1992

Caenorhabditis elegans as a screening organism for naturally occurring neurotoxins in foods

Marilyn Recupero
San Jose State University

Order Number 1348715

Caenorhabditis elegans as a screening organism for naturally occurring neurotoxins in foods

Recupero, Marilyn Annette, M.S.

San Jose State University, 1992

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

CAENORHABDITIS ELEGANS AS A SCREENING ORGANISM FOR NATURALLY OCCURRING NEUROTOXINS IN FOODS

A Thesis

Presented to

The Faculty of the Department of Nutrition and Food Science

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in Nutritional Science

by

Marilyn Recupero

May, 1992

APPROVED FOR THE DEPARTMENT OF
NUTRITION AND FOOD SCIENCE

Dr. Nancy C. Lu (Committee Chair)

Dr. Panfilo Belo

Dr. Miriam Saltmarch

APPROVED FOR THE UNIVERSITY

Abstract

CAENORHABDITIS ELEGANS AS A SCREENING ORGANISM FOR NATURALLY OCCURRING NEUROTOXINS IN FOODS
by Marilyn Recupero

Acute toxicity of naturally occurring food toxins was determined using Caenorhabditis elegans (C. elegans). Five neurotoxins were tested: dihydroxyphenylalanine (DOPA), diaminobutyric acid (DABA), kainic acid (KA), N-methyl-D-aspartic acid (MAA) and quinolinic acid (QA). For range finding, nematodes (10,000/ml) were exposed to each toxin in 1 ml potassium phosphate buffer (pH 5.8) at concentrations of 0.0, 0.016, 0.08, 0.40, 2.0, 10 and 50 mg/ml. Cultures were incubated at 20°C on a tissue culture rotator at 1 rpm for 24 hours. LC_{50} values, extrapolated from dose-response curves, were 1.4, 2.5, 2.8, 3.0 and 8.1 mg/ml for DOPA, DABA, KA, MAA and QA, respectively. In the refined testing, corresponding LC_{50} values were determined to be 1.4, 2.4, 2.7, 2.9 and 7.6 mg/ml. Based on the range finding and refined testing results, the inhibitory neurotransmitter DOPA exhibits greater acute toxicity than the excitatory neurotransmitters DABA, KA, MAA and QA.

ACKNOWLEDGEMENTS

The author appreciatively acknowledges the many people who made significant contributions to the realization of this endeavor: Dr. Nancy Lu, Advisor and Committee Chair, for her discernment, guidance and personal interest; Dr. Panfilo Belo for his assistance with clarity and attention to safety; Dr. Miriam Saltmarch for her incisive perspective and willingness to serve on the committee; Irene Tupper, for her time and skill; Andrew Recupero for his inspiration to embark upon this adventure, unwavering support and his invaluable direction in producing this document; Jennifer and Jeffrey Williams for their affection, encouragement and enthusiasm for my progress.

PREFACE

The following is a publication style thesis. The second chapter is written in journal format according to January, 1988 guidelines and will be submitted to Food and Chemical Toxicology. Chapters 1 and 3 are written according to guidelines outlined in the Publication Manual of the American Psychological Association (3rd. edition), 1986.

Table of Contents

List of Tables

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Historically, toxicological studies have relied on laboratory animals such as mice, rats, guinea pigs and dogs. These mammals require long test cycles and expensive maintenance (Ayres & Kirschman, 1981). More recently, the heightened controversy regarding cruelty to animals has suggested the need for an alternative test organism (Holden, 1986).

Caenorhabditis elegans (C. elegans), a microscopic, hermaphroditic nematode, is cultivated in axenic (germ-free) conditions in a chemically defined medium containing 54 constituents (Lu, et al., 1978). This organism provides the advantages of genetic uniformity and rapid proliferation. A maximum population of approximately 100,000 nematodes/ml can be reached in this chemically defined medium. Therefore, C. elegans has strong potential as an expeditious and inexpensive testing organism.

The objective of this study was to investigate the acute toxicity of the free-living nematode, C. elegans, to purified water-soluble neurotoxins found in food. The results of this study can be used to determine the efficacy of utilizing this organism for further testing of a variety of food related neurotoxins.

Review of Literature

Naturally Occurring Neutotoxins

Significance

Naturally occurring neurotoxins in foods may be related to chronic human illnesses or may contribute to acute episodes of physical distress (Watson, 1987). As localized food shortages increase throughout the world, so will human dependence upon plant protein, particularly oilseeds and legumes which contain 20-50% protein. These plants contain chemical compounds which are toxic to humans and can cause discomfort, paralysis and death (Padmanaban, 1980). A key example is the Lathyrus plant species which includes unusual excitatory amino acids such as b-N-oxalyl-L-a,b-diaminopropionic acid (ODAP) (De Wolff, 1985). The legumes of this species comprise up to 30% of the diet in India and Algeria (Padmanaban, 1980). The excitatory amino acids have been strongly associated with a high incidence of human neurolathyrism, a disease characterized by muscular rigidity, weakness, paralysis and death (Padmanaban, 1980).

Mechanism of Toxicity

Excitatory toxins disrupt normal function of neuromuscular systems by increasing the influx of cations into postsynaptic neurons, thereby lowering membrane potential and increasing nerve impulses. In both mammalian and nematode neuromuscular systems, excess stimulation of

excitatory dendrosomal receptors results in prolonged and persistent flow of acetylcholine across the synapse and induces continuous muscle contraction manifested by spastic movements and eventual paralysis (McGeer & McGeer, 1985; Olney, 1980). When the muscles of gas exchange are involved, suffocation and death may ensue (Croll & Matthews, 1977). In the brain, glutamate carries out 75% of excitatory transmission. Excess stimulation of the glutamate receptors causes neural necrosis. In contrast, inhibitory neurotransmitters induce an efflux of cations and an influx of anions into the postsynaptic neuron, creating a hyperpolarized membrane and reducng neural transmission.

Selected Toxins

The five neurotoxins selected for the present study have been detected in a variety of plant foods. Four are classified as excitatory toxins (DABA, KA, MAA, QA) and the fifth (DOPA) is classified as an inhibitory neurotransmitter. Vicia faba pods contain up to 2.5 mg/g DOPA (Pusztai, 1982). Pusztai (1982) documented episodes of nausea and vomiting following ingestion by humans. Diaminobutyric acid (DABA), a Lathyrogen, is found in the chickpea and has been associated with paralytic episodes in humans (Padmanaban, 1980). Seeds of Lathyrus latifolius and Lathyrus silvestris consist of 5.0-6.7 mg/g and 14.0-27.0 mg/g DABA, respectively (Ressler et al. 1961; Pusztai, 1982).

