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**SUMMER UNDERGRADUATE RESEARCH
PROGRAM:
ENVIRONMENTAL STUDIES**

Projects submitted from the participating students of the 1994 Summer
Undergraduate Research Program

of the

Medical University of South Carolina

ds
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CONTENTS:

Purpose of Program

Student List

Seminar Speakers

Projects

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1994 SUMMER UNDERGRADUATE RESEARCH PROGRAM

Program Description

The purpose of the summer undergraduate internship program for research in environmental studies is to provide an opportunity for well-qualified students to undertake an original research project as an apprentice to an active research scientist in basic environmental research. The students are offered research topics at the Medical University in the scientific areas of pharmacology and toxicology, epidemiology and risk assessment, environmental microbiology, and marine sciences. Students are also afforded the opportunity to work with faculty at the University of Charleston, SC, on projects with an environmental theme. During the ten week period that the students are on campus, several seminars are presented by participating faculty and invited lecturers highlighting aspects of research in the areas of environmental science. At the end of their ten week internship the students are required to give an oral presentation of their research and to turn in a written report on the project. By undertaking an independent research project the students will gain an understanding and appreciation of the process whereby scientific theory is developed, tested, and documented.

Ten well-qualified students from colleges and universities throughout the eastern United States were accepted into the program. Reflecting the emphasis on recruiting minority students for this internship program, four of the ten students were minority students. The students participating in this summer's program selected projects in the areas of pharmacology and toxicology, biostatistics and epidemiology, marine sciences, and biochemistry. The research experience for all these students and their mentors was very positive. The seminars were well-attended and the students showed their interest in the presentations and environmental sciences as a whole by presenting the speakers with thoughtful and intuitive questions. Pre-seminar and post-seminar questionnaires were given to the students to emphasize the educational nature of the talks. The students' oral presentations and written reports were well done and were approached with enthusiasm. Several students have stated that the introduction to and experience in research they gained from this program has provided them with the option of pursuing research in environmental sciences as a career goal. This summer program has shown itself to be a valuable educational tool.

SUMMER UNDERGRADUATE RESEARCH PROGRAM

Summer 1994

Student List

Student: **Christopher Anderson**

College/University: University of Southern Mississippi
Hattiesburg, MS

Major: Biochemistry

Mentor: Thomas Walle, Ph.D.

Department of Cell and Molecular Pharmacology and Experimental Therapeutics

Project Title: *Induction of Taxol Metabolism in the Rat by Dexamethasone*

Student: **Samuel Boyce**

College/University: North Carolina State University
Raleigh, NC

Major: Chemical Engineering

Mentor: Greg Doucette, Ph.D.

National Marine Fisheries Service and
Marine Biomedical and Environmental Sciences

Project Title: *Isolation, Purification and Spectrometric Analysis of PSP Toxins from Moraxella sp., a Bacterium Associated with a Toxic Dinoflagellate*

Student: **Kelly Craig***

College/University: University of Charleston
Charleston, WV

Major: Environmental Science

Mentor: Karen Burnett, Ph.D., and Amy Ringwood, Ph.D.

Marine Biomedical and Environmental Sciences

Project Title: *The Effects of Cadmium on the Growth and Metallothionein Expression of the Bivalve Larvae, Crassostrea virginica*

Student: **Sharon Davis**

College/University: College of Charleston
Charleston, SC

Major: Chemistry

Mentor: Peter Moeller, Ph.D.

National Marine Fisheries Service and
Marine Biomedical and Environmental Sciences

Project Title: *The Isolation and Purification of a Caribbean Maitotoxin*

Student: Mikel Fair*
College/University: The Citadel
Charleston, SC
Major: Physical Education
Mentor: Daniel Lackland, Ph.D.
Department of Biometry and Epidemiology
Project Title: *Increasing the Treatment of Hypertension through Primary Intervention*

Student: Marc Klingshirn
College/University: Ashland University
Ashland, OH
Major: Environmental Science - Chemistry
Mentor: Zhexi Luo, Ph.D.
Department of Biology
College of Charleston
Project Title: *A Comparative Study of the Inner Ear Structures of Artiodactyls and Early Cetaceans*

Student: Sylvia Nelson*
College/University: Xavier University
Cincinnati, OH
Major: Natural Sciences
Mentor: Maria Buse, Ph.D.
Department of Endocrinology and Department of Biochemistry
Project Title: *The Effects of Diabetes on the Activity of the Enzyme Glutamine: Fructose-6-Phosphate Aminotransferase*

Student: S. Elizabeth Stasiukaitis
College/University: Furman University
Greenville, SC
Major: Applied Mathematics
Mentor: Daniel Lackland, Ph.D.
Department of Biometry and Epidemiology
Project Title: *An Analytical Assessment of Population Reaction to Environmental Health Hazards*

Student: Suzanne Tapley
College/University: Alma College
Alma, MI
Major: Biology
Mentor: John Ramsdell, Ph.D.
Marine Biomedical and Environmental Sciences
Project Title: *The Neurological Effects of Brevetoxin on Neonatal Rats*

Student: Elsie Thevenin*
College/University: St. Joseph's College
Brooklyn, NY
Major: Biology
Mentor: JoEllyn McMillan, Ph.D.
Department of Cell and Molecular Pharmacology and Experimental Therapeutics
Project Title: *Trichloroethylene Toxicity in a Human Hepatoma Cell Line*

1994
Summer Undergraduate Research Program

List of Seminars

- Dr. Barry Ledford** -- Professor and Chairman
Department of Biochemistry
MUSC
Title: *ADP-Ribosylation of the Molecular Chaperone GRP78/BiP*
- Dr. Carol Savage, Dr. Donald Wilbur, Dr. Henry Martin** -- MUSC
Title: *Symposium on Graduate Studies*
- Dr. Daniel Lackland** -- Associate Professor
Department of Biometry and Epidemiology
MUSC
Title: *Epidemiological Assessments of Environmental Hazards: Cause and Effect*
- Dr. Michael Schmidt** -- Associate Professor
Department of Microbiology and Immunology
MUSC
Title: *Environmental Microbiology: Bioremediation*
- (Guest Speaker)
Dr. James Bruckner -- Professor
Department of Pharmacology and Toxicology
University of Georgia
Title: *Environmental Contamination by TCE: Assessment of Toxic Hazards and Risk*
- Student Research Presentations** -- All Summer Undergraduate Research Programs
MUSC

Abstract

The antitumor drug taxol was metabolized to two major metabolites (RM1 and RM2) in adult male and female rat liver microsomes. The male rats produced RM1 2.6 fold faster than the females, and they produced RM2 3 fold faster than the females. This correlated well with the sex differences noticed in liver microsomal cytochrome P450 (CYP) 3A content (4.4 fold greater in male) and 6 β -hydroxylation of testosterone (2.4 fold greater in male). Taxol was metabolized to three major metabolites (RM1, RM2, and RM3) in adult male and female rat liver microsomes from rats pretreated with dexamethasone. Production of RM1 and RM2 was increased in these rats (2.3 and 3.3 fold respectively in males; 6.5 and 8.7 fold respectively in females) as compared to the untreated rats. These results compared well with the induction of CYP 3A proteins (3.5 fold in male, 10 fold in female) and induction of 6 β -hydroxylation (1.9 fold in males, 3.8 fold in females). RM3, which was produced only by the rats pretreated with dexamethasone, had a retention time of 0.58 relative to taxol which corresponds to 6 α -hydroxytaxol, the major human metabolite of taxol. This study indicates that taxol metabolism in the rat is likely due to CYP 3A enzymes. Although the evidence points toward CYP 3A1 as the major isoform involved, it does not rule out others. The findings also suggest that CYP 3A1 is responsible for the induced metabolite, RM3.

**Induction of Taxol Metabolism in the Rat by
Dexamethasone.**

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**Department of Cell and Molecular Pharmacology and Experimental
Therapeutics, Medical University of South Carolina.**

***College of Graduate Studies, Summer Undergraduate Research
Program, Medical University of South Carolina.**

Submitted August 3, 1994.

Introduction

Taxol, a relatively new antitumor agent obtained from the Pacific Yew Tree, has shown effectiveness against drug refractory breast and ovarian cancers as well as malignant melanoma (1, 2). Taxol appears to work by enhancing the polymerization of tubulin, even in the absence of GTP and microtubule-associated proteins (2), which alters the normal dynamic equilibrium in the tubulin/microtubule system. Microtubules are an essential component of the mitotic spindle, and they are involved in a wide variety of cellular activities, such as cell motility and transport between organelles. By interfering with the tubulin/microtubule equilibrium, taxol disrupts cell division and normal cellular activity; however, no data is available on the site(s) at which taxol binds to microtubules or the specificity that taxol exhibits toward certain malignant tumors (2).

The therapeutic effectiveness as well as the duration of action of taxol are dependant on metabolism (1). The major *in vivo* rat metabolites (3, 4), and the major *in vitro* human metabolite (5) have been previously identified and described. Studies in humans (6) and to a lesser extent the rat (3) suggest that taxol metabolism is mediated by the cytochrome P450 subfamily 3A enzymes. Based on the known inducibility of the cytochrome P450 superfamily of enzymes (7, 8), it seems reasonable to suggest that taxol metabolism may be induced by glucocorticoids, specifically dexamethasone which is an inducer of cytochrome P450 3A enzymes (9, 10, 11). This has important clinical implications because taxol chemotherapy is often preceded by dexamethasone and other glucocorticoids.

Cytochromes P450 (CYP) are a superfamily of heme-containing monooxygenases. In mammals, there are two general classes of CYPs: those involved in steroid and bile acid biosynthetic pathways and those involved in the metabolism of drugs and the bioactivation of xenobiotics to toxic products (12). The CYP superfamily is composed of families and subfamilies of enzymes defined solely on their amino acid sequence similarities. A CYP family consists of enzymes showing at least a 40% resemblance to each other, and CYP subfamily enzymes are at least 55% similar (12). The prevalent xenobiotic metabolizing CYPs in humans are families CYP 1, CYP 2, and CYP 3 (12). Humans express four isoforms of CYP 3A: 3A3, 3A4, 3A5, and 3A7 (only expressed in fetal liver). Rodents express two isoforms of the subfamily 3A: 3A1 and 3A2. CYP 3A1 is considered the rat orthologue of CYP 3A3 and CYP 3A4 (which differ by only 11 amino acids), and CYP 3A2 is considered the rat orthologue of CYP 3A5. (11).

The monooxygenase reaction catalyzed by CYP requires the input of 2 electrons, usually from NADPH. (13). It then accepts one electron from CYP reductase and binds O₂ giving a ferrous-cytochrome P450-dioxygen complex (13). The transfer of a second electron to this complex (from either CYP reductase or cytochrome b₅ reductase) brings cleavage of the O-O bond with concurrent incorporation of the distal oxygen atom into water and transfer of the other oxygen to the substrate (13).

Methods and Materials

Materials. Taxol was purchased from Calbiochem (San Diego, CA), and [³H]taxol (15-30 Ci/mmol, generally labeled) from Moravek Biochemicals (Brea, CA). Dexamethasone, testosterone, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co.(St. Louis, MO). 6β-hydroxytestosterone used as a reference standard and 11β-hydroxytestosterone used as an internal standard were obtained from Steraloids Inc. (Wilton, NH). All other chemicals were reagent grade.

Animals. Male and female Sprague-Dawley rats ranging in weight from 183g to 223g were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The rats were arranged by sex in groups of 3 and given free access to standard chow and water. One group of males and one group of females were administered dexamethasone (DEX) (100mg/kg, dissolved in corn oil) by subcutaneous injections every 24 hr for 3 days. Control rats were injected with corn oil at the same intervals. The rats were killed 24 hr after the last injection, and the livers were immediately frozen in liquid N₂ and stored at -80°C until use. Preliminary experiments were performed using 3 adult male rats ranging in weight from 187g to 199g. One rat was a control, one was treated with DEX (100mg/kg in corn oil), and the final rat was treated with phenobarbital (PB) (75 mg/kg).

