4.3*).

3.5 Interpretation of kinetic parameters

Michaelis constant (K_m), maximum velocity (V_{max}) and catalytic efficiency (V_{max}/K_m) were determined from LB plot.

4.0 RESULTS

In the present study we determined K_m, V_{max}, IC_{50}, K_i and $V_{i\ (max)}$ of AChE for both rat brain and *C. elegans* homogenate system for the purpose of comparison.

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4.1 Kinetic studies: K_m, V_{max} and substrate (ATCI) saturation

The kinetics of AChE in the presence of ATCI exhibited substrate saturation in both the systems (**Figure 4.1, Table 4.1**). AChE activities were significantly decreased when concentrations of the ATCI substrates were higher than the optimum, showing a characteristic phenomenon of substrate inhibition at higher concentrations. Rat brain homogenate AChE showed a typical saturation at 2mM of the substrate ATCI and in *C. elegans* AChE saturation was obtained above 5 mM of ATCI. Interestingly, kinetic parameters for the ATCI substrate obtained from LB plot which assess affinity of substrate to AChE, as indicated by K_m values revealed K_m for rat AChE as 59 μM and 725.41 μM for *C. elegans* AChE. This showed that affinity of rat brain AChE to ATCI was 12.6 fold higher than that of *C. elegans* AChE. However, the efficiency of substrate being hydrolyzed by AChE, as indicated by V_{max} values, were 112.35 nmol/min/mg protein and 33-nmol/min/mg protein for rat brain and *C. elegans* AChE respectively (**Figure 4.2, Table 4.1**).

4.2 Determination of IC_{50}

The IC_{50} is the concentration of dichlorvos required to inhibit AChE activity by 50% at room temperature in 30 min of incubation. The IC_{50} for *C. elegans* and rat brain AChE were 45.7 ± 2.68 nM and 107.18 ± 3.56nM respectively (**Figure 4.3, Table 4.2**).

4.3 Kinetics of enzyme inhibition

As determined from L-B plot, the K_m, V_{max} and V_{max}/K_m values of the inhibited AChE of rat brain at IC_{50} concentration of dichlorvos were 57.48μM, 42.55nmol of ATCI hydrolyzed/min/mg protein, 0.74 min/mg/protein (**Figure 4.4, Table 4.3**) while for *C. elegans* K_m, V_{max} and V_{max}/K_m at IC_{50} were 734μM, 10.10 nmol of ATCI hydrolyzed/min/mg protein and 0.013 min/mg protein respectively. LB plot revealed the inhibition of AChE in both the system to be of irreversible in nature.

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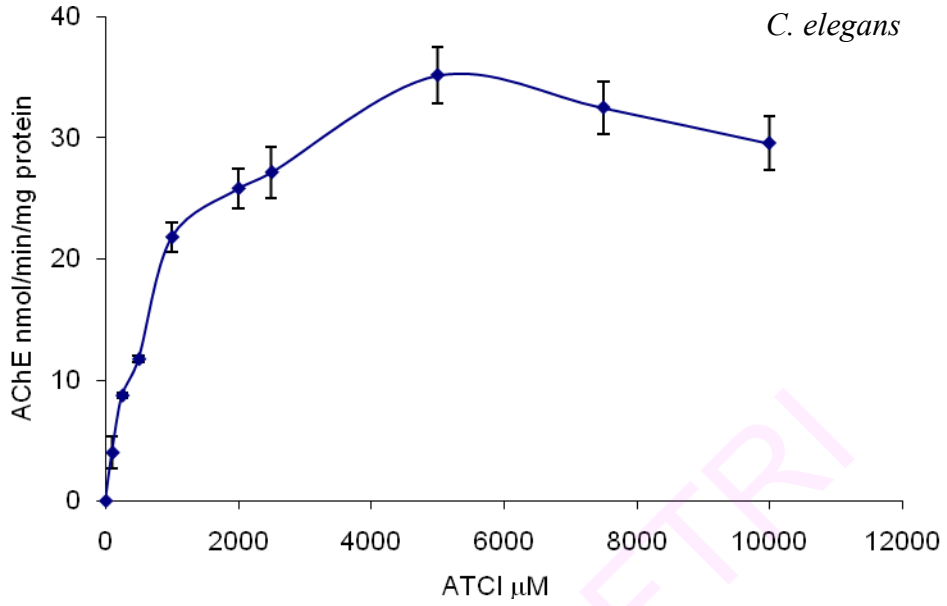

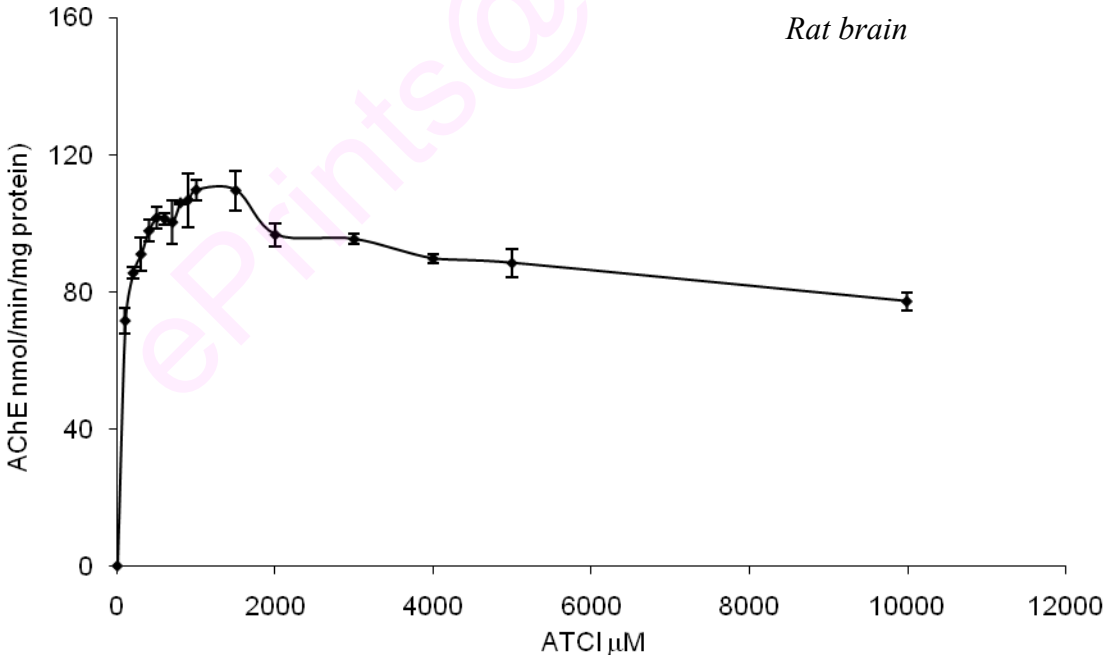

Figure 4.1 Effect of substrate concentrations on rat brain and *C. elegans* AChE.
Values are the mean of two sets with duplicate at each concentration.