Kainic acid (KA) and N-methyl-D-aspartic acid (MAA) activate glutamate receptors during neural transmission (Kizer et al., 1978). Kainic acid, a glutamate analog, is isolated from the Japanese seaweed, Digenea simplex. It causes seizures and brain damage and is 500 times more potent than glutamate (Kizer et al., 1988). N-methyl-D-aspartic acid, a glutamate analog, is a constituent of legumes. It has 100 times the potency of glutamate. Toxicity results in cellular swelling and subsequent neurodegenerative effects on the central nervous system of rats (Menendez et al., 1989). Quinolinic acid (QA) is a tryptophan metabolite found in plant protein foods (McGeer & McGeer, 1985). Toxicity in rats has been shown to cause loss of cholinesterase activity leading to reduction of tremor threshold (Engber & Chase, 1988; Wardley-Smith et al., 1989).

Table A furnishes representative mammalian toxicity data for the aforementioned neurotoxins. Consistent acute toxicity data is limited to intraperitoneal (ip). Lower lethal dosesl (LD_{50}) and convulsive doses (CD_{50}) to 50% of the population are expected for ip administration than for oral dosage (Engstrom & Woodbury, 1988; Litchfield & Wilcoxon, 1949; O'Neal et al., 1968; Wardley-Smith et al., 1989). Intraperitoneal injection of these neurotoxins is not the normal route of ingestion for rats, mice or nematodes. Nematode intake more closely simulates oral

dietary intake due to the presumed impermeability of the outer cuticle. A second limitation of comparison is that the CD_{50} for an organism is assumed to be less than the LD_{50} value because convulsive behavior precedes death.

Table A: Effects of Selected Neurotoxins on Rats and Mice

Toxin	Test Animal	Route	Acute Toxicity mg/kg	Source
QA	rat	ip	349 (LD_{50})	Wardley-Smith et al., 1989
MAA	mouse	ip	350 (CD_{50})	Engstrom and Woodbury, 1988
KA	mouse	ip	80 (CD_{50})	Engstrom and Woodbury, 1988
DABA	rat	ip	840 (CD_{50})	O'Neal et al., 1968
DOPA	mouse	ip	1219 (LD_{50})	Litchfield and Wilcoxon, 1949

LD_{50} = Lethal dose to 50% of the population
CD_{50} = Convulsive dose to 50% of the population
ip = intraperitoneal injection

Nematoda

Physiology

Caenorhabditis elegans belongs to the roundworm phyla pseudocoelomata. It contains a mouth, pharynx, intestine

and reproductive organs. C. elegans are either male or hermaphroditic, producing up to 2000 ova or sperm cells during the life cycle. Over 10,000 types of free-living nematodes have been identified. C. elegans, a microscopic soil dwelling nematode, depends on yeast, fungi, algae and bacteria as its food source. The cylindrical invertebrate is covered by a flexible nonliving, non-cellular collagen cuticle. Movement is accomplished by muscular compression of the cuticle coupled with hydrostatic pressure within the pseudocoele. The sinusoidal movement is achieved by alternating contraction and relaxation of longitudinal muscle blocks (Croll & Matthews, 1977). Each muscle cell consists of three parts: a contractile body attached to the outer cuticle, a nucleated cell body and a muscle arm process extending to the longitudinal portion of the nerve cord to form the synaptic connection.

The central brain mass is formed by a ring of nerve tissue at the pharyngeal region and two longitudinal nerve cords which control somatic muscle by means of catacholaminergic neurons (Willett, 1980). Stimuli are received from anterior sense organs. All 302 neurons have been mapped in detail with 118 cell types identified (Chalfie, 1984; Kenyon, 1988).

Culture

Caenorhabditis elegans can be cultivated in axenic (germ-free) conditions at 20^0 C. The stock medium contains

soy, yeast extract and heated liver extract and presumably supplies amino acid, sterol, mineral, vitamin, heme and carbohydrate requirements (Tomlinson & Rothstein, 1962). The hermaphrodites clone genetically uniform populations within 3-5 days. Initial inoculation of 500 nematodes/ml medium provides a population of 100,000/ml medium within 15 days when test tubes rotate at 1 rpm to facilitate gas exchange. Growth curves indicate a rapid decline in proliferation at 3 weeks due to accumulation of ammonia and decrease of available oxygen.

Efficacy as Animal Model

Utilization of C. elegans in axenic conditions as an animal model has proven to be useful in studies of nutrition, developmental biology and gerontology. Requiring 10% of the cost of laboratory animal experimentation, testing with C. elegans has provided large, genetically uniform populations within a short time and in a small space (Williams & Dusenbery, 1988). Mechanisms controlling the cellular development of C. elegans have been observed throughout the animal kingdom. Molecules responsible for C. elegans development have been shown to be homologous to growth factor precursors and cell surface receptors in vertebrates (Kenyon, 1988). C. elegans utilizes the same neurotransmitters as humans (e.g. serotonin, dopamine, acetylcholine and octopamine) (Horvitz et al., 1982; Kenyon, 1988; Willett, 1980; Wright & Awan, 1976).

Nutrition. Nutritional requirements of C. elegans are analogous to those of the human. Energy requirements are met by carbohydrates which are believed to be oxidized to carbon dioxide and water by the Embden-Meyerhof pathway, TCA cycle and Electron Transport System sequence (Lu & Goetsch, 1987; Chang & Lu, 1990). Lu and Goetsch (1987) confirmed the preference for utilization of glucose and glycogen over sucrose and fructose in the nematode. Protein requirements parallel those of humans. Van Fleteren (1973) reported the 10 essential amino acids in human metabolism to be essential for the nematode. Hieb and Rothstein (1968) and Hieb et al. (1970) established the sterol and heme requirements for C. elegans. Cytochrome c, with its heme moiety, has been associated with significant population growth. Sterol has been shown to be essential for reproduction. Nicholas et al. (1962) reported the need for the vitamins thiamin, riboflavin, folic acid, niacinamide, pantothenic acid and pyridoxine. Lu et al. (1976) further demonstrated specific metabolic requirements for vitamin B_{12} and folic acid. Sun et al. (1986) completed a determination of B_6 metabolites. Studies of mineral requirements have demonstrated that C. elegans require potassium, magnesium, manganese, sodium, calcium and copper (Lu et al., 1983). A combination of Caenorhabditis briggsae Maintenance Medium (CbMM) and other defined compounds permits the axenic study

of C. elegans in a chemically defined medium (Lu et al., 1978).

Developmental Biology. In the field of developmental biology, C. elegans has been a model for elucidation of the mechanism of genetic control of developmental sequence and cell layout. All cell divisions, migrations and deaths have been traced (Sulston & Brenner, 1964). Because the nematode contains many of the same complex cells as higher animals, its study has led to the understanding of cell differentiation. The pathway of a single gene action at the cellular level has been traced. Transparency of the organism has permitted observation of cell division and cell lineages.

Gerontology. Studies of cellular ageing have been enhanced by use of C. elegans as an animal model. It has a lifespan of approximately 30 days and has no capacity for cellular repair or renewal. Therefore, changes in motility, age pigment accumulation and cellular organization which accompany senescence have been successfully observed in this nematode (Johnson, 1983; Kisiel & Zuckerman, 1978). Hodgkin (1991) reported that the identification of a new gene family in C. elegans may contribute to use of the organism for rapid testing of drugs related to degeneration of the human nervous system.