Incubation. Hepatic microsomes were prepared by differential centrifugation (14) and stored at -80°C until use. Protein determinations were made according to

Lowry *et al* (15). Preliminary experiments determined optimal conditions of the incubation with respect to time, and protein amount of the incubation. The taxol incubation system contained 1 mg/ml of microsomal protein, taxol (10 μ M), [3 H]taxol (0.25 μ Ci/ml), and a NADPH generating system (2 mM MgCl₂, 0.4 mM NADP, 4 mM glucose-6-phosphate, and 0.2 U of glucose-6-phosphate dehydrogenase) (3) in HEPES buffer (0.5 M, pH 7.4). The incubation was performed for 1 hour at 37°C. Taxol and metabolites were extracted into 5 volumes of ethyl acetate which was evaporated under a stream of N₂, and the residue was reconstituted in 0.5 ml HPLC mobile phase (35% acetonitrile/water, v/v). The testosterone incubation system contained 1 mg/ml of microsomal protein, testosterone (120 μ M), and a NADPH generating system in HEPES buffer (0.5 M, pH 7.4). The incubation was performed for 15 min. at 37°C, and 0.2 mg of 11 β -hydroxytestosterone was added to each incubate as an internal standard before extraction. Testosterone and metabolites were extracted with 5 volumes of methylene chloride which was evaporated under N₂, and the residue was reconstituted in 250 μ l of HPLC mobile phase (50% Methanol/water, v/v).

HPLC analysis. The HPLC system consisted of a Waters Model 6000A pump (Bedford, MA), a waters U6K injector, a reversed-phase Curosil G 6 μ m (250 X 3.2 mm) column (Phenomenex, Torrance, CA) for taxol analysis, a reversed-phase Nucleosil 5 C18 (250 X 4.6 mm) column (Phenomenex, Torrance, CA) for testosterone analysis, a guard column, a Waters model 440 UV detector with a 229 nm filter for taxol and a 254 nm filter for testosterone, a Kipp & Zonen Chart recorder (Curtin Matheson Scientific, Houston, TX), and a Redifrac fraction collector (Pharmacia, Piscataway, NJ). Taxol

mobile phase consisted of 35% acetonitrile in water, and the testosterone mobile phase was 50% methanol in water. The taxol flow rate was 0.6 ml/min, and the testosterone flow rate was 0.8 ml/min. The quantitation of taxol metabolites was done by liquid scintillation spectrometry of the HPLC elute corresponding to the metabolites. The quantitation of 6 β -hydroxytestosterone was by peak height from the chart recorder.

Immunoblotting. Rat liver microsomes were subjected to discontinuous SDS-PAGE chromatography. The resolved proteins were transferred to nitrocellulose electrophoretically and Western Immunoblotting for CYP 3A was performed using a Rat Cytochrome P450 IIIA ECL Western blotting kit obtained from Amersham Life Science (Buckinghamshire, England). This kit contains rabbit anti-rat CYP 3A1 polyclonal antibodies which have been shown to cross react with CYP 3A2 and an unknown protein similar to the CYP 3A subfamily (9, 17). Quantitations of protein induction from the developed blot were carried out using densitometry scanning.

Statistical Analysis: Instat 1.11 for MacIntosh from Graphpad Software (San Diego, CA) was used to perform two-tailed p-tests on the obtained data. A p-value \leq .05 was considered significant.

Results

Taxol Metabolism: Taxol was metabolized by untreated rat liver microsomes to two major metabolites (fig. 1) in both male and female rats. These metabolites (RM1 and RM2) have retention times of 0.43 and 0.50 respectively relative to taxol. This

corresponds to the two major *in vivo* metabolites previously reported (3). The hepatic microsomes of the dexamethasone pretreated male and female rats produced three major metabolites (fig. 1) with relative retention times of 0.43, 0.50, and 0.58 respectively. The first two induced metabolites correspond to RM1 and RM2. The third metabolite (RM3) has not previously been reported in the rat.

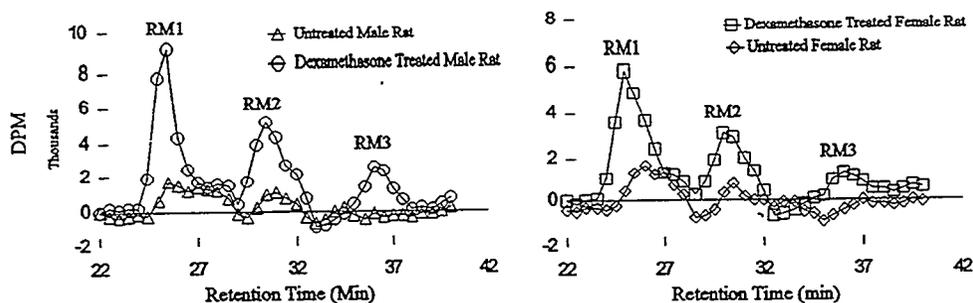


Figure 1: Reversed-phase HPLC of an ethyl acetate extract of male (left) and female (right) rat liver microsomal incubate with $10\mu\text{M}$ Taxol. Thirty second fractions were collected, and the radioactivity measured by liquid scintillation spectrometry. The region shown contains the three major metabolites. Taxol retention time was 58.5 min.

Table I and fig. 2 summarize the metabolite production rate in the treated and untreated rats. The dexamethasone treated rats showed an increased production of RM1 and RM2 in both sexes. The amount of RM1 produced by the male pretreated rats was 2.3 fold greater than that produced by their untreated counterparts. The pretreated female rat showed a 6.5 fold increase of RM1 over the control. RM2 was also increased in the treated males by 3.3 fold over control while RM2 production by the treated females increased by 8.7 fold over control. Neither sex produced a

significant amount of RM3 unless they were pretreated with dexamethasone.

There was a significant sex difference in the production of RM1 and RM2 by untreated rats. Control males produced 2.6 times the amount of RM1 that the control females produced, and they produced 3 times more RM2 than was produced by the female controls. There is no significant sex difference in the production of any of the three major metabolites in the pretreated animals.

Table I: Production rates of the three major metabolites of taxol in male and female, dexamethasone pretreated and control adult rats.

Male			Female		
Rat	Metabolite	Rate of Production pmole/hr/mg	Rat	Metabolite	Rate of Production pmole/hr/mg
DEX*	RM1	261±54	DEX	RM1	286±20
CON**	RM1	115±8.3	CON	RM1	44±23
DEX	RM2	177±38	DEX	RM2	157±11
CON	RM2	54±33	CON	RM2	18±10
DEX	RM3	77±18	DEX	RM3	68±17
CON	RM3	23±22	CON	RM3	3.7±3.7

*DEX: Dexamethasone pretreatment

**CON: Control, Corn oil pretreatment

All *p*-values < 0.05.

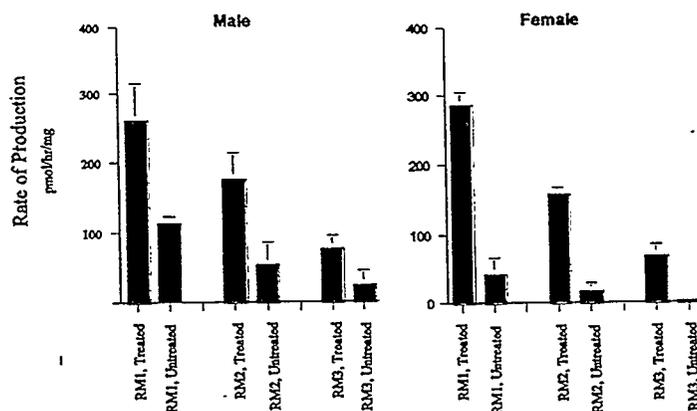


Figure 2: Induction of taxol ($10\mu\text{M}$) metabolism in adult male (left) and female (right) rats. Mean values \pm standard error of 4 male rats and 3 female rats are shown. All p -values are < 0.05 .

The statistical studies on taxol metabolism include 8 male rats (4 treated, 4 untreated), but only 3 of each class for the females. This is because the preliminary experiment which was carried out under the same conditions was included in this experimental data. No preliminary studies were performed using a female rat.

Testosterone Metabolism: It is well known that testosterone is a model substrate for the CYP 3A enzymes (9, 12, 17). It has been clearly shown that CYP 3A oxidizes testosterone to 6β -hydroxytestosterone (9, 17).

The metabolism of testosterone ($120\mu\text{M}$) to 6β -hydroxytestosterone was determined in rat liver microsomal incubations. The production of 6β -hydroxytestosterone was measured by HPLC. Table II summarizes the results of these experiments. The dexamethasone treated male rats showed 1.9 fold induction of 6β -

hydroxytestosterone production over the untreated rats. The pretreated female rats produced 3.8 times as much 6 β -hydroxytestosterone than did the female control.

A sex difference was also observed in the control rats. The male controls produced 2.4 times more 6 β -hydroxytestosterone than the female control rat. There was no significant sex difference in the production of 6 β -hydroxytestosterone in the treated rats.

Table II: Production rates of 6 β -hydroxytestosterone in dexamethasone pretreated and control rat liver microsomes.

Male		Female	
Rat	6 β -hydroxytestosterone Production nmole/hr/mg	Rat	6 β -hydroxytestosterone Production nmole/hr/mg
DEX*	65.5 \pm 4.8	DEX	54.9 \pm 3.8
CON**	34.2 \pm 4.1	CON	14.5 \pm 2.2

*DEX: Dexamethasone pretreatment

**CON: Control, Corn oil pretreatment

All *p*-values < 0.05.

Induction of Cytochrome P450 3A: There are two known isoforms of CYP 3A in the rat: 3A1 and 3A2 (11). CYP 3A1 is not constitutively expressed in adult rat liver, but it is highly inducible by treatment with dexamethasone (9, 10, 11). CYP 3A2 is constitutively expressed in male adult rats but not in adult female rats, and even though dexamethasone is known as a specific inducer of CYP 3A1, it has been shown to give a slight induction of CYP 3A2 (10, 11).



Figure 3: Induction of liver microsomal CYP 3A in female and male rats treated with dexamethasone. 5 μ g of rat liver microsomal protein was subjected to SDS-PAGE chromatography and then analyzed by Western immunoblotting as described in Materials and Methods.

- A: Dexamethasone pretreated female
- B: Control (corn oil pretreated) female
- C: Dexamethasone pretreated male
- D: Control (corn oil pretreated) male

The pretreated male rat showed an induction of CYP 3A of 3.5 fold over the control, and the pretreated female showed a 10 fold induction over the control. The control male contained 4.4 times more CYP 3A than does the control female rat. A 50 kDa CYP 3A like protein that has been reported to react with the polyclonal CYP 3A1 antibodies (9, 17). A band believed to be this protein was induced in the rats pretreated with dexamethasone, although the band is not visible in fig. 3. Quantitations of the level of induction of this individual band were impossible, however the band was included in the total CYP 3A induction calculations.

Discussion

Findings in this study correspond with previous reports in respect to both induction and the sex differences in rats of CYP 3A (10, 11). Proteins recognized by polyclonal antibodies against CYP 3A1 were expressed 4.4 fold in the male control rat as compared to the female control rat. This correlates well with the sex difference in 6 β -hydroxylation of testosterone and the production of the taxol metabolites RM1 and RM2 in the rats (2.4, 2.6, and 3 fold respectively). Production of RM1, RM2 and 6 β -hydroxytestosterone was induced by dexamethasone pretreatment of male rats by 2.3, 3.3, and 1.9 fold respectively which correlates well with the 3.5 fold increase of CYP 3A proteins in these animals. The pretreated female animals showed a fair correlation in respect to the induction of RM1, RM2, and CYP 3A proteins (6.5, 8.7, and 10 respectively), but not so well when 6 β -hydroxytestosterone production was considered (3.8 fold increase). However, 6 β -hydroxylation of taxol is only one of three oxidations that CYP 3A enzymes perform on testosterone (9, 17).

The production of a third metabolite (RM3) of taxol in the dexamethasone pretreated animals was unexpected. Preliminary studies showed that pretreatment of male rats with phenobarbital also induces the production of RM3. The observed retention time of RM3 was 0.6 with respect to taxol. This retention time corresponds to the relative retention time of 6 α -hydroxytaxol, the major human metabolite of taxol (5). Moreover, injection of 6 α -hydroxytaxol, produced by a human liver microsomal incubation, onto the HPLC system revealed an identical retention time for 6 α -

hydroxytaxol and RM3 (fig. 3). We are currently obtaining HPLC-mass spectrometric analysis of RM3 to provide more definitive structural information.