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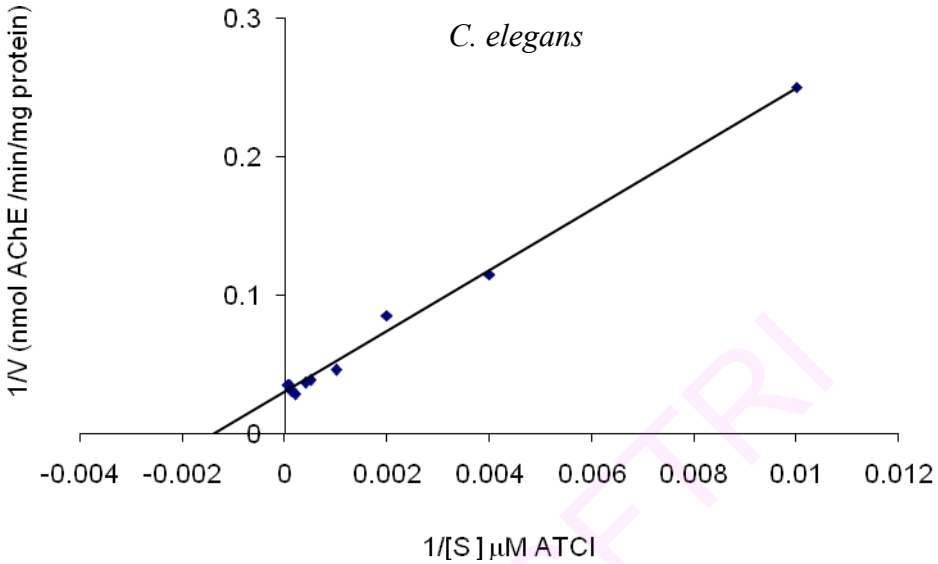

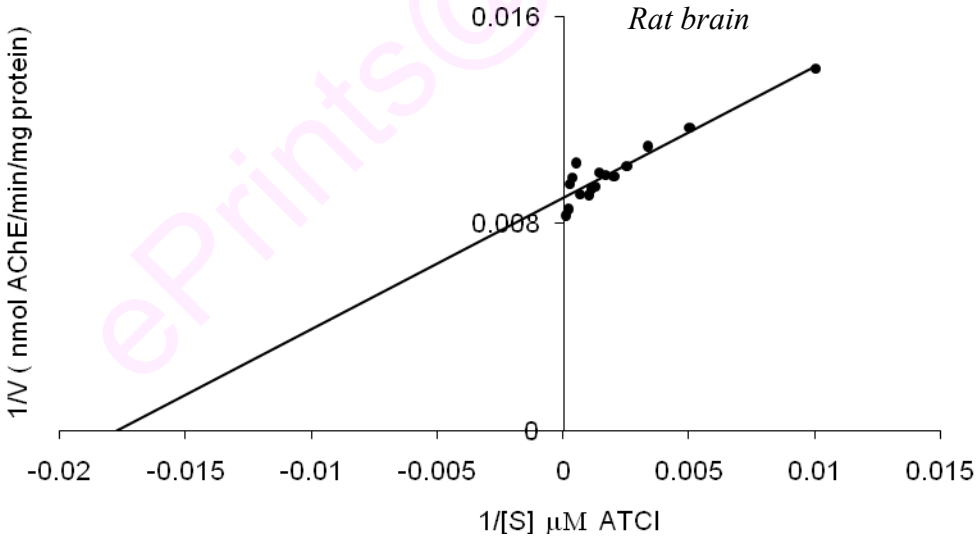

Figure 4.2 Lineweaver - Burk plot for AChE activity in *C. elegans* and rat brain

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Table 4.1 Substrate specificity and the kinetic characteristics of *C. elegans* and rat brain AChE

Homogenate	Substrate concentration (mM)	K_m (μM)	V_{max} (nmol/min/mg protein)
C. elegans	> 5	725.41	33.00
Rat brain	> 2	59.01	112.35

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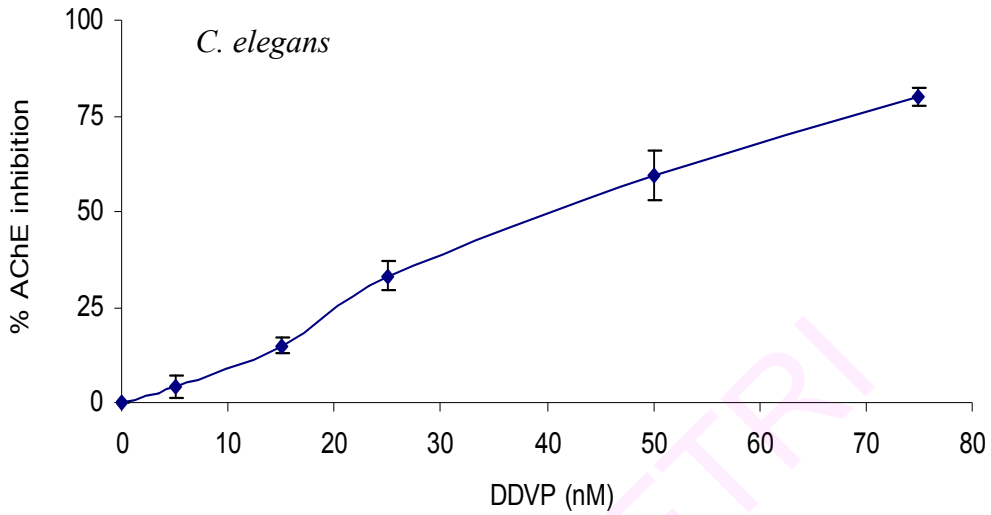