Toxicology. Few toxicological studies have been performed using C. elegans. Completed studies have reported

measureable neurotoxic damage in C. elegans including reduced size, diminished reproductive ability, decreased proportion of larvae reaching adult stage, inhibition of enzyme action and mutagenesis (Kampfe et al., 1986; Kitts & Lu, 1987; Morgan & Cascorbi, 1985; Ohba & Ishibashi, 1984; Popham & Webster, 1979; Samoiloff, 1980; Williams & Dusenbery, 1988; Wong, 1986). Because there is knowledge of postembryonic cellular development, and the effects of known inhibitors on these developmental events, researchers have used C. elegans to detect toxic effects of environmental agents such as benzene and methyl mercury (Samoiloff, 1980). These studies point to a variety of possible emphases and to the practicality and applicability of utilizing this organism for toxicity testing.

To build a convincing argument for the use of C. elegans as a prescreening organism for substances which may be neurotoxic to humans, Morgan and Cascorbi (1985) compared their results of the effects of 6 neurotoxins on nematodes with effects on dogs. Six volatile anesthetics (methoxyflurane, chloroform, halothane, enflurane, isoflurane, and fluroxene) and 1 convulsant (flurothyl) were tested by placing the nematodes on sealed agar plates containing nematode growth medium and injecting liquid forms of the anesthetics into the chamber through a steel needle. While nematodes required higher doses of the chemicals to exhibit toxic effects, the dose-response curves of dogs and

nematodes were parallel. In both data sets, there was a positive relationship between the two variables of concentration and depressed muscular activity. Additionally, there was an initial increase in motor activity followed by a sequential and reversible loss of muscle function in both groups of organisms. The work strongly suggested the existence of a comparable neuromuscular mechanism in nematodes and in mammals.

Williams and Dusenbery (1988) conducted an acute toxicity study to determine the validity of utilizing C. elegans to predict effects of the selected toxins on mammals. Salts of 8 metals (mercury, aluminum, copper, beryllium, lead, zinc, cadmium and strontium) were tested. Lethal concentration (LC_{50}) values were extrapolated from the dose/response curves. LC_{50} values for C. elegans were higher than respective values for rats and mice for all toxins tested. However, the standard error of the estimate ($S_{x,y}$) of the log/log curve of LC_{50} values for rats, guinea pigs, rabbits and hamsters versus the C. elegans LC_{50} for each chemical tested was parallel to the $S_{x,y}$ of the log/log curve of the LC_{50} values for mammalian species minus rats versus the LC_{50} value for rats and to the $S_{x,y}$ of the log/log curve of the LC_{50} values for mammalian species minus mice versus the LC_{50} values for mice for each chemical tested. Statistical analysis of data indicated that C. elegans are

equivalent to rats and mice in their ability to predict the human toxicity of the 8 metal salts.

Acute toxicity testing of cadmium on C. elegans demonstrated an inverse relationship between length of exposure and nematode growth (Popham & Webster, 1979). The researchers found modified mitochondria in the esophagus and intestines. Mitochondria appeared coagulated and degenerated in both structures in electron micrographs. Both growth and fecundity were decreased, presumably due to interference of cadmium with nutrient uptake and assimilation capabilities.

Neurotoxic Lathyrus substances such as ODAP are degraded to toxic metabolites (e.g isoniazide, hydrazinehydrate, thiosemicarbazide and phenylhydrazine) in animals. Kampfe et al. (1986) performed axenic tests of the effects of these metabolites on Caenorhabditis briggsae (C. briggsae). Toxic effects included degeneration of the nervous system as measured by impeded motility. In addition, the nematode demonstrated retarded development and decreased reproduction. Repetition of tests in nonaxenic conditions resulted in bacterial degradation of the toxic metabolites and in consequent sparing of C. briggsae.

Caenorhabditis elegans served as a test organism for non-fumigant nematicides for control of plant nematodes (Ohba & Ishibashi, 1984). Testing of the neurotoxins methomyl, aldoxycarb and methylisothiocyanate caused reduced

reproductivity and consequent population decline as well as irreversible transformation into dumpy mutant form. Progeny of the dumpy nematodes showed reduced proliferation. The researchers concluded that the nematicides suppressed egg hatching and larval development. They further postulated that previously observed hypercontraction of musculature, cessation of pharyngeal movement and death resulted from inhibition of acetylcholinesterase by the nematicides. Testing mutant strains of C. elegans, Opperman and Chang (1991) investigated nematode recovery from acetylcholinesterase inhibitors. The nematode possesses two forms of acetylcholinesterase. The soluble form is located at the neuromuscular synapse and is destroyed by one class of inhibitory neurotoxins. The insoluble form contains a class of acetylcholinesterase believed to be unique to nematodes. It may be less susceptible to inhibitory neurotoxins and may serve a backup function for the nematode. Opperman and Chang (1991) demonstrated that the latter class of acetylcholinesterase was reactivated during the recovery period due to the instability of the enzyme-inhibitor bonding.

In a study of the response of C. elegans to saxitoxin and to tetrodotoxin from shellfish, Kitts and Lu (1987) reported statistically significant sensitivity at a concentration of 0.01 ug/ml. Minimum lethal injections of 0.25ug and 0.30ug are used for saxitoxin and tetrodotoxin

respectively in the standard mouse lethality assay for Paralytic Shellfish Poisoning. Under the experimental conditions, maximum lethality involved 23-35% of the population prohibiting determination of the LC_{50}. The researchers postulated that toxin potency was diminished by the pH level of 5.9 of the cultivation medium and recommended a lower pH for further testing. Future repetitions of this test were recommended to validate a linear dose-response curve.

Caenorhabditis elegans exhibited a linear response of decreased survival in response to increasing concentrations of five food additives (benzoic acid, sodium propionate, ethylenediaminetetraacetic acid (EDTA), ascorbic acid and sodium bisulfite) (Wong, 1986). Results of the controlled study were compared with LD_{50} values for rats. Rat toxicity rankings were sodium bisulfite>EDTA>benzoic acid>ascorbic acid>sodium propionate. Toxicity rankings for C. elegans were benzoic acid>sodium bisulfite>sodium propionate> ascorbic acid>EDTA. Because C. elegans demonstrated toxic responses to the chemicals, Wong concluded that the nematode may be used as a test organism for the selected food additives.

Acute toxicity of C. elegans for neurotoxins is currently unknown. Completed studies support further investigation into the use of this organism for rapid testing. Time, expense, equipment and laboratory animals

can be conserved by advancement of this approach to toxicity testing. Productive areas for future studies include examination of naturally occurring food and bacterial toxins suspected or known to be toxic to humans. In addition, use of C. elegans mutants with altered sensitivity to a chemical or with modification of genes of known function can assist in the study of the mechanisms of neurotoxicity (Williams & Dusenbery, 1987).

Chapter 2

JOURNAL ARTICLE

Authors Title Page

CAENORHABDITIS ELEGANS AS A SCREENING ORGANISM FOR NATURALLY OCCURING NEUROTOXINS IN FOODS[1]

M. RECUPERO [2], N.C. LU, P.S. BELO and M. SALTMARCH

Department of Nutrition and Food Science

San Jose State University

One Washington Square

San Jose, CA 95192

1) Presented at the 73rd Annual Meeting of the American Dietetic Association; supported in part by the San Jose State University Foundation Research Grant.