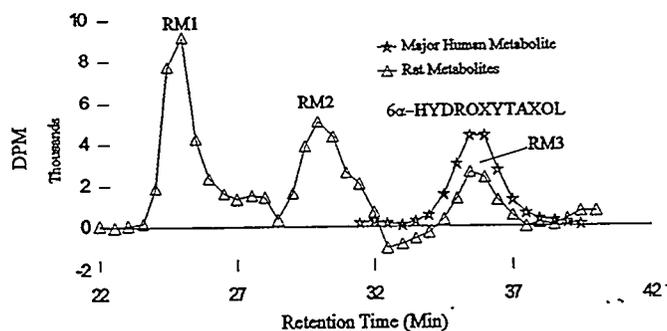


Figure 3: Reversed-phase HPLC chromatogram of a treated male rat liver microsomal incubate ($10\mu\text{M}$ taxol) overlaid by the reversed-phase HPLC chromatogram of previously obtained 6α -hydroxytaxol. Thirty second fractions were collected and subjected to liquid scintillation spectrometry. Taxol retention time was 58.5 min.

This study shows that rats pretreated with dexamethasone will metabolize taxol at higher rates than untreated rats. The metabolism increase is likely due to the CYP 3A protein increase in these rats, although the specific isoform involved is not fully known. However, it is evident that CYP 3A1 plays a major role in this increase because dexamethasone primarily induces the 3A1 isoform (10, 11). This also suggests that RM3 production in the rat is due to the 3A1 isoform of CYP; however, the possible role of the 50kDa CYP 3A like protein can not be ruled out. Further studies are needed in order to extrapolate this information to human taxol metabolism. However, these

findings have important implications for the chemotherapy with taxol because of the use of glucocorticoid drugs during such therapy. Because of the strong sequence and function similarities of the human and rat isoforms of CYP 3A, human metabolism of taxol may likely be induced by these drugs.

Acknowledgements

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Isolation, Purification and Spectrometric Analysis of PSP Toxins from Moraxella sp., a Bacterium Associated with a Toxic Dinoflagellate.

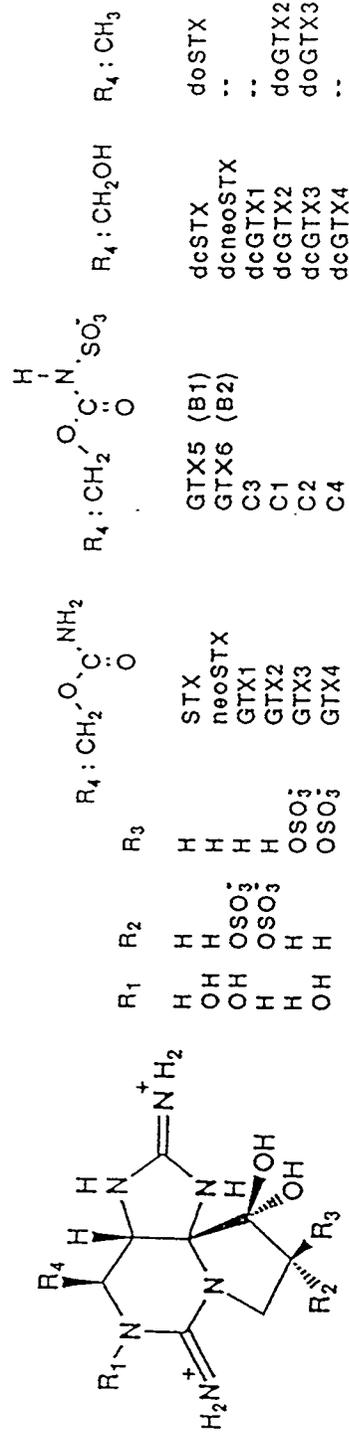
Samuel D. Boyce
Dr. Greg J. Doucette, Advisor

Introduction

Paralytic shellfish poisoning (PSP) is a seafood intoxication syndrome caused by the ingestion of shellfish contaminated with toxins produced by algae known as dinoflagellates. The PSP toxins, saxitoxin and its derivatives, act to block voltage-dependent sodium channels and can cause paralysis and even death at higher doses (3). It is well documented that bacteria coexist with many harmful or toxic algal species, though the exact nature of the association in relation to toxin production is unknown (1). Recently, the bacterium Moraxella sp. was isolated from the PSP toxin producing dinoflagellate Alexandrium tamarense (4). Through HPLC analysis and saxitoxin receptor binding assays performed on crude bacterial extracts, it appears that Moraxella sp. is capable of producing saxitoxin and several of its derivatives (2). However, physical confirmation (e.g. mass spectrometry) of these results is still needed.

PSP toxins are classified into four general groups, the carbamoyls, the sulfocarbamoyls, the decarbamoyls, and the deoxydecarbamoyls. Each of these toxins have a similar purine ring based structure with different side chains (Fig. 1). The B1, B2, C1, and C2 toxins have a low toxicity, but can be converted to more toxic forms through acid hydrolysis at 100 C (3). They can be separated and purified by the use of column chromatography, by first placing the sample on a size exclusion column to remove most impurities such as lipids or proteins and then by treating the sample with an ion-exchange resin (6). While the isolation of the toxins is not difficult, detection and identification of the toxins is a problem. Three methods that have been used successfully to identify the toxins are HPLC analysis, receptor binding assays, and ion-spray mass spectrometry. HPLC and receptor binding assays are mainly used to determine relative amounts of toxins in the sample as compared to a reference with HPLC yielding the best quantitative data (3). While these

STRUCTURES OF PARALYTIC SHELLFISH TOXINS (Oshima et al. 1993)



STX = SAXITOXIN;
 neoSTX = NEOSAXITOXIN;
 GTX1 - GTX6 = GONYAUTOXINS 1 - 6;
 dcSTX = DECARBAMOYLSAXITOXIN;
 dcGTX2 & dcGTX3 = DECARBAMOYLGONYAUTOXINS 2 & 3;
 doSTX = 13-DEOXYDECARBAMOYLSAXITOXIN;
 doGTX2 & doGTX3 = 13-DEOXYDECARBAMOYLGONYAUTOXINS 2 & 3

Figure 1

analytical methods provide strong evidence for the presence of saxitoxin, an analysis using mass spectrometry would provide a positive identification. Mass spectrometry identifies the sample based on its atomic mass. For example, saxitoxin has an atomic mass of 299 and thus gives a peak at 300 m/z on an ion-spray mass spectrometer in positive ion mode (9).

A concentrated sample of purified, bacterial PSP toxin required for mass spectrometry analysis has yet to be obtained. Thus, the objective of this research project was to produce material suitable for such physical confirmation of PSP toxin production by the bacterium Moraxella sp. The approach involved: culturing bacteria under conditions thought to maximize PSP toxin production (i.e., phosphorus limitation); chromatographic isolation and purification of the toxins; and analysis of samples by ion-spray mass spectrometry.

Materials and Methods

Bacterial Cultures

Cultures of the bacterium Moraxella sp. (strain PTB-1), were first grown up from cryopreserved material in 13*100 mm culture tubes with 3 ml of sea water complete (SWC) (see Table 1). Growth of the cultures was monitored on a spectrophotometer at a wavelength of 660 nm until the absorbance reached 1.2-1.9. At this point, 300 ml Bellco erlenmeyer flasks containing 100 ml SWC were inoculated with up to 3 ml from the culture tubes, providing an initial absorbance reading of 0.01 - 0.05. Bacterial growth in these flasks was also monitored spectrophotometrically as above. When these cultures reached an absorbance of 1.5, they were used to inoculate a fernbach flask containing 1500 ml of a low phosphorus, minimal medium (see Table 2) to an initial absorbance of about 0.01. Two experiments were performed, one with succinate as the carbon source (Toxin 26) and the other with α -ketoglutarate as the carbon source (Toxin 28). The fernbach cultures were monitored daily by spectrophotometer and were harvested when growth slowed due to phosphorus limitation at an absorbance of 0.85 ± 0.05 . The harvest began by removing and freezing two 0.5 ml samples for a protein determination. The remaining culture was divided into six 250 ml aliquots and centrifuged for 15 minutes at 8000 rpm (10° C). The supernatant was decanted and the bacterial pellets from each centrifuge bottle were resuspended in 0.5 N glacial acetic acid, transferred to a 50 ml conical tube with 1.5 ml 0.5 N glacial acetic acid, and weighed.

Samples were stored at -20 C until extracted.

Table 1
SWC Composition

Natural Seawater	833 ml
M Q Water	167 ml
Tryptone	5.0 g
Yeast Extract	3.0 g
Glycerol (50%)	6.0 ml

Table 2
Low Phosphorus Minimal Media Composition (amt / L medium)

NaCl	15.5 g
KCl	0.75 g ^g
MgSO ₄ * 7H ₂ O	12.35 g
CaCl * 2 H ₂ O	2.90 g
NH ₄ Cl	1.00 g
M Q Water	980 ml
Na ₂ HPO ₄ (2.4 mM)	41.67 ul
NaHCO ₃	10 ml
Ferric Sequestrene (0.22 g / 100 ml)	10 ml
a-Ketoglutarate (1 M)	30 ml
or	
Succinate (1 M)	37.5 ml

Toxin Extraction

The toxin contained in the bacteria was extracted by placing zirconium beads (0.1 mm diameter) and the cell pellets into polyallomar centrifuge tubes, and adding 0.5 N glacial acetic acid to achieve total volume of 20 ml. This preparation was then sonicated 3 times for one minute intervals in an ice bath. The tubes were centrifuged for 5 minutes at 9000 rpm (10° C) and the supernatant was removed. The extraction process was repeated with an additional 20 ml of 0.5 N glacial acetic acid to remove any remaining toxin. The samples were acidified to a pH of 1 with HCl and heated to 99° C for 7 minutes. Any precipitates were removed by filtration. The solution was freeze dried and then redissolved in 3-4 ml 0.1 N glacial acetic acid.

Toxin Chromatography

The concentrated sample was loaded onto a Bio-Gel P2 column (5). The column was operated at a flow rate of 0.5 ml/min of 0.1 N glacial acetic acid and 80, 9.5 ml fractions were collected. These fractions were freeze dried, resuspended in 1 ml of water, and analyzed for PSP toxin activity using a receptor binding assay (10). The active fractions were combined, freeze dried, and resuspended in water. The sample was titrated to pH 7 using 1 M Tris-HCl and then placed on a Bio-Rex 70 column (30*1 cm). Chromatography involved a pH gradient from 0-3.0 N glacial acetic acid with a flow rate of 0.3 ml/min. 6 ml fractions were collected and a receptor binding assay was performed to identify active samples. The presence of PSP toxins in active samples was further verified using HPLC according to the method of Oshima et al (8).

Mass Spectrometry Analysis

The samples were analyzed using Capillary Electrophoresis/Electrospray Mass Spectrometry. The electrophoresis was performed using a Beckman P/ACE 2100 system with a 75-50 um id column with an overall length of 97 cm and an inner wall coated with linear polyacrylamide. A Perkin Elmer SCIEX API/III+ triple quadrupole mass spectrometer was used to perform the mass spectrometry analysis. A constant voltage of +30 kV was applied to the injector end of the capillary during separations and the electrospray needle was maintained at 5 kV (6).

Results and Discussion

The growth curves for PTB-1 supplied with the carbon source succinate (Toxin 26) and a-ketoglutarate (Toxin 28) are shown in Figures 2 and 3, respectively. The log of the absorbance at 660 nm is plotted versus the time in hours. Each point along the curve represents a time at which the absorbance was measured.

While both cultures have similarly shaped growth curves that begin to level off after about 70 hours, those provided succinate grew faster and were harvested at a slightly higher absorbance than those grown on a-ketoglutarate. This was due to the slight jump in the growth rate of Toxin 26 immediately before harvesting. Both cultures were harvested at the point where the phosphorus limitation controlled the growth of the bacteria. By comparing the growth rate curves, it appears that PTB-1 prefers succinate rather than a-ketoglutarate as a carbon source.