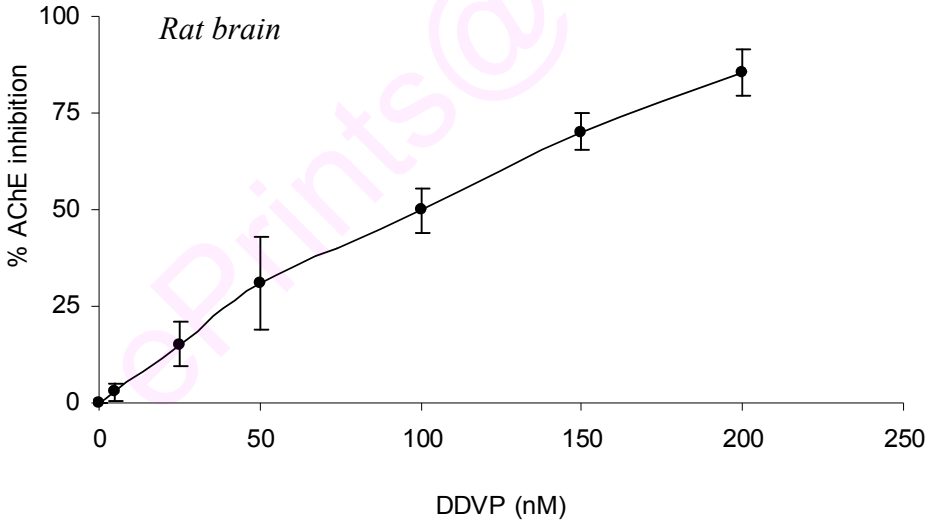

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Figure 4.3 Inhibition pattern of acetylcholinesterase by dichlorvos (DDVP) in *C. elegans* and rat brain homogenate.

Table 4.2 Inhibitory concentrations of dichlorvos in *C. elegans* and rat brain AChE

Homogenate	Dichlorvos (nM)		
	IC_{25}	IC_{50}	IC_{75}
C. elegans	22.85 ± 1.34	45.7 ± 2.68	68.56 ± 4.02
Rat brain	49.18 ± 2.68	107.18 ±3.56	165.17 ±6.42

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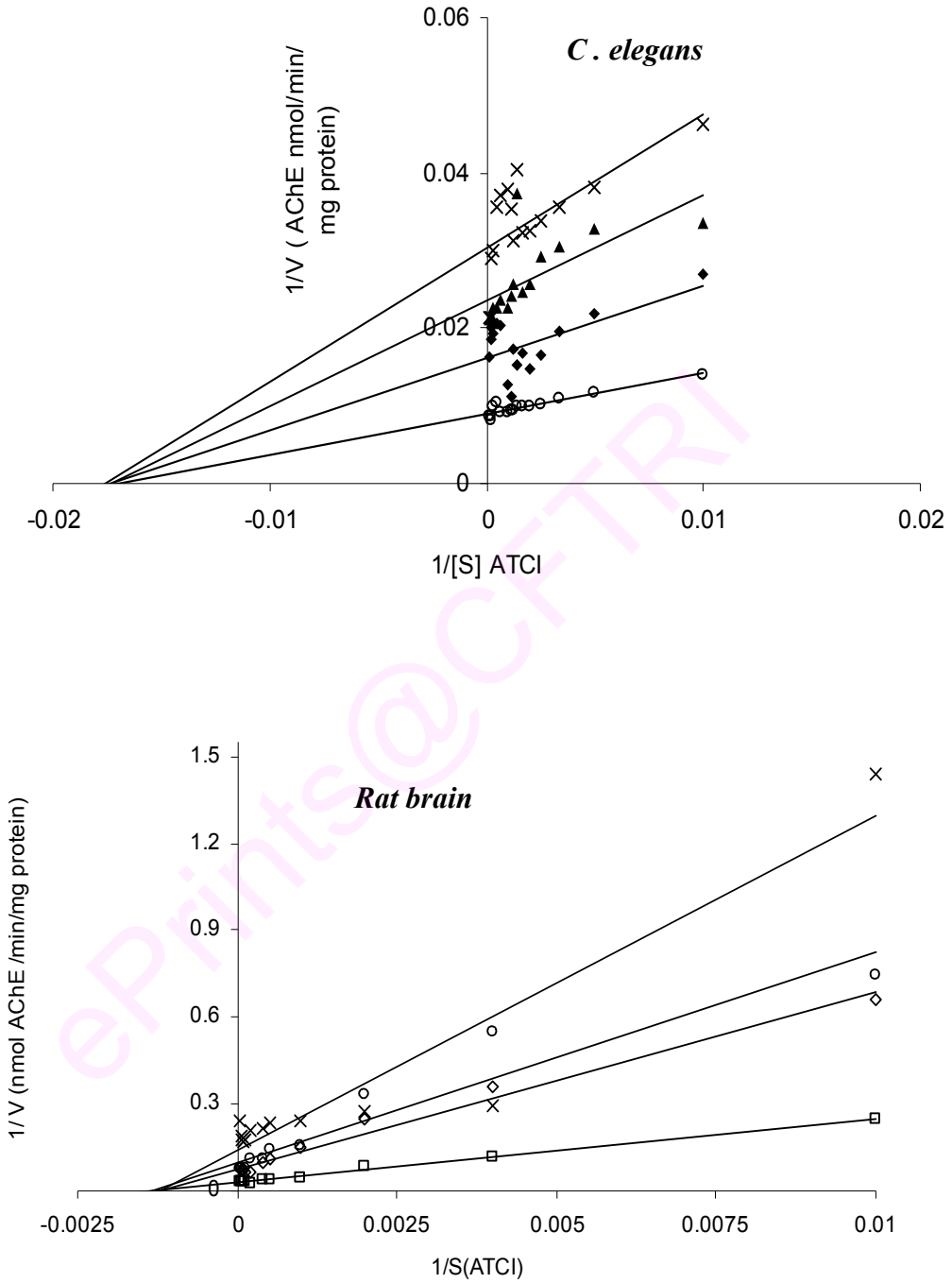

Figure 4.4 Inhibition of AChE activity in *C. elegans* and rat brain homogenates with IC_{25}, IC_{50} and IC_{75} concentrations of Dichlorvos.

Plot of reciprocal of initial velocities versus reciprocal concentrations of ATCI.