2) Address questions and comments to:

Marilyn Recupero

7016 Burnside Drive

San Jose, CA 95120, (408) 268-3420

ABSTRACT

Acute toxicity of purified naturally occurring food toxins was determined using Caenorhabditis elegans (C. elegans) and five neurotoxins: dihydroxyphenylalanine (DOPA), diaminobutyric acid (DABA) and N-methyl-D-aspartic acid (MAA), legume components; kainic acid (KA), a Japanese seaweed component; quinolinic acid (QA), a tryptophan metabolite. Two levels of acute toxicity testing were performed. For range finding, nematodes (10,000/ml) were exposed to test medium (1 ml) containing neurotoxins in concentrations of 0.0, 0.016, 0.08, 0.40, 2.0, 10 and 50 mg/ml. Cultures were incubated at 20°C on a tissue culture rotator at 1 rpm for 24 hours. LC_{50} values, extrapolated from dose-response curves, were 1.4, 2.5, 2.8, 3.0 and 8.1 mg/ml for DOPA, DABA, KA, MAA and QA respectively. In refined testing, concentrations were narrowed to 0.0, 1.0, 1.5, 2.0, 2.5 mg/ml (DOPA); 0.0, 2.0, 2.5, 3.0, 3.5 mg/ml (DABA, KA, MAA); 0.0, 7.0, 7.5, 8.0, 8.5 mg/ml (QA). LC_{50} values were determined at 1.4, 2.4, 2.7, 2.9 and 7.6 mg/ml for DOPA, DABA, KA, MAA and QA, respectively. DOPA, an inhibitory neurotoxin, created greater toxic response than DABA, KA, MAA and QA, excitatory neurotoxins. Consistent results from the two testing levels suggest that C. elegans can be used for rapid, inexpensive acute toxicity testing of isolated natural food toxins.

INTRODUCTION

Historically, acute toxicological studies have required laboratory animals which demand long test cycles and expensive maintenance (Ayres & Kirschman, 1981). Recently, the issue of cruelty to laboratory animals has added to the need for alternative test organisms (Holden, 1986).

Caenorhabditis elegans, a hermaphroditic microscopic nematode, has demonstrated efficacy as an animal model for studies of nutrition (Lu et al., 1978, 1983), developmental biology (Kisiel & Zuckerman, 1978) and gerontology (Johnson, 1983; Sulston & Brenner, 1974). This organism provides the advantages of genetic uniformity and rapid proliferation. Few toxicological studies have been performed using C. elegans. However, completed research indicates the potential of utilizing this organism for acute toxicity testing. Morgan and Cascorbi (1985) reported that 6 neurotoxins (5 anesthetics and 1 convulsant) had similar depressive effects on muscular activity and function in both C. elegans and in higher animals. Williams and Dusenbery (1988) stated that the neurotoxic effects of 8 metal salts on C. elegans were parallel to effects on rats and mice and concluded that the organism was a reliable predictor of mammalian acute lethality for these chemicals. Cadmium toxicity resulted in modified mitochondrial structure in the esophagus and intestine of C. elegans (Popham & Webster, 1979). Both growth and fecundity were decreased in

nematodes, presumably due to interference with nutrient uptake and assimilation. Lathyrogenous substances (e.g., L-2,4-diaminobutyric acid) were degraded to neurotoxic metabolites in C. elegans (Kampfe et al., 1986). The accumulation resulted in decreased reproduction and delayed development. Repetition of tests in nonaxenic conditions resulted in bacterial degradation of toxic metabolites and consequent sparing of C. elegans.

The objective of the present study was to investigate the acute toxicity of C. elegans to selected purified water-soluble neurotoxins found in food. The results of this study can be used to determine the efficacy of utilizing C. elegans as an acute toxicity testing organism for a variety of food related neurotoxins.

MATERIALS AND METHODS

Test Organism

C. elegans is a microscopic nematode. It is cultivated in axenic conditions. Populations of 100,000 nematodes/ml culture medium can be developed within 15 days. The hermaphroditic organism provides genetic uniformity for controlled testing.

Populations of *C. elegans* were cultivated in an axenic stock medium consisting of 4% Hi Soy, 1% yeast extract and 10% heated liver extract (HS-YE-HLE) for 15 days prior to the acute toxicological testing (Tomlinson & Rothstein, 1962). To prepare the inoculum, *C. elegans* were harvested, washed three times and resuspended in 1/15 M potassium phosphate buffer solution (pH 5.8). The inoculum (0.1ml) containing 10,000 nematodes was combined with neurotoxins at various concentrations (0.9ml) resulting in a final population of 10,000 nematodes per ml test medium.

Materials

Five neurotoxins selected for this study were obtained from Sigma Chemical Company (St. Louis, MO). Dihydroxyphenylalanine (DOPA), L-2,4-diaminobutyric acid (DABA), N-methyl-D-aspartic acid (MAA) and quinolinic acid (QA) were isolated, purified chemicals comparable to the toxins found in foods. Kainic acid was isolated from the Japanese Seaweed, *Digenea simplex*.

Experimental design and statistical analysis

Range finding study. An initial range finding study was performed to establish the approximate lethal concentration to 50% of the population (LC_{50}) for each neurotoxin. Concentrations tested for DOPA, KA and MAA were 10, 2.0, 0.40, 0.08 and 0.016 mg/ml potassium phosphate buffer. Concentrations tested for DABA and QA were 50, 10, 2.0, 0.40 and 0.08 mg/ml potassium phosphate buffer. Quadruplicate culture tubes were prepared for each concentration of each toxin. Each culture tube (10x100mm) contained 0.9 ml of test medium and 0.1 ml of inoculum with approximately 10,000 nematodes. Control tubes contained 0.9 ml of potassium phosphate buffer and 0.1 ml of inoculum. The culture tubes were incubated at 20°C on a tissue culture rotator with the drum set at 75° from the horizontal at 1 rpm for 24 hours.

At the end of the 24 hour incubation period, survival percentage was determined. The number of surviving nematodes was counted under a dissecting microscope. Individual counts of three 0.1 ml aliquots from the population of each culture tube were executed. Dose-response curves were prepared by linear regression and LC_{50} values were extrapolated from each curve.

Refined Testing. Further study was performed for the purpose of verifying the LC_{50} of each neurotoxin. The procedure was similar to that of the range-finding study.

Four concentrations within the approximated LC_{50} values were tested for each toxin. Concentrations of 1.0, 1.5, 2.0 and 2.5 mg/ml were prepared for DOPA; 2.0, 2.5, 3.0 and 3.5 mg/ml for DABA, KA and MAA; 7.0, 7.5, 8.0 and 8.5 mg/ml for QA. Controls with no neurotoxin were carried out for each testing. Linear regression curves were calculated for dose-response data. LC_{50} values were then extrapolated.