The elution profiles from the BioGel-P2 column for Toxins 26 and 28 are given in Figures 4 and 6, respectively. The elution profile from the BioRex-70 column for Toxin 26 is given in Figure 5. All profiles were generated from a saxitoxin receptor binding assay data for each fraction for the columns. The receptor binding assay that was used detects compounds that bind to the same biological receptor as saxitoxin. A BioRex-70 column separation was not performed on Toxin 28 due to time limitations.

Both Toxin 26 and Toxin 28 BioGel-P2 elution profiles show activity around fraction 50. However, the Toxin 26 sample appears to contain much more toxin. When toxin totals are estimated from receptor assay data, it reveals that Toxin 26 has about 30 times more toxin than in Toxin 28. This is probably due to the different carbon substrates on which each culture was raised. Toxin 26 also shows some activity in fractions 64-68 and 73-77. This activity is most likely due to small concentrations of C toxins that were not completely hydrolyzed to their corresponding carbamate form. Toxin 28 appears to have been almost completely hydrolyzed as there were no active fractions above number 52. Another possibility is that the a-ketoglutarate supplied bacteria did not produce any, or produced very minute amounts, of the C toxins.

The elution profile for Toxin 26 shows several active fractions, increasing in toxicity until fraction 21 and then dropping off. In addition, there were two extremely active samples

(fractions 3 and 4) that eluted off the column. However, the pH of these two fractions was very low (about 1), and a low pH can cause false positive responses in the receptor assay.

The active fractions from all the columns were analyzed on an HPLC, and the HPLC data for fraction 15 from Toxin 26 BioRex-70 column is given in Figure 7. The GTX4 peak for both the toxin standard and the bacterial extract have a very close retention time and therefore are probably the same compound. From this HPLC analysis and from the receptor assay data, it is believed that the bacterial extract does contain some PSP toxins.

While analysis of purified samples by mass spectrometry (MS) was not performed due to time limitations, the type of data we expect to obtain are given in Figure 8. These results (8) show the mass spectrum of saxitoxin (Figure 8A) and the fragmentation pattern of the toxin when subjected to MS/MS analysis (Figure 8B). The series of daughter ions generated by MS/MS provide a "fingerprint" that allows unambiguous identification of a compound exhibiting a given atomic mass.

Summary

The column chromatographic procedures that were developed for the purification of algal PSP toxins were used effectively to purify bacterial PSP toxins. The saxitoxin receptor binding assay that was used to produce the elution profiles from the columns, is evidence that the bacterial extracts did contain PSP toxins. Further evidence that the bacterial extracts contained toxin was the HPLC analysis on the active fractions from the columns.

Future Research

While the analytical data produced from this research shows that PSP toxins were produced by the bacteria, the samples still need to be analyzed further. An ion-spray mass spectrometry analysis will be run on the samples. Also, the column chromatography methods will continue to be used in labelling studies of PSP toxins.

GROWTH CURVE

Toxin 26

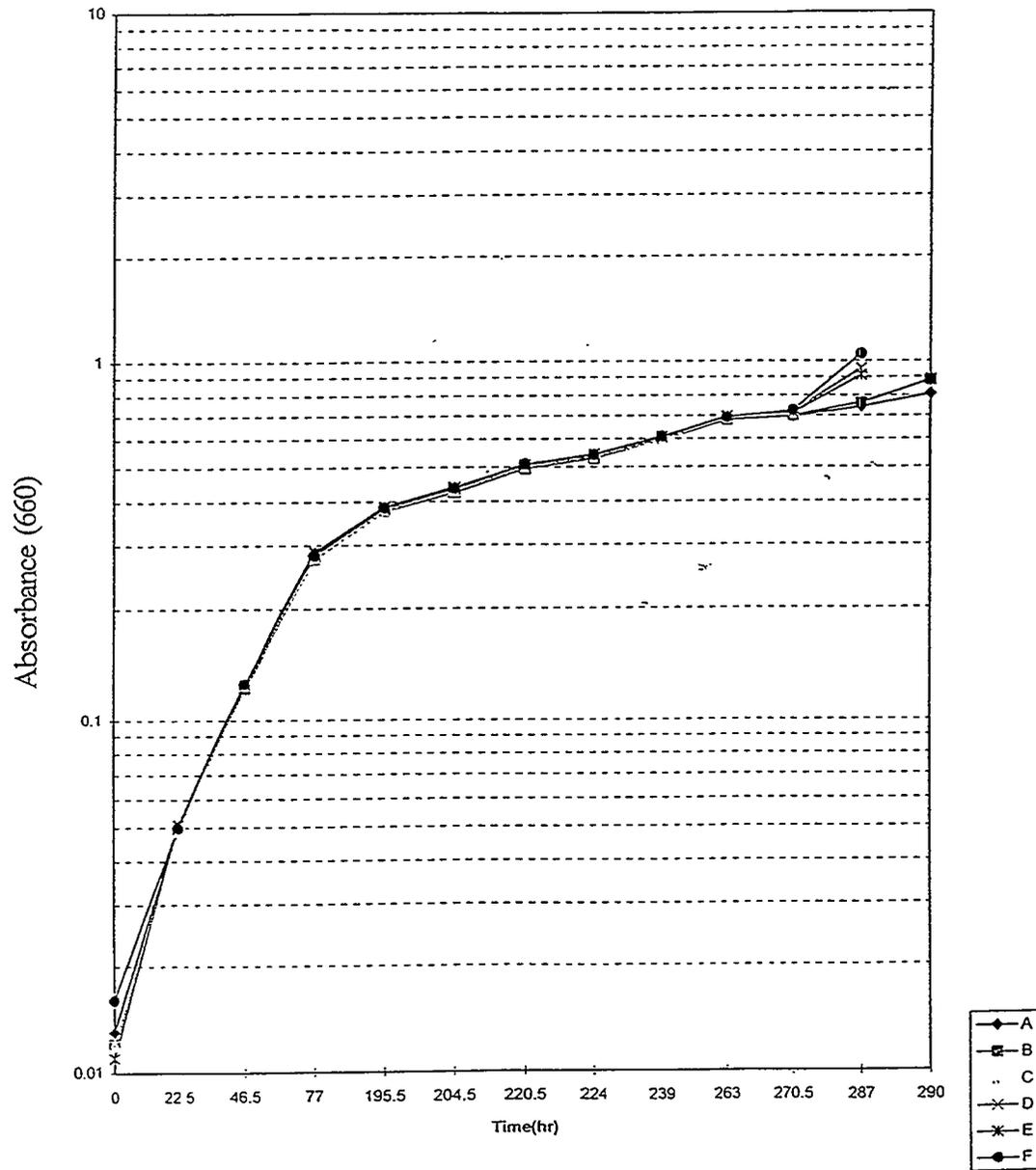


Figure 2 - Growth curves for Toxin 26 bacteria cultures grown in low phosphorus medium with succinate.

GROWTH CURVE

Toxin 28

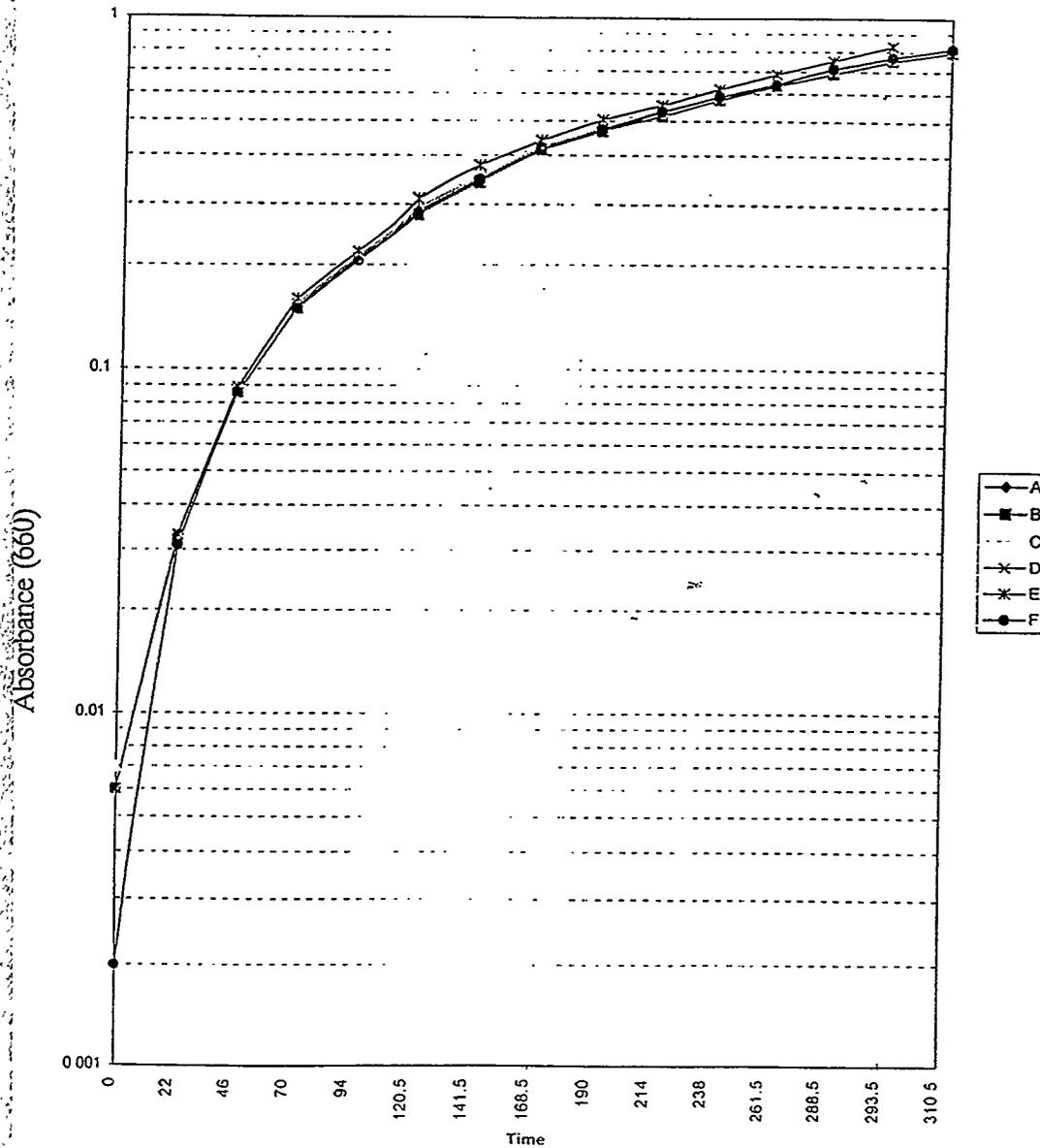


Figure 3 - Growth curves for Toxin 28 bacteria cultures grown in low phosphorus medium with α -ketoglutarate.