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Table 4.3 Kinetic characteristics of *C. elegans* and rat brain AChE in the presence of dichlorvos

Dichlorvos (nM)	*C. elegans*			Rat brain		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
IC_{25}	789.27	13.00	0.016	57.31	61.72	1.07
IC_{50}	734.70	10.10	0.013	57.48	42.55	0.74
IC_{75}	800.27	6.94	0.0008	56.83	33.00	0.60

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6.0 DISCUSSION

In the present study, the rates of acetylthiocholine hydrolysis by both *C. elegans* and rat brain homogenate system showed typical Michaelis-Menten patterns with increasing concentration of ATCI resulting in increased rates followed by attainment of zero order kinetics. Acetylcholinesterase activities in both homogenates exhibited inhibition at higher substrate concentrations. While *C. elegans* AChE hydrolysed ATCI with a K_m of 725μM and V_{max} of 33 nmol/min/mg protein, interestingly, the K_m of rat brain AChE for ATCI was 59.01mM indicating a greater affinity of rat brain AChE for substrate by 12 folds compared to that of *C. elegans* AChE.

The model for enzyme action, first elucidated by Michaelis and Menten, suggests the binding of free enzyme to the reactant forming an enzyme-reactant complex which undergoes a transformation, releasing product and free enzyme. The free enzyme is then available for another round of binding to new reactant. K_m is termed the Michaelis constant and V_{max} denotes the maximum steady-state reaction velocity. The V_{max} can be expressed as the total enzyme concentration times the rate constant for the product formation. K_m is an indicator of the affinity that an enzyme has for a given substrate, and hence the stability of the enzyme-substrate complex and is equal to the substrate concentration that gives 1/2 of the maximal velocity. The lower the value of K_m, the greater the affinity of the enzyme – substrate complex formation. Therefore it is related to the affinity or strength of binding of a substrate to the enzyme.

Lineweaver–Burk plot is obtained by plotting the inverse of substrate concentration against the inverse of the initial velocity. Analysis of the Lineweaver-Burk plot of rat brain AChE revealed that maximum velocity attained under the experimental conditions was 112.35 ± 3.5 nmol/min/mg protein (V_{max}).

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Although acetylcholinesterase enzyme is widely distributed across the animal kingdom and performs the conserved action of hydrolysis of ACh, the affinity of AChE for the substrate varies from species to species. The work of Filho et al., (2004) demonstrates considerable variations in K_m values of brain AChEs among different neotropical fishes. Analysis of Kinetic parameters of insect and mammalian AChE revealed 3 fold greater affinity of mammalian AChE for ATCI than insect AChE (Ali et al., 2003). The present work also reports a similar trend with mammalian AChE rat brain showing greater affinity for substrate than an invertebrate *C. elegans* AChE.

A characteristic of true AChE activity is the inhibition of the enzyme activity at high substrate concentrations (Silver. 1974). Of the two AChEs tested in the present study, the rat brain AChE exhibited inhibition at lower concentration of ATCI (2mM) than the *C. elegans* AChE (5mM). Our findings of inhibition of rat brain AChE by substrate are in close agreement to the study of Mortensen et al., (1998) who reported substrate inhibition of rat brain AChE of adult rats at ATCI concentration of 2.5mM. Survey of the relevant literature provides information suggesting that cholinesterases from nematodes are inhibited by the substrate relatively higher substrate concentrations. Inhibitory substrate concentrations reported previously are 10mM for *N. brasiliensis* (Sanderson et al., 1967), 10mM for *M. apri* (Reiner et al., 1978), and 20mM for *S. dentatus* (Rhodes, 1981). Our results of inhibition of *C. elegans* AChE at concentrations above 5 mM in comparison that of rat brain at 2mM are in agreement with these findings.

In laboratory assessments, IC_{50} values (Inhibitor concentration required to inhibit enzyme activity by 50%) serve as a tool to understand the relative sensitivity of an enzyme to the inhibitor. In the present study, regression analysis of percent inhibition of both AChEs by DDVP revealed 45.7± 2.68 and 107.18 as IC_{50} for *C. elegans* and rat brain AChE, respectively. Interestingly, *C. elegans* AChE was 2.3 fold times more sensitive inhibition to inhibitory action of dichlorvos as determined by the compound selectivity (Compound selectivity

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= rat brain AChE [IC_{50}] / *C. elegans* AChE [IC_{50}] (Ali et al., 2005). As is the case of variations in the toxicities of an OPI and varies from species, the relative sensitivities of AChE, from different species and different organs from the same organism differ. Extensive account of such variations could be obtained from the literature (Murphy et al., 1968; Anderson et al., 1972, 1977, 1978; Singh., 1985).

Previous reports (Benke and Murphy., 1976) suggest that binding of anti-cholinesterases to non-critical proteins is an important way of achieving detoxification *in vivo*. This might partly explain the differential sensitivities of different AChEs to inhibitors. However, work of Wang and Murphy (1982) and Singh (1985) suggest that the differential sensitivities of AChEs cannot be sufficiently explained on the basis of binding of inhibitor to non-specific proteins. This has lead scientists to pursue studies to understand the role of kinetic parameters in different species and there differential sensitivities. Differences in the affinity of the inhibitor for enzymes active site and rates of phosphorylation provide most satisfactory explanation (Wang and Murphy., 1982; Singh, 1985) for differences in sensitivities of different AChEs to inhibitors. It's important to note that the K_m of *C. elegans* was much higher (lower affinity for substrate) than rat brain AChE. This finding is on the same lines with the report of Filho et al., (2004) whose work demonstrated that the neotropical fish brain AChE with higher K_m values were more sensitive to methyl parathion inhibition *in vitro*. However, differences in the sensitivities of *C. elegans* and rat brain AChE cannot be explained merely on the basis of K_m values and requires further studies to understand the basis of relative differences in the sensitivity. The double reciprocal Lineweaver - Burk plot provides information necessary for identifying the mechanism of enzyme inhibition. The double reciprocal groups for inhibited AChEs of both *C. elegans* homogenate and rat brain homogenate system revealed similar pattern of inhibition by dichlorvos characterized by concentration related decrease in V_{max} and unaltered K_m values; these changes are hall mark of irreversible inhibition.