Statistical analysis of data. Statistical analysis was performed by the Statistical Package for the Social Sciences (SPSS) computer program for both the range-finding and the refined testing. Data for individual chemicals were subjected to one way analysis of variance (ANOVA) to determine mean and standard deviation. Calculations of significant differences between experimental concentrations and controls were determined by the Scheffé procedure for each chemical separately ($p \leq 0.01$). Final dose-response curves were established by linear regression. LC_{50} values were extrapolated from the dose-response curves.

RESULTS

Range finding Study

The concentrations of neurotoxin lethal to 50% of the nematode population (LC_{50}) for dihydroxyphenylalanine (DOPA), L-2,4-diaminobutyric acid (DABA), kainic acid (KA), N-methyl-D-aspartic acid (MAA) and quinolinic acid (QA) were estimated to be 1.4, 2.5, 2.8, 3.0 and 8.1 mg/ml, respectively. A linear relationship was demonstrated between increased neurotoxin concentration and decreased nematode survival for all five tested. Differences at $p \leq 0.01$ were statistically significant between some concentration levels in the range-finding tests (Table 1).

Refined Testing

The LC_{50} values determined during the refined testing of DOPA, DABA, KA, MAA AND QA were 1.4, 2.4, 1.7, 1.9 and 7.6 mg/ml, respectively. A linear relationship was again demonstrated between increased neurotoxin concentration and nematode survival. Statistically significant differences at $p \leq 0.01$ were seen between controls and all concentrations (see Table 2).

Acute Toxicity

C. elegans exhibited acute toxic responses to all five neurotoxins. The test organism demonstrated the greatest toxic response to DOPA, a midrange response to DABA, KA, and MAA and the least response to QA based on LC_{50} values from both range finding and refined testing studies.

Results of the two tests were very consistent (see Table 3). The congruity of the LC_{50} data for both investigations suggests that utilization of the organism and procedure developed for this study yields highly reproducible results.

DISCUSSION

Naturally occurring neurotoxins in foods have been shown to be related to chronic human illnesses and to contribute to acute episodes of physical distress (Watson, 1987). As localized food shortages increase throughout the world, so will human dependence upon plant protein. These plants contain toxic chemical compounds (Padmanaban, 1980). A well-known example is the Lathyrus plant species which contains unusual excitatory amino acids, including diaminobutyric acid (DABA), which have been strongly associated with a high incidence of human neurolathyrism, a disease characterized by muscular rigidity, weakness, paralysis and death (De Wolff, 1985; Padmanaban, 1980). The legumes of this species (e.g., chickpea) comprise up to 30% of the diet in India and Algeria (Padmanaban, 1980).

All five neurotoxins selected for the present study have been detected in plant foods. Dihydroxyphenylalanine (DOPA) is a constituent of Vicia faba pods. Pusztai (1982) documented episodes of nausea and vomiting following ingestion by humans. Vicia faba pods contain as much as 2.5 mg/ml DOPA (Pusztai, 1982). Diaminobutyric acid (DABA), a Lathyrogen, is found in the chickpea and has been associated with paralytic episodes in humans (Padmanaban, 1980). Lathyrus silvestris seeds contain up to 27.0 mg/ml DABA (Pusztai, 1982). Seeds of Lathyrus latifolius contain

5.0-6.7 mg/ml DABA (Ressler et al., 1961). Kainic acid (KA) and N-methyl-D-aspartic acid (MAA), both glutamate analogs, activate glutamate receptors during neural transmission. In the brain, glutamate carries out 75% of excitatory transmission. Kainic acid is isolated from the Japanese seaweed, Digenea simplex. It has demonstrated 500 times the potency of glutamate with resultant seizures and brain damage (Kizer et al., 1978). N-methyl-D-aspartic acid is found in legumes. Potency has been measured to be 100 times that of glutamate. Its toxicity has been indicated by cellular swelling and subsequent neurodegenerative effects on the central nervous system of rats (Menendez et al., 1989). Quinolinic acid is a product of tryptophan metabolism (McGeer & McGeer, 1985). Toxicity in rats has been shown to cause loss of cholinesterase activity resulting in reduction of tremor threshold (Engber & Chase, 1988; Wardley-Smith et al., 1989).

Four of the neurotoxins tested in this study were excitotoxins (DABA, KA, MAA, QA). Excitatory neurotoxins disrupt normal function of nerve transmission by inducing the prolonged and persistent flow of acetylcholine across the synapse, thereby provoking continuous muscle contraction manifested by spastic movements and eventual paralysis (McGeer & McGeer, 1985; Olney, 1980). When the muscles of gas exchange are involved, suffocation and death may ensue (Croll & Matthews, 1977). In contrast, DOPA, an inhibitory

neurotransmitter, induces reduced neural transmission and subsequent paralysis.

Few toxicological studies have been completed using C. elegans. However, completed research points to the practicality and applicability of utilizing this organism for acute neurotoxicity testing (Hodgkin, 1991; Kampfe et al., 1986; Kitts & Lu, 1987; Morgan & Cascorbi, 1985; Opperman & Chang, 1991; Popham & Webster, 1979; Williams & Dusenbery, 1988). C. elegans is a microscopic cylindrical invertebrate, covered by a flexible collagen cuticle. The central brain consists of a ring of nerve tissue and two longitudinal nerve cords (Willett, 1980). The functional neurotransmitters employed by C. elegans are identical to those used by humans and include serotonin, dopamine, acetylcholine and octopamine (Horvitz et al., 1982; Kenyon, 1988; Willett, 1980; Wright & Awan, 1976). Neurotoxic damage in C. elegans can be measured by reduced size, diminished reproductive ability, decreased proportion of adult to larval stage organisms in a given population, inhibition of enzyme activities and mutagenesis.

In this study, C. elegans exhibited acute toxicity to all five neurotoxins tested. For each toxin, the dose-response curve was linear. In the range finding study, the survival percentages at various concentrations showed significant differences (Table 1.) In the refined study, the survival percentages of the experimental concentrations

were significantly different from the survival percentage of the controls (Table 2). The demonstrated relative potencies in this study were as follows: DOPA>DABA>KA>MAA>QA. Reported relative potencies for rat and mouse experiments were KA>QA>MAA>DABA>DOPA (Engstrom & Woodbury, 1988; Litchfield and Wilcoxon, 1949; O'Neal et al., 1968; Wardley-Smith, 1989; Weil, 1952). The data from this study suggest that C. elegans is more susceptible to toxic effects from the inhibitory neurotoxin than from the excitotoxins.

The consistent demonstration of acute toxicity in C. elegans both in range-finding and in refined testing of the neurotoxins indicates the potential usefulness and reproducibility of both the test procedure and the test organism employed in this research. Further, the axenic (germ-free) condition used in this study provides a direct relationship between neurotoxin concentration and survival of the testing organism, C. elegans. The elimination of bacteria as an additional variable facilitates reproducibility of results. In contrast, Kampfe et al. (1986) reported that bacteria degraded toxic metabolites spared the nematodes.