EXPERIMENT: TOXIN #26

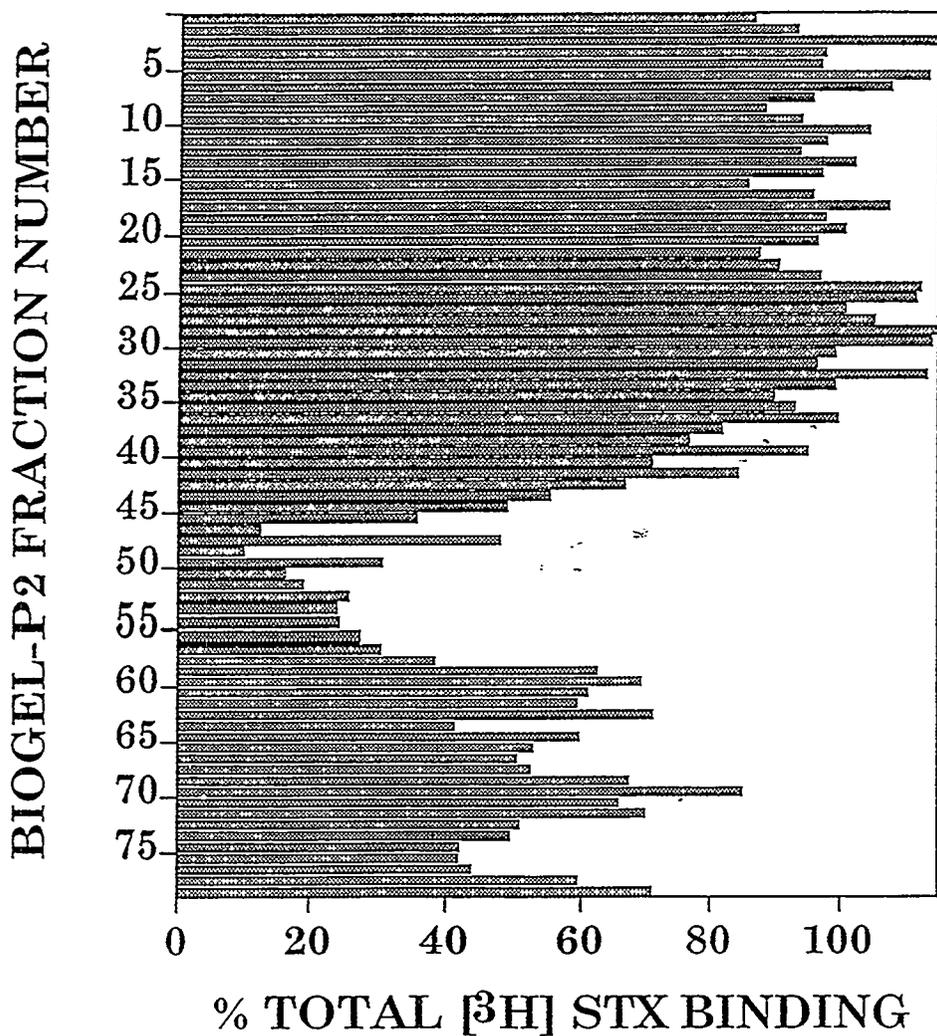


Figure 4 - Elution profile for Toxin 26 BioGel-P2 column. Activity of fractions determined by saxitoxin receptor binding assay. Low percent total binding indicates high toxin content in the fraction.

EXPERIMENT: TOXIN #26

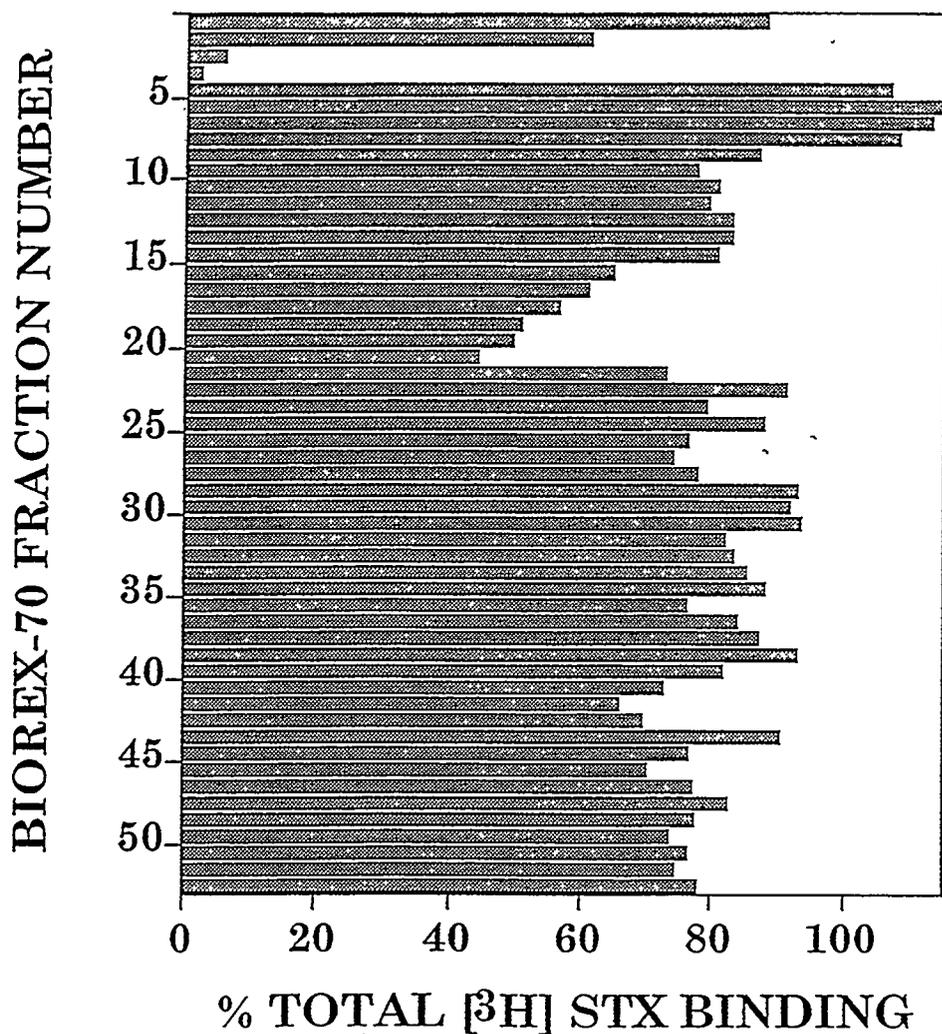


Figure 5 - Elution profile for Toxin 26 BioRex-70 column. Activity of fractions determined as in Figure 4. See text for explanation of apparent activity in fractions 3 and 4.

EXPERIMENT: TOXIN #28

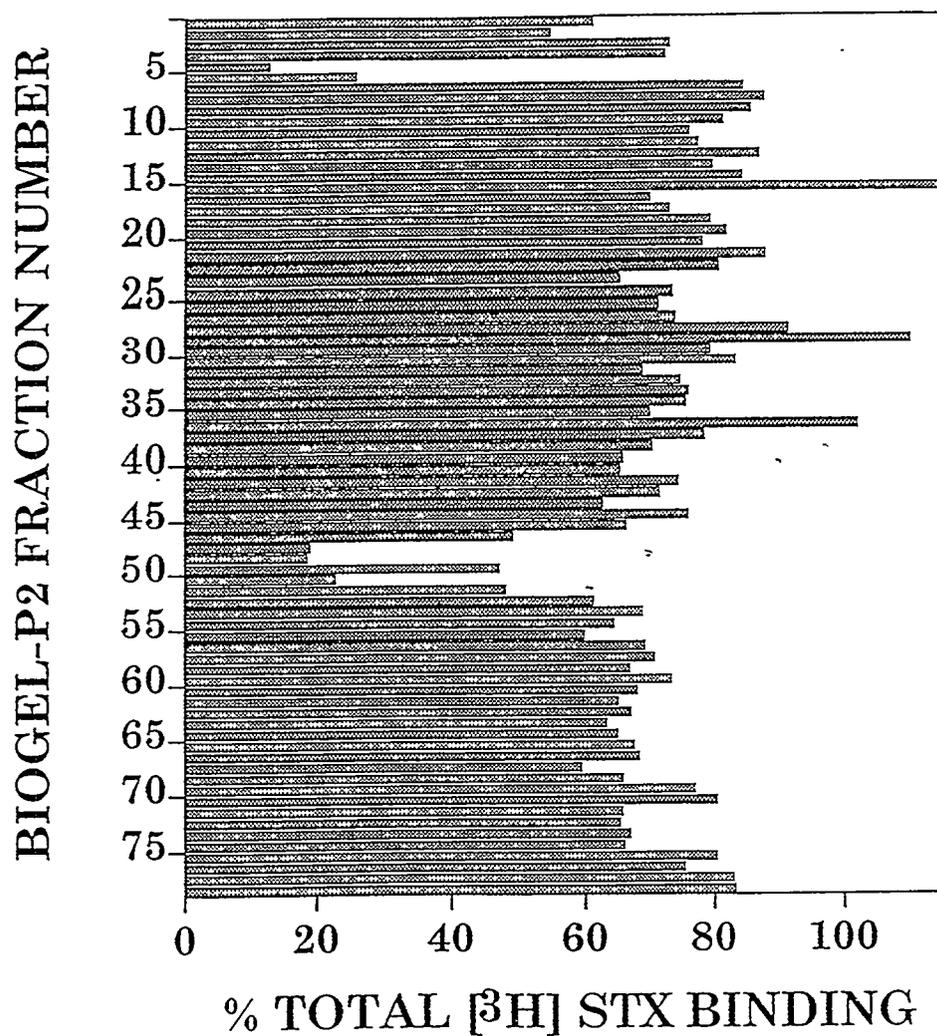


Figure 6 - Elution profile for Toxin 28 BioGel-P2 column. Activity of fractions determined as in Figure 4.

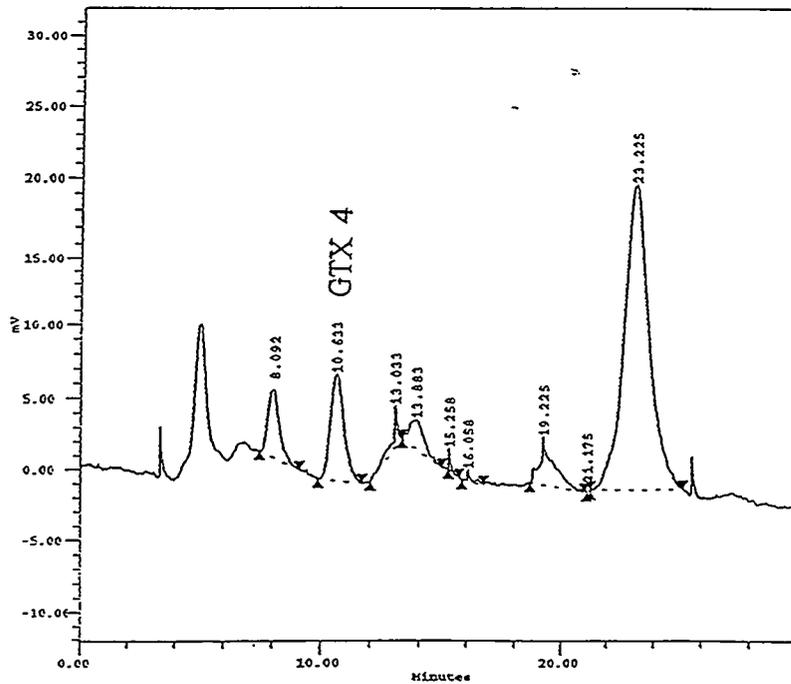
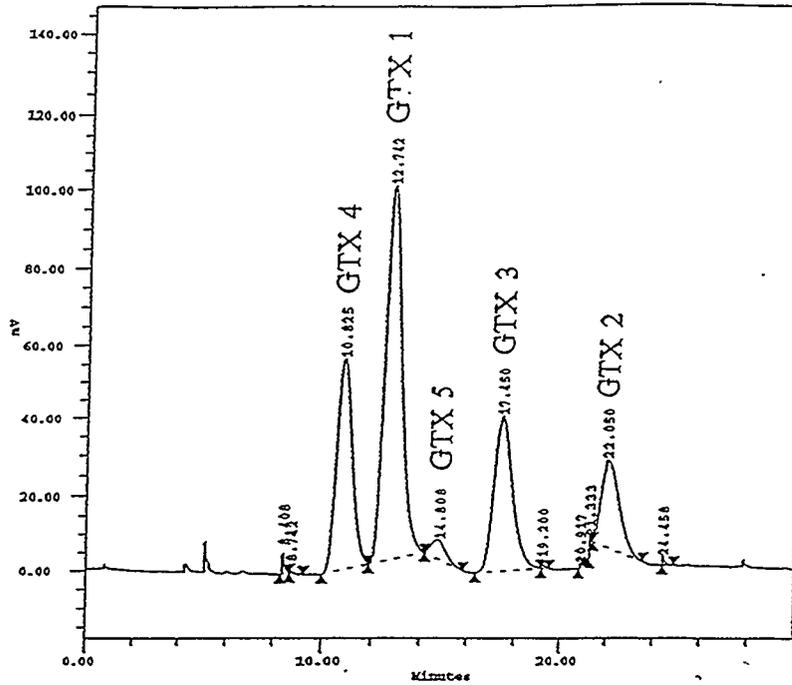


Figure 7 - A: HPLC analysis of gonyautoxin(GTX) standards. B: HPLC analysis of fraction 15 form Toxin 26 BioRex-70 column. Data indicate presence of toxin GTX4 in bacterial sample.