7.0 SUMMARY

1. The kinetics of AChE in the presence of acetylthiocholine iodide exhibited the substrate saturation in both the systems.
2. Rat brain homogenate AChE showed a typical saturation at 2mM of the substrate ATCI and in *C. elegans* AChE saturation was obtained above 5 mM of ATCI.
3. Interestingly, kinetic parameters for the ATCI substrate obtained from Lineweaver-Burk (LB) plot K_m for rat AChE as 59μM and 725.41μM for *C. elegans* AChE. This showed that affinity of rat brain AChE to ATCI was 12.6 folds higher than that of *C. elegans* AChE.
4. The efficiency of substrate being hydrolyzed by AChE, as indicated by V_{max} values, were 112.35 nmol/min/mg protein and 33-nmol/min/mg protein for rat brain and *C. elegans* AChE respectively.
5. The IC_{50} for *C. elegans* and rat brain AChE were 45.7 ± 2.68 nM and 107.18 ± 3.56nM respectively.
6. The K_m, V_{max} and V_{max}/K_m values of the inhibited AChE of rat brain at IC_{50} concentration of dichlorvos were 57.48μM, 42.55nmol of ATCI hydrolyzed/min/mg protein, 0.74 min/mg/protein.
7. For *C. elegans* K_m, V_{max} and V_{max}/K_m at IC_{50} were 734μM, 10.10 nmol of ATCI hydrolyzed/min/mg protein and 0.013 min/mg protein respectively.
8. Lineweaver - Burk plot revealed the inhibition of AChE in both the system to be of irreversible in nature.

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SECTION IV B

Kinetic studies on carboxylesterase of *C. elegans* exposed *in vitro* to dichlorvos

1.0 INTRODUCTION

Organophosphorus insecticides (OPI) exert their main toxicological effects through non-reversible phosphorylation of esterases in the central and peripheral nervous system (Aldridge and Reiner, 1972). AChE is a key enzyme that terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine in the nervous system. Inhibition of this enzyme causes over- stimulation of nicotinic and muscarinic acetylcholine receptors. The acute toxic effects of OPI are related to the inhibition of acetylcholinesterase (AChE) (WHO, 1986).

Several types of enzymes are involved in the detoxification of OPI of which the most important are the esterases. Carboxylesterases (CaE) are a class of esterases that hydrolyze ester-containing compounds to the corresponding alcohol and acid (hydrolysis products) (Satoh and Hosokawa, 1998; Wheelock et al., 2005). These enzymes are important in the metabolism and subsequent detoxification of OPI. CaE are considered to be involved in detoxification of OP compound via scavenging mechanism and this has been considered as an efficient way of detoxification (Prabhakaran and Kamble, 1996). The work of Wheelock et al., (2005) showed that carboxylesterases are more sensitive to inhibition by OPI than AChE.

Dichlorvos exerts its toxic effects by inhibiting esterases, especially AChE. In addition, production of different forms of carboxylesterases is also reported to be the cause of dichlorvos toxicity and resistance in several insect species (Wang et al., 2004). Our earlier studies (Chapter I, II, III and IV A) have clearly demonstrated the impact of dichlorvos on AChE in *C. elegans*. Further, as described in our earlier studies (Chapter IIC), we have observed *in vivo* inhibition of carboxylesterase in *C. elegans* following exposure to sub lethal

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concentrations of dichlorvos. Hence it was of interest to investigate employing *C. elegans* homogenate system was carried out to determine basic kinetic parameters of *C. elegans* carboxylesterase and establish nature of inhibition by dichlorvos.

2.0 MATERIALS AND METHODS

2.1 Chemicals

BSA (fraction V) was procured from Sigma Chemical Co., (St. Louis, MO, USA). p-nitrophenylacetate (PNPA) was procured from Sisco Research Lab., (Mumbai, India). Dichlorvos (Technical grade; 97.6%) was a gift from Hyderabad Chemical Supplies Ltd., (Hyderabad, India). All other reagents used were of analytical grade.

2.2 Pesticide solution

Stock solution (5mM) of Dichlorvos (DDVP) was prepared in double distilled water and further diluted to obtain working solutions for *in vitro* assays.

2.3 Worm culture and maintenance (Brenner, 1974)

The wild-type *Caenorhabditis elegans* Bristol strain N2 was obtained from Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA) which is funded by the NIH National Center for Research Resources (NCRR). The worms were cultured and maintained as described in Chapter IA (*Section 2.3.1 – 2.3.3*). Adult worms (3.5 d old) were used for the studies.

3.0 Preparation of *C. elegans* homogenate

Worms (gravid stage/ 3.5d old) were harvested and homogenized in 50mM Tris-HCl, pH 7.4. Homogenates were centrifuged at 10,000 rpm for 10 min and supernatant was stored at –20°C until the assay.

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4.0 EXPERIMENTAL PROCEDURE

4.1 Substrate saturation

Homogenate of *Caenorhabditis elegans* was used as enzyme source to determine kinetic parameters for carboxylesterase (CaE) activity. Carboxylesterase was assayed with the substrate, p-nitrophenylacetate (PNPA) by the method of Sidhu and Blair (1975). Enzyme sample (50µl) was added to 3 ml of Tris-HCI buffer (50mM pH-7.4) and preincubated for 10 min at 30ºC in a water bath. K_m and V_{max} for the substrate PNPA were determined by assaying the activity with increasing substrate concentration (2.5 - 500µM disolved in acetone). The reaction mixture was incubated for 30 minutes at 30°C. The absorbance of the reaction product p-nitrophenol was measured at 405 nm against a reagent blank containing PNPA and buffer in a UV-VIS spectrophotometer (Shimadzu). Enzyme activity (CaE-PNPA) was calculated using the extinction co-efficient for p-nitrophenol (€405 = 1.83 X 10^4 M^{-l} cm^{-l}). The rate (V) was plotted against the substrate concentrations by the inverse plot of Lineweaver and Burk, (1934) to obtain Michaelis constant (K_m), maximum velocity (V_{max}) and catalytic efficiency (V_{max}/K_m).

4.2 Determination of IC_{50}

Sensitivity of the *Caenorhabditis elegans* carboxylesterase (CaE) to dichlorvos was studied *in vitro*. The enzyme (50µl) in 50 mM Tris-HCI buffer (pH.7.4) was incubated with different concentrations of inhibitor dichlorvos ranging from 10 - 200nM at 30°C for 30min. The residual CaE activity was assayed with increasing substrate concentration (2.5 - 500µM dissolved in acetone), as described earlier. The median inhibitory concentration IC_{50} was determined by linear regression of the inhibition curve (Finney, 1997).

4.3 Kinetic properties of carboxyl esterase (CaE) by dichlorvos

The nature of inhibition of the CaE activity *in vitro* by dichlorvos was studied. Enzyme source was incubated with IC_{25}, IC_{50} and IC_{75} concentrations

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of dichlorvos in 3.0 ml of Tris-HCl buffer for 30 min at 30ºC. The residual activity for each of the inhibitor concentration was determined with increasing substrate (PNPA) concentrations. The K_m, V_{max} and nature of inhibition was determined from the Lineweaver-Burk plot (1/V against 1/S).