Caenorhabditis elegans possesses good potential as a test organism for water-soluble food neurotoxins. The economy and rapidity of developing large populations of C. elegans clones provide the researcher with valuable genetic uniformity. Populations of 100,000 nematodes can be

cultivated within 15 days in one 1ml aliquot of a stock medium, requiring inexpensive medium components and trivial laboratory space. Once water-soluble purified neurotoxins and potassium phosphate buffer are prepared, the testing can be completed within 24 hours. Time, expense, equipment and laboratory animals are significantly conserved by advancement of this approach to testing potential food toxins.

TABLE 1. Range-finding: Percentage survival of C. elegans in various concentrations of toxins

Concentration (mg/ml)	*DOPA* †	*DABA* †	*KA* †	*MAA* †	*QA* †
50	-----‡	0.8± 1.7^{a}	------- ‡	------- ‡	5.78±4.8^{a}
10	0.0±0.0	31.0±4.2^{b}	20.0±7.3^{a}	23.5±4.7^{a}	44.3±3.4^{b}
2.0	0.0±0.0	54.2± 6.8^{c}	60.0±2.4^{b}	54.0±4.2^{b}	68.3±5.7^{c}
0.40	29.3±1.5^{a}	77.5± 11^{d}	78.5±1.7^{c}	70.8± 8.3^{bc}	72.8±5.1^{cd}
0.08	70.8±9.7^{ab}	87.5± 4.1^{de}	85.0±2.3^{cd}	76.8± 2.9^{c}	89.0± 7.1^{de}
0.016	77.5±11^{ab}	--------‡	93.3±3.3^{de}	87.5± 5.6^{cd}	------- ‡
0.0	100.0±9.6^{bc}	100.0±11^{e}	100.0±5.0^{e}	95.0± 5.8^{d}	95.0±8.1^{e}
$LC_{50(mg/ml)}$*	1.4	2.5	2.8	3.0	8.1

Values are mean SD of data from four replicas, each inoculated with 10,000 nematodes.

Superscripts with different letters in columns are significantly ($p \leq 0.01$) different.

†DOPA = dihydroxyphenylalanine
DABA = L-2, 4-diaminobutyric acid
KA = kainic acid
MAA = N-methyl-D-aspartic acid
QA = quinolinic acid

‡ Not tested

*Values extrapolated from dose-response linear regression curves

TABLE 2. Refined testing: Percentage survival of C. elegans in various concentrations of toxins

Concentration (mg/ml)	*DOPA*†	*DABA*†	*KA*†	*MAA*†	*QA*†
8.5					20.8± 5.9
8.0					30.0± 6.2
7.5					61.5± 3.0
7.0					65.0± 4.4
3.5		31.0 ±4.9	39.0 ±4.2	34.0 ±4.2	
3.0		47.0 ±1.4	46.8 ±2.9	51.5± 1.7	
2.5	21.8 ± 3.5	48.5 ±1.7	53.3 ±2.9	63.0± 0.0	
2.0	38.5 ±6.0	53.3 ±1.2	58.0 ±5.8	66.8 ±2.9	
1.5	50.0 ±2.4				
1.0	58.5 ±3.0				
0.0	99.0 ± 8.0	100.0 ± 7.5	100.0 ± 2.1	100.0± 2.1	100.0± 7.5
LC_{50} (mg/ml)*	1.4	2.4	2.7	2.9	7.6

Values are mean SD of data from four replicas, each inoculated with 10,000 nematodes

†DOPA = dihydroxyphenylalanine
DABA = L-2, 4-diaminobutyric acid
KA = Kainic acid
MAA = N-methyl-D-aspartic acid
QA = quinolinic acid

Control values are significantly different from experimental values ($p \leq 0.01$).

Values extrapolated from dose-response linear regression curves

TABLE 3. Lethal concentrations of (LC_{50}) of neurotoxins tested on C. elegans

Toxin	*Range Finding LC_{50} (mg/ml)*	*Refined Testing LC_{50} (mg/ml)*
DOPA*	1.4	1.4
DABA*	2.5	2.4
KA*	2.8	2.7
MAA*	3.0	2.9
QA*	8.1	7.6

*DOPA = dihydroxyphenylalanine
DABA = L-2,4-diaminobutyric acid
KA = kainic acid
MAA = N-methyl-D-aspartic acid
QA = quinolinic acid

References

Ayres, J.C. & Kirschman, J.C. (1981). Impact of toxicology on food processing. Westport, CT: AVI Publishing Company.

Croll, N.A. & Matthews, B.E. (1977). Nematodes as models and model nematodes. Biology of Nematodes. New York: John Wiley & Sons.

De Wolff, F.A. (1985). Nutritional toxicology: The significance of natural toxins. Human Toxicology, 7, 443-447.

Engber, T.M. & Chase, T. N. (1988). Dextromethorphan does not protect against quinolinic acid neurotoxicity in rat striatum. Neuroscience Letters, 95, 269-274.

Engstrom, F.L. & Woodbury, D.M. (1988). Seizure susceptibility in DBA and C57 mice: The effects of various convulsants. Epilepsia, 29, 389-395.

Hodgkin, J. (1991). Nematodes in nervous decline. Nature, 349, 564-565.

Holden, C. (1986). A pivotal year for lab animal welfare. Science, 232, 147-150.

Horvitz, H.R., Chalfie, M., Trent, C., Sulston, M.E. & Evans, P.D. (1982). Serotonin and octopamine in the nematode. Science, 216, 1012-1014.

Johnson, T.E. (1983). Aging in Caenorhabditis elegans. In M. Rothstein (Ed.), Review of biological research in aging (pp.37-49). New York: Alan R. Liss, Inc.

Kampfe, V.L., Kreil, V. & Ullrich, H.L. (1986). Effects of lathyrogenous compounds and of the synergist piperonyl butoxide (PBO) on the free-living nematode species. Journal of Zoological Physiology, 90, 257-271.

Kenyon, C. (1988). The nematode Caenorhabditis elegans. Science, 240, 1448-1453.

Kisiel, M.J. & Zuckerman, B.M. (1978). Effects of centrophenoxine on the nematode Caenorhabditis briggsae. Age, 1, 17-20.

Kitts, D.D. & Lu, N.C. (1987). Response of the Free Living Nematode C. elegans to Saxitoxin and Tetrodotoxin Seafood Toxins. Unpublished manuscript. University of British Columbia, Department of Food Science and San Jose State University, Department of Nutrition and Food Science.

Kizer, J.S., Nemeroff, C.B. & Younglood, W.W. (1978). Neurotoxic amino acids and structurally related analogs. Pharmacological Reviews, 29, 301-318.

Litchfield, J.T. & Wilcoxon, F. (1949). Simplified method of evaluating dose-effect experiments. Journal of Pharmacological and Experimental Therapy, 96, 99-113.

Lu, N.C., Cheng, A.C. & Briggs, G.M. (1983). A study of mineral requirements in Caenorhabditis elegans. Nematologica, 29, 425-434.

Lu, N.C., Hugenberg, G., Briggs, G.M. & Stokstad, E.L.R (1978). The growth-promoting activity of several lipid related compounds in the free-living nematode Caenorhabditis briqqsae. Proceedings of the Society for Experimental Biology and Medicine, 158, 187-191.