ANALYSIS OF SAXITOXIN USING ION-SPRAY MASS
SPECTROMETRY (data of Quilliam et al., 1989)

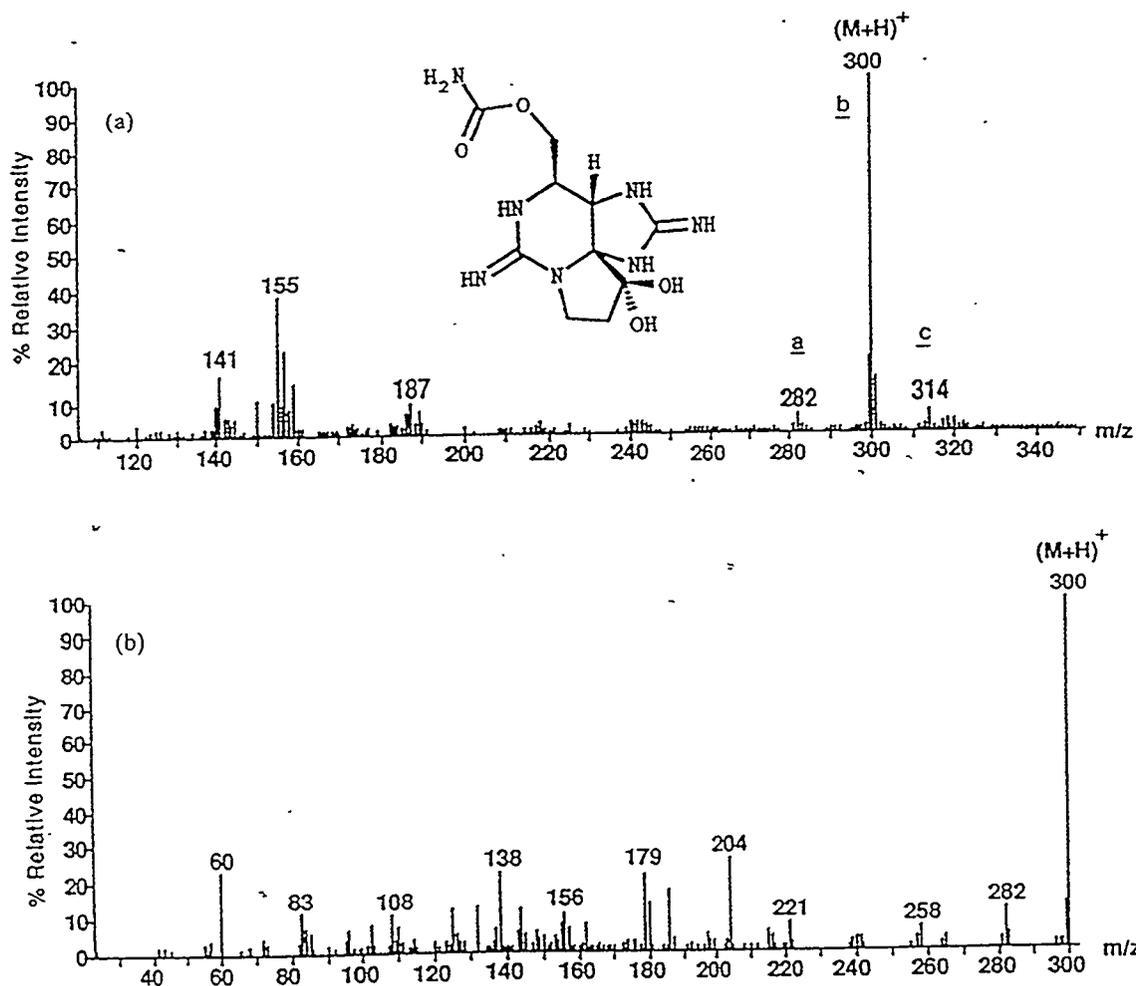


Figure 8 - A: Expected Mass Spectrometry results for samples containing saxitoxin. B: Expected MS/MS results for samples containing saxitoxin. (Actual results will be available when mass spectrometry analysis of samples is completed).

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ABSTRACT

Oyster larvae, *Crassostrea virginica*, were exposed to 20 ppb of cadmium(Cd) and fed (mixture of *Isochrysis galbana* & *Chaetoceros gracilis*, 40mL) in the laboratory for 10 days. On the 0, 4, 7 and 10 day the larvae samples were taken and frozen. Then they were homogenized, centrifuged, ultrafiltered through a membrane separation technique used to segregate substances according to the molecular weight and size, and then the cytosolic protein was first partially purified by gel permeation, then by PAGE (Polyacrylamide Gel Electrophoresis). The controls and metal exposed larvae were evaluated on total wet weight and the metallothioneins(MT) were identified from the preparations using silver staining techniques.

No significant changes could be detected in controls. However, there was a great number dead at the beginning of experiment. Cd accumulation began at time of exposure this suggests that surface area may play a role in determining short-term accumulation rates(Ringwood, 1991). Cd effects on growth(wet weight) was slightly different, the exposed weighed less than or equal to the controls. In addition to, the Cd uptake via food played an insignificant role compared to direct uptake from sea water (Riissard, H. U., et al. 1987). Between day 0 and 7 there was a number of mortalities for the controls and exposed. In addition to, there was a major weight change with the exposed, they appeared to weigh less than the controls on day 7, where as on day 4 they weighed more. So weight is a very sensitive indicator of toxic stress.

INTRODUCTION

In marine pollution monitoring systems, bivalve mollusc have been used extensively for environmental indicators for trace metal pollution. It is essential to conduct studies of the effects of toxic metals on the physiological and biochemical responses of the organisms, so that stressed populations can be identified (A.

Crassostrea virginica

1

The Effects of Cadmium on the growth and Metallothionein
expression of the bivalve larvae, *Crassostrea virginica*

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Running Head: *Crassostrea virginica*

Viarengo, M. Pertica, G. Mancinelli, S. Palmero, G. Zancchi, and M. Orunesu, 1981).

Metals tend to be insidious toxins, whose effects are often chronic, rather than acute, so although levels high enough to cause sudden kills of organisms are rarely achieved, chronic long-term effects may be serious. When animals are stressed by altered environmental conditions, homeostasis is disrupted, and animals must adjust, behaviorally or physiologically, to adapt to the surrounding conditions. If the adaptations for re-establishment of homeostasis are insufficient, the organisms will suffer chronic stress or die (Ringwood, 1992). Reports have indicated that metals induce rapid biosynthesis of low molecular weight proteins. These proteins are capable of binding the metal and have properties similar to metallothioneins (MT) (Viarengo, et. al., 1980). Metallothioneins (MT) are a class of low molecular weight, soluble proteins characterized by a high metal affinity, heat stability and by an unusually high content of cysteine (30-35%) (Margoshes & Vallee, 1957; Kagi & Vallee, 1967; Weser et. al., 1973); the inducible nature of these proteins seems to imply that MT's play a role in the detoxification of heavy metals (Piscator, 1964; Shaikh et. al., 1971; Squibb & Cousins, 1974).

In this study, bivalve larvae (*C. virginica*), will be exposed to cadmium (Cd) and the effects of the exposure on the growth and metallothioneins (MT) expression will be determined. It has been hypothesized that the initial severe effects occur prior to the induction of MT's, and that partial recovery coincides with MT production. In previous studies, young larvae exposed to Cd have shown severe adverse effects on growth initially followed by a period of partial recovery, and sustained adverse effects with continued exposure. However, preliminary studies have suggested that MT's are not produced by bivalve larvae until between 3 and 7 days after Cd exposure (Ringworm, unpublished).

The present work will demonstrate that bivalve larvae are capable of MT expression, although MT's have been identified in adults and embryos, that has not been demonstrated in the larval stage. Concurrent studies evaluating the effect on growth will enable consideration of the toxicological significance of MT expression and insights into the adverse effect of metals on larval growth. Growth is a particularly useful stress index because it is the result of integrated biochemical and physiological responses of an organism to environmental conditions (Widdow, 1985). Another goal of chronic toxicity studies is to identify responses that could be used as a signal of adverse environmental conditions before the biota are irreversibly damaged. It is important to use species or life stages and responses that are sufficiently sensitive (Ringwood, 1992).

MATERIALS AND METHODS

Bivalves (oysters, mussels, clams) can be spawned in the laboratory, providing precise control over the time of fertilization and culture conditions. Furthermore, the high degree of synchronous development, especially during the early stages, and the inherent markers of distinct developmental periods make this system amenable to experimental manipulation. The description of development that follows occurs in oysters, *Crassostrea virginica*, but the events that characterize embryogenesis, larval development, and metamorphosis are remarkably conserved among bivalve taxa. Embryogenesis consists of a pre-hatching phase, dominated by cell division, that lasts approximately 6-8 hr, and a post-hatching phase, involved primarily in differentiation. Rates of transcription of the post-hatching stages are more rapid than during cleavage stages. By approximately 16 hr after fertilization, embryos develop into trochophores, an unshelled nonfeeding stage that is short-lived. Formation of the first shell begins during the late trochophore stage, and originates from an aboral region referred to as the shell field

invagination (SFI) (Kniprath 1979, Eyster, and Morse, 1984). Although the functions of SFI are not understood, they are assumed to be involved in matrix deposition, and nucleation and initiation of calcification. By 24 hours, the first shell fully encases the larva, now referred to as a veliger (Ringwood, 1988).

The gametes of adult oysters were spawned and stripped and the embryos and larvae were fed and reared in filter seawater. The initial concentration was 31.5 larvae mL (total volume 20L), but then was diluted to 10-15 larvae mL (total volume 30L) into 5L containers. The larvae were exposed to 20 ppb of Cd and samples were taken over a period of 10 days. The seawater was changed every other day, the larvae were fed a mixture of *Isochrysis galbana* & *Chaetoceros gracilis*, 40mL, and fresh Cd was added to the exposed. On days 0, 4, 7, and 10 small samples were taken and weighed for the effects on growth and samples were taken for MT analysis. All samples were then frozen at -20 C. Then samples were thawed, homogenized, centrifuged, ultrafiltrated through a membrane separation technique used to segregate substance according to molecular weight and size, and then the cytosolic protein was first partially purified by gel permeation then by PAGE (Polyacrylamide Gel Electrophoresis). The use of controls and metal exposed larvae were evaluated on the basis of total wet weight.

RESULTS

The 10 days of exposure to cadmium (Cd) for the bivalve larvae, *Crassostrea virginica* showed effects on weight in the short-term experiment (Figure 1). Days before Cd exposure a great number of larvae were dead. After exposure more deaths were observed for both controls and exposed. Larvae of *C. virginica* accumulated Cd upon immediate exposure. There was no lag period similar to that which has been observed with embryos. It can be seen that

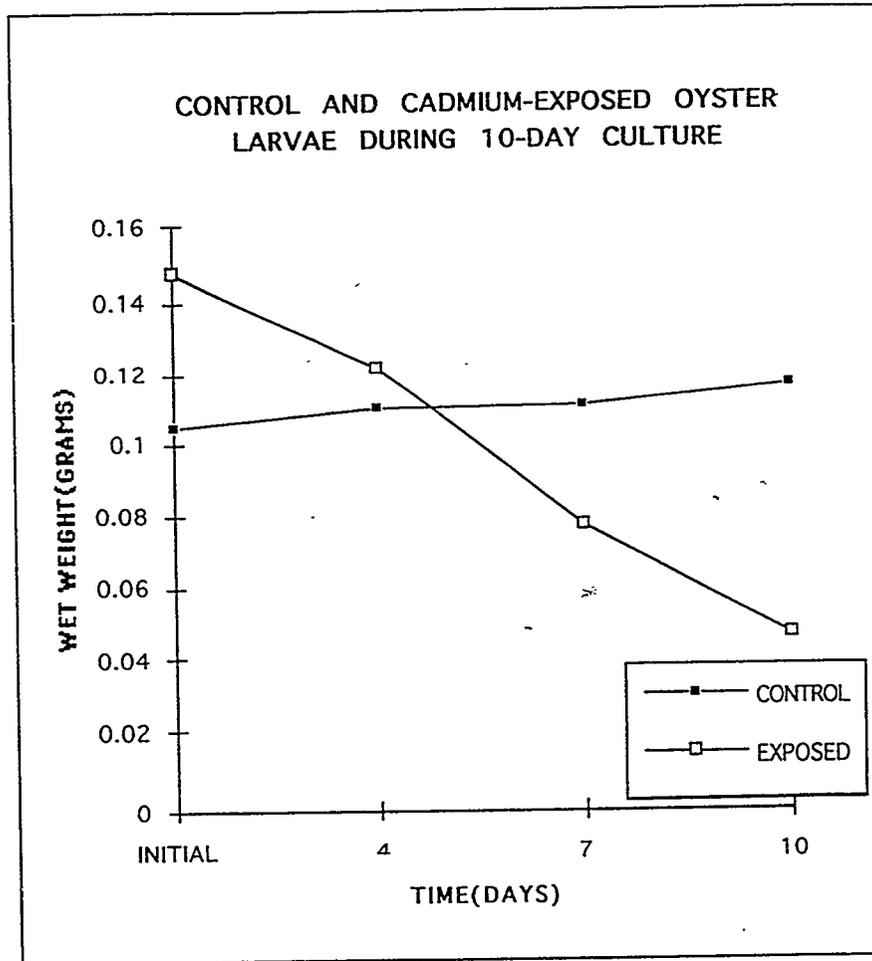


Figure 1. Weights of control and cadmium-exposed oyster larvae, *Crassostrea virginica*, cultured for 10 days in filtered seawater. Larvae were exposed to 20 ppb cadmium.

between 4 and 7 days of exposure, adverse effects on growth and with continued exposure growth declined. The exposure periods corresponds to the severe effects on growth.