4.4 Protein estimation (Lowry et al., 1951)

10 µl of *C. elegans* homogenate was added to 490 µl of distilled water and incubated with 2.5ml alkaline copper sulfate solution for 10 min at room temperature 250 µl of Folin's reagent (1:1 diluted with water) was added and the color was read after 30min at 670nm. Bovine serum albumin (BSA) was used as the standard.

4.5 Interpretation of kinetic parameters

Michaelis constant (K_m), maximum velocity (V_{max}) and catalytic efficiency (V_{max}/K_m) were determined from Lineweaver-Burk plot by plotting reaction velocity (1/V) versus 1/S.

5.0 RESULTS

5.1 Kinetic studies: K_m, V_{max} and substrate saturation

The carboxylesterase (CaE) from *C. elegans* hydrolyzed substrate p-nitrophenyl acetate. The results showed that the rate of hydrolysis of p-nitrophenyl acetate by the CaE was inhibited at higher concentration suggesting substrate saturation (350µM). Lineweaver-Burk plots obtained for the enzymatic hydrolysis of PNPA with a K_m value of 20.95µM, V_{max} of 4.62 nmol/min/mg protein and substrate saturation (350µM) (**Figure 4.5 and 4.6**).

5.2 Determination of IC_{50}

The concentration of dichlorvos required to inhibit the CaE activity by 50% at room temperature in 30 min. The IC_{50} for carboxylesterase of *C .elegans* homogenate (**Figure 4.7**) IC_{50} obtained was 81.16nM at excess K_m concentration of the substrate, p-nitrophenyl acetate.

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5.3 Kinetics of enzyme inhibition

As estimated from LB plot, the K_m, V_{max} and V_{max}/K_m values of the inhibited CaE were 19.57µM, 2.88 nmol/min/mg protein and 0.14min/mg/protein respectively **(Figure 4.8,Table 4.4)** at IC_{50} concentration of dichlorvos. Inverse of Line-weaver Burk plot revealed that the inhibition of CaE in *Caenorhabditis elegans is* irreversible in nature in the presence of dichlorvos.

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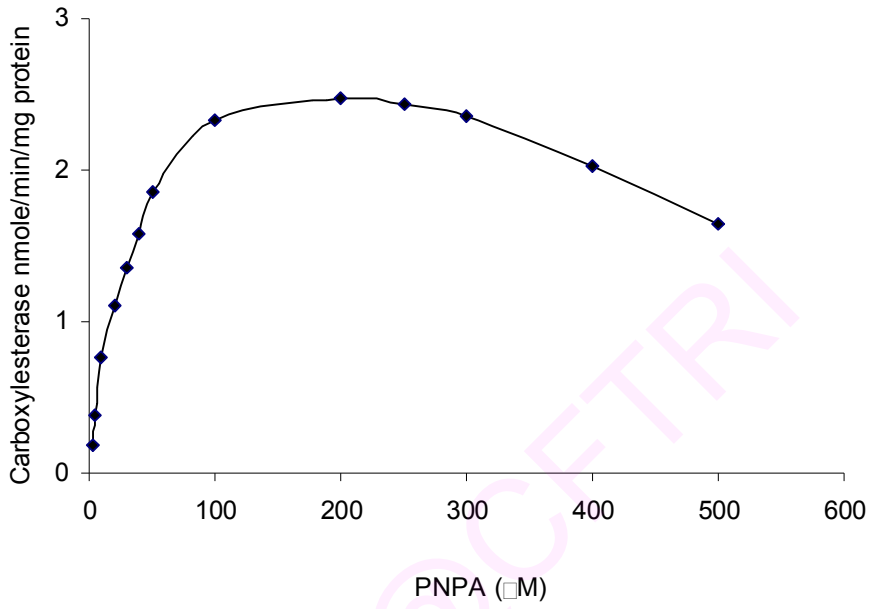

Figure 4.5 Effect of substrate (PNPA) concentrations on carboxylesterase activity in *C. elegans*.

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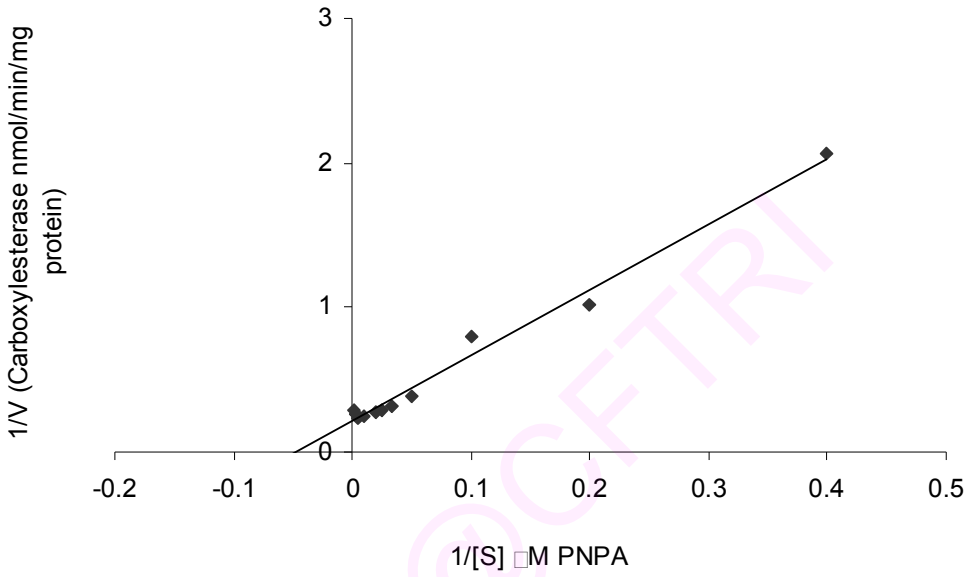

Figure 4.6 Lineweaver-Burk plot for carboxylesterase activity in *C. elegans*.