McGeer, E.G. & McGeer, P.L. (1985). Neurotoxin-induced animal models of human diseases. In K. Blum and L. Manzo, (Eds.), Neurotoxicology (pp. 515-533). New York: Marcel Dekker, Inc.

Menendez, N., Herreras, O., Solis, J.M., Herranz, A.S. & del Rio, R.M. (1989). Extracellular taurine increase in rat hippocampus evoked by specific glutamate receptor activation is related to the excitatory potency of glutamate agonists. Neuroscience Letters, 102, 64-69.

Morgan, P.G. & Cascorbi, H.F. (1985). Effect of anesthetics and a convulsant on normal and mutant C. elegans. Anesthesiology, 62, 738-744.

Olney, J.W. (1980). Excitatory neurotoxins as food additives: An evaluation of risk. Neurotoxicology, 2, 163-192.

O'Neal, R.M., Chen, C.H., Reynolds, C.A., Meghal, S.K. & Kopeep, R.E. (1968). The neurotoxicity of L-2-4-diaminobutyric acid. Biochemistry Journal, 106, 699-706.

Opperman, C.H. & Chang, S. (1991). Effects of Aldicarb and Fenamiphos on acetylcholinesterase and motility of Caenorhabditis elegans. Journal of Nematology, 23, 20-27.

Padmanaban, G. (1980). Lathyrogens. In I.E. Liener (Ed.). Toxic constituents of plant foodstuffs (pp. 239-263). New York: Academic Press.

Popham, J.D. & Webster, J.M. (1979). Cadmium toxicity in the free-living nematode, Caenorhabditis elegans. Environmental Research, 20, 183-191.

Pusztai, A. (1982). The role in food poisoning of toxins and allergens from higher plants. In R.K. Robinson (Ed.), Developments in food microbiology (Vol. 1, pp. 179-194). New York: Elsevier Science Publishing Co., Inc.

Ressler, C., Redstone, P.A. & Erenberg, R.H. (1961). Isolation and identification of a neuroactive factor from Lathyrus latifolius. Science, 134, 188-190.

Sulston, J.E. & Brenner, S. (1974). The DNA of Caenorhabditis elegans. Genetics, 77, 95-104.

Tomlinson, G.A. & Rothstein, M. D(1962). Nematode biochemistry. I. Culture methods. Biochemistry Biophysics Acta, 63, 465-470.

Wardley-Smith, B., Halsey, M.J., Hawley, D. & Joseph, M.H. (1989). The effects of kynurenic acid, quinolinic acid, and other metabolites of tryptophan on the development of the high pressure neurological syndrome in the rat. Neuropharmacology, 28, 43-47.

Watson, D.H. (Ed.). (1987). Natural toxicants in food. New York: VCH Publishers.

Weil, C.S. (1952). Tables for convenient calculation of median effective dose (LD_{50} or ED_{50}). Biometrics, 8, 249-263.

Willett, J.D. (1980). Control mechanisms in nematodes. In B.M. Zuckerman (Ed.), Nematodes as biological Models, (Vol 1., pp. 197-225).

Williams, P.L. & Dusenbery, D.B. (1988). Using the nematode Caenorhabditis elegans to predict mammalian acute lethality to metallic salts. Toxicology and Industrial Health, 4, 469-478.

Wright, D.J. & Awan, F.A. (1976). Acetylcholinesterase activity in the region of the nematode nerve ring: Improved histochemical specificity using ultrasonic pretreatment. Nematologica, 22, 326-331.

CHAPTER 3

SUMMARY AND RECOMMENDATIONS

Summary

Acute toxicity tests were performed to determine the relative acute toxicity of the nematode Caenorhabditis elegans exposed to five naturally occurring food toxins. Two levels of tests were completed. The range finding experiment examined concentrations over a broad scope to determine the approximate concentration of each toxin lethal to 50% (LC_{50}) of the population. The refined experiment tested concentrations within the target range for verification of the LC_{50}. The relative potency of the toxins was DOPA>DABA>KA>MAA>QA. Reported mammalian relative toxicity is KA>QA>MAA>DABA>DOPA. Caenorhabditis elegans showed the greatest toxic response to DOPA, an inhibitory neurotoxin and less toxic response to DABA, KA, MAA and QA, excitatory neurotoxins. Linear dose-response relationships were manifested for each chemical. The LC_{50} values for the range finding test were similar to those for the refined test under the experimental conditions in this study. Caenorhabditis elegans appears to have good potential as a test organism for acute toxicity determination of purified, isolated neurotoxins found in foods.

Recommendations

The following suggestions are made for further investigation of the efficacy of utilizing C. elegans for acute toxicity testing of food related neurotoxins:

1. Range finding and refined testing LC_{50} values were similar. Therefore, range finding testing alone may be adequate for use of C. elegans as organism for acute toxicity testing.

3. Reproducibility could be further verified with higher populations (e.g., 20,000 or 100,000 nematodes per tube).

4. Toxicity could be further verified with different incubation periods (e.g., 12 hr or 48 hr).

5. Demonstration of strong toxic response to the inhibitory neurotoxin DOPA suggests the need for further experimentation with other inhibitory neurotoxins.

6. Use of the organism could be extended to acute toxicity testing of neurotoxic pesticide residue on foods.

7. Electron micrographs could investigate correlation of neurotoxicity with regions of neuromuscular damage.

References

Ayres, J.C. & Kirschman, J.C. (1981). Impact of toxicology on food processing. Westport, CT: AVI Publishing Company.

Chalfie, M. (1984). Neuronal development in Caenorhabditis elegans. Trends in Neuroscience, 7, 197-202.

Chang, C. & Lu, N.C. (1990). Heme requirement and effect of medium surface area exposure on population growth and production of Caenorhabditis elegans. Unpublished master's thesis. San Jose State University, San Jose, California.

Croll, N.A. & Matthews, B.E. (1977). Nematodes as models and model nematodes. Biology of Nematodes. New York: John Wiley & Sons.

De Wolff, F.A. (1985). Nutritional toxicology: The significance of natural toxins. Human Toxicology, 7, 443-447.

Engber, T.M. & Chase, T. N. (1988). Dextromethorphan does not protect against quinolinic acid neurotoxicity in rat striatum. Neuroscience Letters, 95, 269-274.

Engstrom, F.L. & Woodbury, D.M. (1988). Seizure susceptibility in DBA and C57 mice: The effects of various convulsants. Epilepsia, 29, 389-395.

Hieb, W.F. & Rothstein, M. (1968). Sterol requirement for reproduction of a free-living nematode. Science, 160, 778-790.

Hieb, W.F., Stokstad, E.L.R. & Rothstein, M. (1970). Heme requirement for reproduction of a free-living nematode. Science, 168, 143-144.

Hodgkin, J. (1991). Nematodes in nervous decline. Nature, 349, 564-565.

Holden, C. (1986). A pivotal year for lab animal welfare. Science, 232, 147-150.