Larvae data were compared on the basis of total wet weight during the 10 day experiment. Because the shells and tissues of the larvae were analyzed simultaneously, the determination of tissue accumulation of Cd by larvae required extrapolation of the data. It appears that weight is very sensitive and corresponds with increase in mortalities therefore, could be used to explain variations in the growth patterns. Initially, there was a great deal of mortality, which may have skewed initial weight measurements.

DISCUSSION

During the 10 day experiment the growth of the exposed was significantly lower than that of the control larvae. However, at the beginning of the experiment a number of mortalities were observed, before exposure began. But throughout the 10 days the effects were observed more for the exposed.

In the open ocean or in nonpolluted seawater, Cd concentrations are less than or equal to 0.01 ug , but in polluted estuaries, values as high as 50 ug L have been reported (Holmes et al. 1974, Coombs 1979, Fasset 1980). Therefore the concentration used in this experiment (20 ug L Cd) is environmentally realistic. These results indicate that even moderately elevated concentrations of Cd in coastal environments could adversely affect larval growth and MT expression(Calabrese et al; 1971, Beaumont et al, 1987). So therefore growth and MT expression could correspondently be used as indicators for toxicity. When animals encounter changing environmental conditions they respond in an adaptive manner. The immediate response involves behavioral and/or physiological changes and is often characterized by a response such as growth being dramatically depressed. The immediate response is followed by a period of readjustment , and finally establishment of a new steady state. However, with continued environmental degradation

the organisms will suffer chronic long-term stress or die (Ringwood, 1992).

Accumulation of Cd by larvae of *Crassostrea virginica* occurred upon exposure, adverse affects were observed on growth between days 4 and 7 and with continued exposure the growth declined even more. The growth results of the experiment were consistent with other findings. The MT expressions were not observed in the larval stages due to gel electrophoretic problems. Accumulation rates may be due to a variety of factors including differences in (1) metabolic rates, (2) surface area and (3) accumulation mechanisms. The biological roles of metals during the development of larvae marine invertebrates are unknown. Short-term uptake rates of larvae were significant and MT expression was not observed in larvae due to technical problems.

It seems reasonable to conclude that the bivalve larvae were affected by the concentration of Cd (20ug L⁻¹). Moreover, the weight appears to be a very sensitive indicator of adaptive response and stress. But that the toxicity depends on how much of it enters the organism. If the toxicity exceeds the capabilities of the detoxification system, irreversible damage occurs or death. Organisms that can sequester the metal uptake may be able to partially recover with depuration, but if there is continued exposure the ability to continue to regulate would decline.

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The Isolation and Purification of a Caribbean Maitotoxin

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Introduction

The phenomenon known as red tide has been a topic of great interest in the past several years. When these blooms of algae are toxic, they pose a major threat to human and marine life. There is a "conviction among many experts that the scale and complexity of this natural phenomenon are expanding. They note that the number of toxic blooms, the economic losses from them, the types of resources affected and the kinds of toxins and toxic species have all increased" (Anderson, 1994). A bloom develops when single cell algae photosynthesize and multiply rapidly. A serious impact of these toxic blooms also occurs when clams, mussels, oysters or scallops ingest the algae as food and sequester the toxins in their tissues (Anderson, 1994). Ingestion of the toxins result in poisoning known as ciguatera seafood poisoning. Extensive research began 15-20 years ago when there was a serious outbreak of ciguatera seafood poisoning. Takeshi Yasumoto was commissioned to investigate the outbreak. He detected toxin in the surgeonfish *Ctenochaetus striatus* and named the toxin for the Tahitian name of the fish, maito (Yasumoto *et al.*, 1976). Yasumoto also observed the diet of the fish and of the morey eel (in which toxin was also detected). The fish and eel were feeding on smaller fish which fed on dinoflagellates, specifically *Gambierdiscus toxicus*. Through mass culturing of the dinoflagellate Yasumoto was able to obtain a partial structure of a Pacific maitotoxin (Murata *et al.*, 1992) and eventually a complete structure (Murata *et al.*, 1993).

It is known that the benthic dinoflagellate, *Gambierdiscus toxicus*, produces a variety of polyether toxins that contaminate seafood and result in human illness. Many different toxins including maitotoxin, ciguatoxin, and

**The Isolation and Purification of a Caribbean
Maitotoxin**

brevetoxin originate from *Gambierdiscus toxicus*. Maitotoxin (MTX) is one of the toxins that have been implicated in ciguatera seafood poisoning (Yasumoto *et al.*, 1976). Some believe that ciguatoxin could be a precursor or degradation product of maitotoxin. A necessity exists for the development of a much broader understanding of the nature of the poisoning toxins. MTX congeners can be difficult to isolate due to its size and chemical nature. To date it is the largest fully characterized naturally occurring product known (figure 1). However, isolation and purification of MTX is essential. A major goal of the natural products chemistry program at the National Marine Fisheries Service is to obtain a purified standard of a Caribbean MTX so that more efficient assays can be developed. The assays would be used to test seafood for the presence of toxins and thus avoid human harm. However, separation of MTX is a very complicated procedure. The toxins are not single chemical entities but families of compounds having similar chemical structures and effects (Anderson, 1994). It is vital that the individual congeners of toxin be separated from each other in as few steps as possible. With every additional column the toxin is run through, more toxin is lost. The fastest most efficient procedure is being sought.

Purpose

To isolate, purify, and structurally characterize a large, polar Caribbean maitotoxin congener.

Procedure

Gambierdiscus toxicus cells from the Caribbean are obtained from mass culture and sonicated in methanol on full power for ten minutes. The cells are contained within a tough exterior covering, called theca, that must be broken in order for the toxin to be released. A pre-purification step involving a solvent partition protocol is carried out using a liquid/liquid extraction technique. The methanol is first extracted with non-polar hexane to remove many non-toxic materials. It is then taken down to dryness and dissolved in 60% methanol and 40% water. A methylene chloride extraction is next employed on the methanol extract to remove the ciguatoxin present in *Gambierdiscus toxicus*. The

remaining methanol extract is then prepared for liquid chromatography. It is taken down to dryness and then dissolved in 50% methanol (the first mobile phase). The methanol extract is then filtered using a glass fiber filter and a teflon syringe filter. The filtered extract is then subjected to medium pressure liquid chromatography.

The sample is auto injected onto a C-18 Biosil column (40 x 350 mm, 35-40 μm , Biorad Labs, Richmond, CA). After several runs it was deduced that 1 liter of 50% methanol and then 1 liter of 60% methanol could be run through the column and collected in bulk since no toxin was removed with these eluants. At 70% methanol 25.6 mL fractions are collected in 30 mL test tubes. This collection is continued for 1 liter of 80% methanol and the first 500 mL of 90% methanol. The second 500 mL of 90% methanol and 1 liter of 100% methanol are collected in bulk. The bulk collections are taken down to dryness and dissolved in methanol and tested for toxicity.

Each collected fraction is tested for toxicity against one or more bioassay techniques. A cytotoxicity test involves preparing 96 Well Cluster plates. Cultured cells are removed from a culturing flask using sterile PBS/EDTA for GH4C1 cells. First the growth media is suctioned out. Then 4 mL of PBS/EDTA is added and the flask is allowed to sit for 1 minute. The solution is again suctioned out and the flask is placed back in an incubator for 4 minutes. Next 8 mL of media is rinsed over the cells in order to harvest them. Then 9 μL of the media is pipetted onto a hemacytometer and the cells in a 4x4 area are counted. The number of cells counted are multiplied by the amount of media added (8) and by 10,000. That number is then divided by 500,000. This formula calculates the total amount of media that should be mixed with the cells. Then 100 μL of the media is pipetted into each well of the 96 Well Cluster plates except the first column. Plain media is placed in the first column as a control. Also as a control, no samples are tested in the second column. The fractions are tested by pipetting 2 μL of each into the prepared 96 Well Cluster plates. Approximately 18 hours later, 15 μL of MTT/PBS (500 mg C₁₈H₁₆N₅SBr and 100 mL PBS) dye is added

and then four hours after that 100 μ L of a solubilization solution (50 g sodium lauryl sulfate, 495 mL H₂O, 5 mL 1 N HCl) is added. In several hours the plates are read using a Titertek Multiskan^R PLUS plate reader. Since each well of the plate contains live cells those that are destroyed indicate the presence of toxin. The wells are read as positive, partially positive, partially negative, or negative. It is through this technique that the toxins can be further separated. Performing cytotoxicity tests on dilutions of the samples is also useful. This involves pipetting 10 μ L of sample into 90 μ L of methanol. This sample is then tested for toxicity. Data from the dilutions indicates where the strongest toxins are eluting. The toxic fractions are pooled together and taken down to dryness and dissolved in the appropriate mobile phase. As the purification procedure progresses it becomes necessary to use greater precaution in the pooling of the samples. Fraction tubes are rinsed several times with methanol and water and then sonicated to insure that all the toxin is being obtained. Also, as the procedure progresses, the amount of mobile phase needed to dissolve the dry toxic fractions in becomes smaller. This is because each step is separating out more impurities.

MTX is a Ca²⁺ activator (Yokoyama et al., 1988). The toxic fractions were also tested by a Calcium Flux assay (Van Dolah, In Press). To insure that the toxin was actually MTX this assay was utilized. A positive test indicated the presence of MTX.

At this stage the toxic areas are split into four distinct toxic bands by chromatography. The toxic bands are separated and further purified by high pressure liquid chromatography. First the crude toxin is applied to an Alltech Econosil C-18 semi-prep column (22.5 x 250 mm, 10 μ m) using an acetonitrile/water gradient as eluant. The gradient starts at 20% acetonitrile for 20 minutes and then increases to 43% acetonitrile over a period of 92 minutes. Finally the gradient reaches 100% acetonitrile at 169 minutes. All the toxic bands were run by this procedure at 8 mL per minute and 160 1 minute fractions were collected. The fractions were each tested for toxicity using the same cytotoxicity technique described above. Peaks were detected using a Waters 991

Photodiode Array Detector. This information is useful in correlating where the toxin is eluting in various samples.

Next a Keystone Hypersil SAS C-1 column (4.6 x 150 mm, 5 μ m) is used in an isocratic run of 35% acetonitrile and water with appropriate buffers (87.7 mM KH_2PO_4 , 12.3 mM K_2HPO_4 , and .20 mM $\text{CaCl}_2\cdot\text{H}_2\text{O}$). The buffers maintain a pH of 6. Fractions (105) are collected at .5 minute intervals with the column running at 1 mL per minute. Cytotoxicity tests were again performed to guide fractioning.