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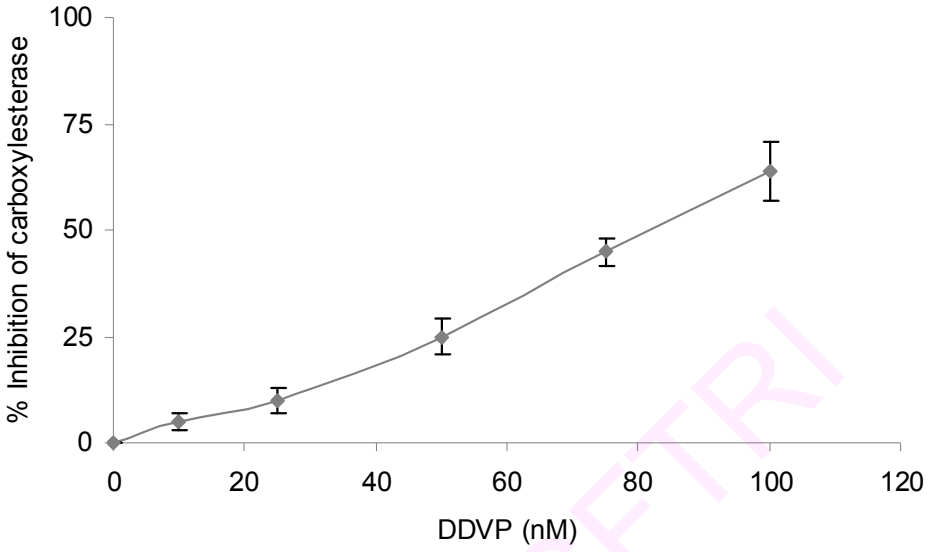

Figure 4.7 Inhibition pattern of carboxylesterase by dichlorvos (DDVP) in *C. elegans* homogenate.

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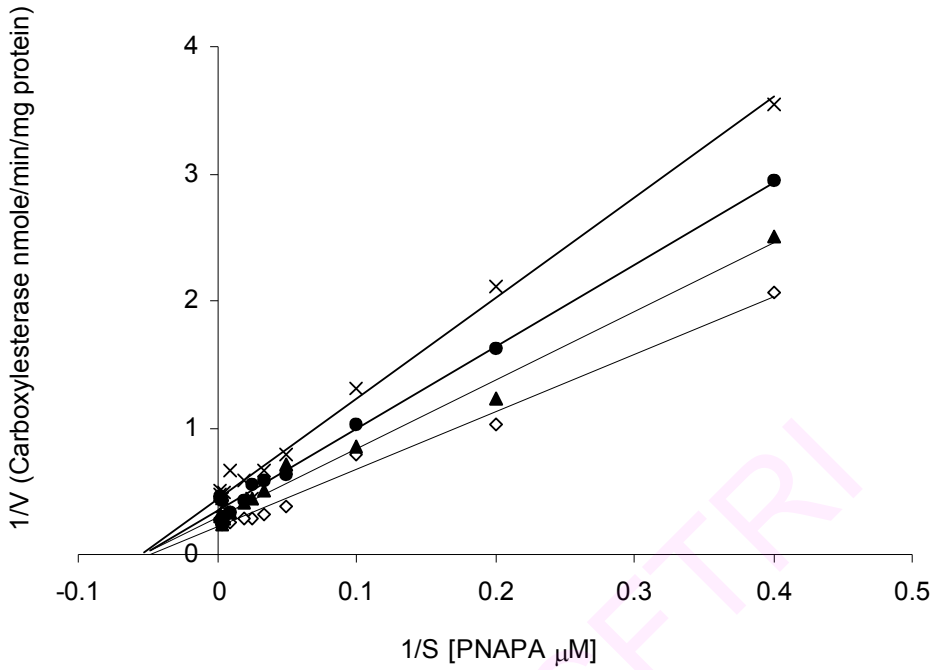

Figure 4.8 Inhibition of *C. elegans* CaE activity with IC_{25}, IC_{50} and IC_{75} concentrations of dichlorvos.
Plot of reciprocal of initial velocities versus reciprocal concentrations of ATCI.

Table 4.4 Kinetic charecteristics of *C. elegans* CaE in the presence of dichlorvos

Dichlorvos (nM)	K_m (μM)	V_{max} (nmol/ min/ mg protein)	V_{max}/K_m
IC_{25}	18.69	3.45	0.18
IC_{50}	19.57	2.88	0.14
IC_{75}	17.58	2.28	0.12

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6.0 DISCUSSION

Carboxylesterase from *C. elegans* homogenate system hydrolyzed the substrate (PNPA) in a typical Michaelis-Menten pattern with the rate of hydrolysis being directly proportional to the substrate concentrations in the initial phase followed by attainment of zero order kinetics with respect to substrate concentrations. Interestingly, higher concentrations of PNPA (>350 μM) inhibited the enzyme activity. The double reciprocal Lineweaver-Burk plot revealed K_m of the enzyme for substrate hydrolysis as 20.95 μM while the maximum velocity attained (V_{max}) was 4.62nmol/min/mg protein.

Dichlorvos inhibited the enzyme activity in vitro in a concentration dependent manner with regression analysis of revealing IC_{50} of dichlorvos against Carboxylesterase activity as 81.16nm. The double reciprocal Lineweaver-Burk plot revealed similar pattern of Carboxylesterase inhibition by dichlorvos as compared to inhibition of AChE reported in the previous chapter. In vitro exposure of *C. elegans* homogenate system to different concentrations (IC_{25}, IC_{50} and IC_{75}) of dichlorvos resulted in alterations in enzymes' kinetic parameters typical of irreversible inhibition. The V_{max} of inhibited enzyme progressively decreases while K_m values remain unchanged. As a consequence the catalytic efficiency (V_{max}/K_m) decreased with increasing of dichlorvos.

Despite the relevance of carboxylesterase in toxicity of OPI, it is surprising that the enzyme as a target for inhibition been received less attention. Carboxylesterase, particularly the B-esterses, are known to be inhibited by OP compounds, a property of the enzyme, which has given the status of detoxification mechanism to the enzyme with regard to OPI toxicity. Chlorpyrifos has been shown to inhibit carboxylesterase in Chinook Salmon at concentrations that failed to inhibit AChE. This nature of greater affinity of the enzyme for OP compound has earned them the reputation of "sink" for OP compounds, which has been proposed as a protective mechanism for OP

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toxicity (Maxwell, 1992). In mammals (especially in serum and liver), there is a pool of B-esterases, with no known functions. These have been considered as enzymes involved in detoxification of OPI and Carbamates, since each molecule of these enzymes can scavenge minimum of one insecticide molecule before it reaches its target in nervous system (Sogorb and Vin, 2002).