Horvitz, H.R., Chalfie, M., Trent, C., Sulston, M.E. & Evans, P.D. (1982). Serotonin and octopamine in the nematode. Science, 216, 1012-1014.

Johnson, T.E. (1983). Aging in Caenorhabditis elegans. In M. Rothstein (Ed.), Review of biological research in aging (pp.37-49). New York: Alan R. Liss, Inc.

Kampfe, V.L., Kreil, V. & Ullrich, H.L. (1986). Effects of lathyrogenous compounds and of the synergist piperonyl butoxide (PBO) on the free-living nematode species. Journal of Zoological Physiology, 90, 257-271.

Kenyon, C. (1988). The nematode Caenorhabditis elegans. Science, 240, 1448-1453.

Kisiel, M.J. & Zuckerman, B.M. (1978). Effects of centrophenoxine on the nematode Caenorhabditis briggsae. Age, 1, 17-20.

Kitts, D.D. & Lu, N.C. (1987). Response of the Free Living Nematode C. elegans to Saxitoxin and Tetrodotoxin Seafood Toxins. Unpublished manuscript. University of British Columbia, Department of Food Science and San Jose State University, Department of Nutrition and Food Science.

Kizer, J.S., Nemeroff, C.B. & Younglood, W.W. (1978). Neurotoxic amino acids and structurally related analogs. Pharmacological Reviews, 29, 301-318.

Litchfield, J.T. & Wilcoxon, F. (1949). Simplified method of evaluating dose-effect experiments. Journal of Pharmacological and Experimental Therapy, 96, 99-113.

Lu, N.C., Cheng, A.C. & Briggs, G.M. (1983). A study of mineral requirements in Caenorhabditis elegans. Nematologica, 29, 425-434.

Lu, N.C. & Goetsch, K.M. (1987). Carbohydrate utilization in Caenorhabditis elegans - Developing an animal model for glycemic index. Symposium on recent advances in biological and medical sciences. Taipei, Taiwan.

Lu, N.C., Hieb, W.F. & Stokstad, E.L.R. (1976). Effect of vitamin B_{12} and folate on biosynthesis of methionine from homocysteine in the nematode Caenorhabditis briggsae. Proceeding of the Society of Experimental Biology and Medicine, 151, 701-704.

Lu, N.C., Hugenberg, G., Briggs, G.M. & Stokstad, E.L.R. (1978). The growth-promoting activity of several lipid related compounds in the free-living nematode Caenorhabditis briggsae. Proceedings of the Society for Experimental Biology and Medicine, 158, 187-191.

McGeer, E.G. & McGeer, P.L. (1985). Neurotoxin-induced animal models of human diseases. In K. Blum and L. Manzo, (Eds.), Neurotoxicology (pp. 515-533). New York: Marcel Dekker, Inc.

Menendez, N., Herreras, O., Solis, J.M., Herranz, A.S. & del Rio, R.M. (1989). Extracellular taurine increase in rat hippocampus evoked by specific glutamate receptor activation is related to the excitatory potency of glutamate agonists. Neuroscience Letters, 102, 64-69.

Morgan, P.G. & Cascorbi, H.F. (1985). Effects of anesthetics and a convulsant on normal and mutant C. elegans. Anesthesiology, 62, 738-744.

Nicholas, W.L., Hansen, E. & Dougherty, E.C. (1962). The B-vitamins required by Caenorhabditis briggsae. Nematologica, 8, 129-135.

Ohba, K. & Ishibashi, N. (1984). A nematode, Caenorhabditis elegans, as test organism for nematicide evaluation. Journal of Pesticide Science, 9, 91-96.

Olney, J.W. (1980). Excitatory neurotoxins as food additives: An evaluation of risk. Neurotoxicology, 2, 163-192.

O'Neal, R.M., Chen, C.H., Reynolds, C.A., Meghal, S.K. & Koeppe, R.E. (1968). The neurotoxicity of L-2,4-diaminobutyric acid. Biochemistry Journal, 106, 699-706.

Opperman, C.H. & Chang, S. (1991). Effects of Aldicarb and Fenamiphos on acetylcholinesterase and motility of Caenorhabditis elegans. Journal of Nematology, 23, 20-27.

Padmanaban, G. (1980). Lathyrogens. In I.E. Liener (Ed.). Toxic constituents of plant foodstuffs (pp. 239-263). New York: Academic Press.

Popham, J.D. & Webster, J.M. (1979). Cadmium toxicity in the free-living nematode, Caenorhabditis elegans. Environmental Research, 20, 183-191.

Pusztai, A. (1982). The role in food poisoning of toxins and allergens from higher plants. In R.K. Robinson (Ed.), Developments in food microbiology (Vol. 1, pp. 179-194). New York: Elsevier Science Publishing Co., Inc.

Ressler, C., Redstone, P.A. & Erenberg, R.H. (1961). Isolation and identification of a neuroactive factor from Lathyrus latifolius. Science, 134, 188-190.

Samoiloff, M.R. (1980). Action of chemical and physical agents on free-living nematodes. In B.M. Zuckerman, (Ed.), Nematodes as biological models (Vol. 1). New York: Academic Press.

Sulston, J.E. & Brenner, S. (1974). The DNA of Caenorhabditis elegans. Genetics, 77, 95-104.

Sun, S.J., Lu, N.C., Tseng, R. & Smith, E. (1986). Federation Proceedings, 45, 823.

Tomlinson, G.A. & Rothstein, M. (1962). Nematode biochemistry. I. Culture methods. Biochemistry Biophysics Acta, 63, 465-470.

Van Fleteren, J.R. (1973). Amino acid requirements of the free-living nematodes, Caenorhabditis briggsae. Nematologica, 19, 93-101.

Wardley-Smith, B., Halsey, M.J., Hawley, D. & Joseph, M.H. (1989). The effects of kynurenic acid, quinolinic acid, and other metabolites of tryptophan on the development of the high pressure neurological syndrome in the rat. Neuropharmacology, 28, 43-47.

Watson, D.H. (Ed.). (1987). Natural toxicants in food. New York: VCH Publishers.

Willett, J.D. (1980). Control mechanisms in nematodes. In B.M. Zuckerman (Ed.), Nematodes as biological Models, (Vol 1., pp. 197-225).

Williams, P.L. & Dusenbery, D.B. (1987). Screening test for neurotoxins using Caenorhabditis elegans. In A. Shahan & A.M. Goldberg (Eds.), Model systems in neurotoxicology: Alternative approaches to animal testing (pp. 163-170). New York: Alan R. Liss, Inc.

Williams, P.L. & Dusenbery, D.B. (1988). Using the nematode Caenorhabditis elegans to predict mammalian acute lethality to metallic salts. Toxicology and Industrial Health, 4, 469-478.

Wong, C. & Lu, N.C. (1986, May). The use of Caenorhabditis as a screening organism for food additives. Abstract presented at a meeting of the meeting of the Institute of Food Technologists,

Wright, D.J. & Awan, F.A. (1976). Acetylcholinesterase activity in the region of the nematode nerve ring: Improved histochemical specificity using ultrasonic pretreatment. Nematologica, 22, 326-331.