The worm *Caenorhabditis elegans*, in natural conditions is inhabitant of soil ecosystem, particularly where moist microenvironments exist (Boyd and Williams, 2003). These microenvironments are at high risk of contamination with the run- off agrochemicals particularly insecticides. The present work clearly demonstrates that dichlorvos inhibit carboxylesterase of *C. elegans* by irreversible mechanisms. Although sensitivity of CaE to dichlorvos is much lower than that of AChE in *C. elegans* (as reflected by IC_{50} values), it is extremely important to note that carboxylesterase still operates as a target for OPI in *C. elegans*. Presence of such target machinery could serve as a detoxification mechanism that may protect the worms from OPI toxicity.

7.0 SUMMARY

1. CaE from *C. elegans* was inhibited at higher concentration suggesting substrate (PNPA) saturation (350µM).
2. LB plot obtained for the enzymatic hydrolysis of PNPA with a K_m value of 20.95µM, V_{max} of 4.62nmol/min/mg protein and substrate saturation (350µM).
3. The IC_{50} for CaE was 81.16nM at excess K_m concentration PNPA.
4. LB plot revealed the K_m, V_{max} and V_{max}/K_m values of the inhibited CaE as: 19.57µM, 2.88 nmol/min/mg protein and 0.14min/mg/protein respectively at IC_{50} concentration of dichlorvos.
5. Inverse of LB plot revealed that the inhibition of CaE in *C. elegans* is irreversible in nature in the presence of dichlorvos.

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CONCLUSIONS

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CONCLUSIONS

1. Initially, three OPI (randomly selected viz., dichlorvos, dimethoate and monocrotophos) were screened for assessment of their toxicity in *C. elegans* (N2) in terms of 4h-LC_{50}. ***Of the three OPI tested, dichlorvos was relatively the most toxic*** (LC_{50}: 16.0 mM) followed by dimethoate (LC_{50}:18.2 mM) and monocrotophos (LC_{50} : 69.0 mM).
2. The 4h-LC_{50} values of dimethoate and monocrotophos in the transgenic worms were 17.4 mM and 66.6 mM respectively. The LC_{50} values of dichlorvos for the four exposure periods, 4, 24, 36 and 72h were: 17.00, 2.20, 1.04 and 0.70 mM respectively. ***Dichlorvos was equitoxic in both the strains.***
3. Equimolar concentrations (5, 40 and 80 µM) of the three OPI were employed for heat shock protein induction studies in transgenic worms (PC-72). ***β-galactosidase staining was elicited only in the pharyngeal region of the worms by the three OPI*** similar to $CdCl_2$ (positive stressor). Hsp induction was significantly high and concentration-dependant on exposure to the three OPI. ***The order of induction was: monocrotophos (6.4 fold) > dichlorvos (4 fold) > dimethoate (1.6 fold). Linear regression analysis plots showed a good correlation between feeding inhibition and AChE inhibition and between Hsp induction and AChE inhibition in dichlorvos treated worms.***
4. ***Dichlorvos at sublethal concentrations induced cessation in feeding and paralysis in C. elegans (N2).*** These worms also hyper contracted their nose muscles in a time- and dose-dependent manner. Worms exposed to the highest concentration (200µM) of dichlorvos showed marked decrease in egg laying. The treated worms exhibited significant reduction in the AChE activity and increase in ACh content.

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5. Worms exposed to both dichlorvos ± atropine showed inhibition in feeding. Worms exposed for 4h to dichlorvos ± atropine and then washed and exposed to 2-PAM for 90 min revealed varying degrees of recovery in feeding. Worms exposed to dichlorvos + atropine showed a concentration related decrease in the extent of paralysis. Worms treated with dichlorvos + atropine followed by treatment with 2-PAM showed 100% recovery from AChE inhibition. ***These results clearly demonstrated that co-treatment of dichlorvos with atropine and further post-treatment with 2-PAM completely protected C. elegans from dichlorvos-induced toxicity.***
6. ***Exposure to dichlorvos at sublethal concentrations significantly decreased the brood size and life span, increased reactive oxygen species and altered antioxidant status in the worms.***
7. ***Exposure of eggs to dichlorvos in agar (100 µM) resulted in drastic reduction in the growth of the worms. Significant differences in fatty acid composition were evident in dichlorvos exposed worms.*** Qualitative analysis of the fatty acid methyl esters derived from lipids of the worms showed that the control and dichlorvos treated worms contained several identical fatty acid components. However, the relative quantities of the individual acids varied. Dopamine content was markedly increased in the treated worms.
10. In untreated worms, the AChE activity increased up to 5d and then decreased after 10 d. ***However in worms exposed to 200nM dichlorvos, the AChE activity decreased after 5d. Maximum inhibition of AChE activity due to exposure to dichlorvos was evident on day 10. An increase in carboxylesterase activity was evident in untreated worms up to day 5, beyond which the enzyme activity decreased drastically.*** Interestingly, in worms exposed to dichlorvos, the enzyme activity was significantly higher than control worms on day1.

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11. Age-dependent increase in lipofuscin content was observed in the control worms. ***Interestingly, remarkable concentration and age dependent increase in lipofuscin accumulation was observed on exposure to dichlorvos.*** While lipofuscin accumulation in control worms was restricted to intestine only, exposure to dichlorvos resulted in significant fluorescence throughout body of the worm.
12. Comparative studies on the AChE kinetics in rat brain homogenate (RBH) and *C. elegans* homogenate (CEH) revealed substrate saturation in both the systems in the presence of acetylthiocholine. Affinity of rat brain AChE to ATCI was 12.6 fold higher than that of *C. elegans* AChE. ***AChE of C. elegans appears to be more sensitive to inhibition by dichlorvos than rat brain AChE. Lineweaver-Burk plot revealed the inhibition of AChE in both the system to be of irreversible in nature.***
13. The carboxylesterase (CaE) from *C. elegans* hydrolyzed substrate p-nitrophenyl acetate. The IC_{50} for carboxylesterase of *C. elegans* homogenate was 81.16nM at excess K_m concentration of the substrate, p-nitrophenyl acetate. ***Inverse of LB plot revealed that the inhibition of CaE in C. elegans is irreversible in nature in the presence of dichlorvos.***
14. ***In conclusion, data obtained from the present series of investigations support our premise that the nematode, Caenorhabditis elegans is a good model organism for studying the neurotoxicity of organophosphorus insecticides. This organism offers several advantages such as simple and easily measurable physiological and biochemical endpoints for studying the mechanistic toxicity of OPI.***

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