The C-18 semi-prep column is again employed to remove salts and residual buffer. An acetonitrile and water gradient was again employed. The column ran at 15% acetonitrile for 20 minutes and increased to 60% acetonitrile over 10 minutes and remained there for 10 more minutes. Then the concentration is dropped back down to 15% acetonitrile over ten more minutes. Fractions were collected from 20 minutes until 45 minutes at 8 mL per minute.

After more toxicity tests a Tosohaas TSK-Gel G2500PW_{X1} size exclusion column (7.8 x 300 mm, 6 μ m) is utilized. An isocratic run of 35% acetonitrile and 65% water is run at 1 mL per minute and fractions are collected every .5 minute. After toxicity testing, the C-1 column is employed as the final step. The column is run at 1 mL per minute using the same buffer solution and .5 minute fraction are collected. The toxic fractions are again desalted and then analyzed for their purity. The purified toxin is then analyzed by nuclear magnetic resonance on a 500 MHz Bruker NMR.

Results

Liquid/Liquid Extraction:

This is the first step in purifying and isolating MTX. Liquid/Liquid extraction removes a large bulk of impurities and also separates ciguatoxin [congeners] from maitotoxin [congeners]. The extractor apparatus used constantly provides a fresh supply of the extracting liquid for maximum efficiency. The extraction technique is also very important because it is mild and does not damage the toxin. The non-polar hexane extraction removes non-toxic

literature called for .50 M. The CaCl₂ concentration was reduced to .2 M so that there would be less precipitate to filter. Also, the acetonitrile and water concentration was changed to accomodate MTX and provide better retention.

The relative polarity of the different MTX congeners can be determined by their elution times. Figure 4 shows the division of the toxic areas into 3S1, 3S2, 3S3, and 3S4. Again, the different toxic areas were separated and run individually on the subsequent step. The buffers are removed by the desalting step.

TSK-Gel Column:

This is the only size exclusion column used. Since MTX is so large it elutes from the column quickly. The first procedure used 20% methanol and water. However, due to hydrophobic interactions, toxin retention in the column was too long and was then being released gradually (figure 5). A size exclusion column should theoretically have no chemical interactions. Ideally it should only retain by size. A new procedure of 35% acetonitrile and water was tried. This time the toxin eluted in a sharper band (figure 6). This procedure was utilized for the rest of the runs. It was hoped that this procedure would be the last step but the toxin was still not pure enough. MTX is a white powder so any color in it indicates impurities. Another step had to be added to the procedure to remove colored impurities.

C-1 Column with Buffers:

This column was utilized as a final step. The separation it provides is good but many partial positives and partial negatives appear. The chromatograms of the toxic areas were examined closely. MTX does not have a strong chromophore. Adsorption at 224 nm is only in the range of .006 to .020 adsorption units (figures 7 and 8).

Nuclear Magnetic Resonance:

Discussion

The primary goal of this project is to obtain large amounts of a pure maitotoxin. The source of the toxin, *Gambierdiscus toxicus*, contains several

non-polar impurities. The methylene chloride extraction removes toxic non-polar impurities. This is the step which separates the two toxic families. The less polar ciguatoxin remains in the methylene chloride while the more polar maitotoxin remains in the methanol.

C-18 Biosil Column:

This column is not very efficient but it is inexpensive and quite useful because the sample is still very impure. This reversed phased chromatography uses water as the weak solvent and methanol as the stronger [eluting] solvent (ie. the methanol gradient is slowly increased) to separate by polarity. The toxic area was divided into four distinct bands based on the cytotoxicity results. The bands were labeled S1, S2, S3, and S4 and were run individually on the next step.

C-18 Semi-prep Column:

This column also separates materials based on their polarity. On the run the gradient used was acetonitrile and water. As the acetonitrile concentration increased, the toxin was eluted. Figure 2 and 3 show how the toxic areas were split into four fractions (2S1, 2S2, 2S3, and 2S4) based on where the toxin was eluting. Each toxic area was run separately on the next step. This column provides better separating resolution than the C-18 Biosil column and therefore provides better separation. The column is reversed phase which is well suited for polar compounds. MTX demonstrates good retention times.

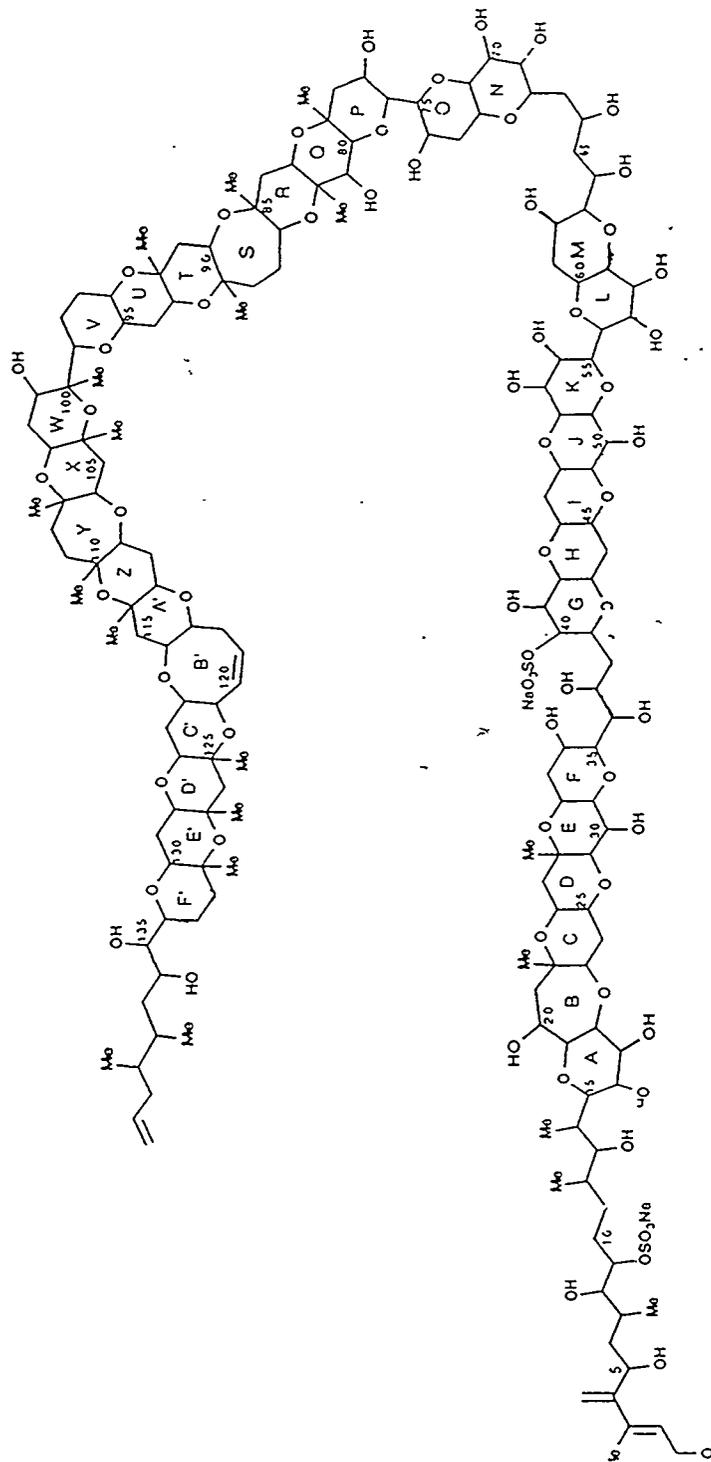
C-1 Column with Buffers:

This column also separates by polarity and it is identical to a column used in literature on the separation of MTX (Murata, 1991). The buffers utilized in this step, however, are different than the ones utilized in the literature. The buffers maintain a pH of 6, MTX is not stable in stronger acid. The pH of 6 is not acidic enough to destroy the toxin but is acidic enough to interact with the toxin. The buffers interaction stabilizes the toxin, thus helping to sharpen the peaks on the chromatogram. Sharper peaks require fewer collection tubes and subsequently better resolution. The concentrations of the buffers used is very different from the literature. CaCl_2 is saturated in water at .19 M whereas the

suites of toxins including ciguatoxin and maitotoxin congeners. However, it is difficult to know how many congeners of MTX or other toxins are present. After the first liquid chromatography run the toxic area was split into four distinct, active fractions. Some overlap of the different toxic bands was expected. However, after the second and third step in the procedure each toxic band split into four fractions again. This may be due to different conformations of the toxins or to different reactivities on different columns. As the techniques are further developed to separate specific targeted maitotoxins, other toxic congeners will inevitably be isolated as well. Thus the procedure described will also be useful as a starting point for the purification of other toxins. The HPLC protocols used in the procedure also require fine tuning. The size exclusion column showed varying results. Most of the time it showed good separation based on the fact that several non-toxic tubes had color. However, at other times, every tube with color was toxic; hence there did not appear to be efficient separation. Different concentrations of solutions and buffers as well as different elution times will need to be optimized. The C-1 column produced many partial positives and partial negatives fractions in toxicity. This creates a dilemma because if the partials are included it may make the sample less pure. However, if the partials are not included valuable sample would be lost. It is difficult to decide if the fractions are partial because they are dilute or impure. MTX associates with the pigments in *Gambierdiscus toxicus*. The pigments are very difficult to remove because they co-elute with the toxin. More time is required in order to eventually reach the optimal protocol for the isolation and purification of MTX. Pure MTX is currently in high demand so at times it is necessary to proceed with a procedure even if it has not yet been optimized. It is general procedure to have columns that separate by different methods to insure the best separation possible. After the size exclusion run the product was still slightly impure so the sample was run through the C-1 column again. Future work will include a size exclusion column before the C-1 column to see if the repetition of the C-1 column can be avoided.

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Mw 3422 (1.5minal)
 $C_{164}H_{256}O_{68}S_2Na_2$

Figure 1. The structure of a maitotoxin.

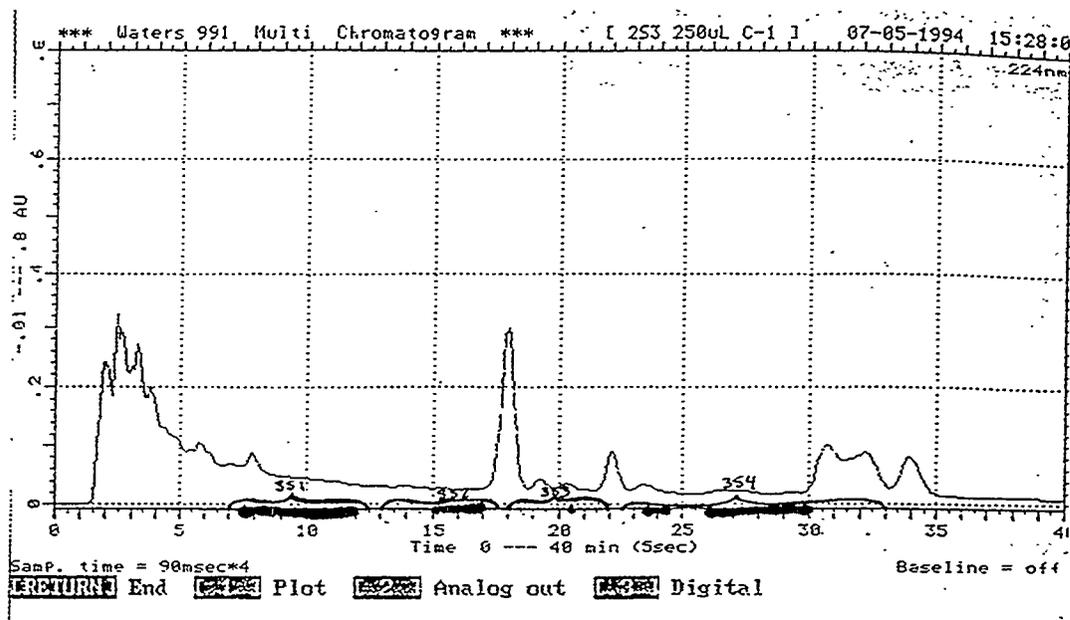


Figure 4. Chromatogram of a C-1 column with buffers run.

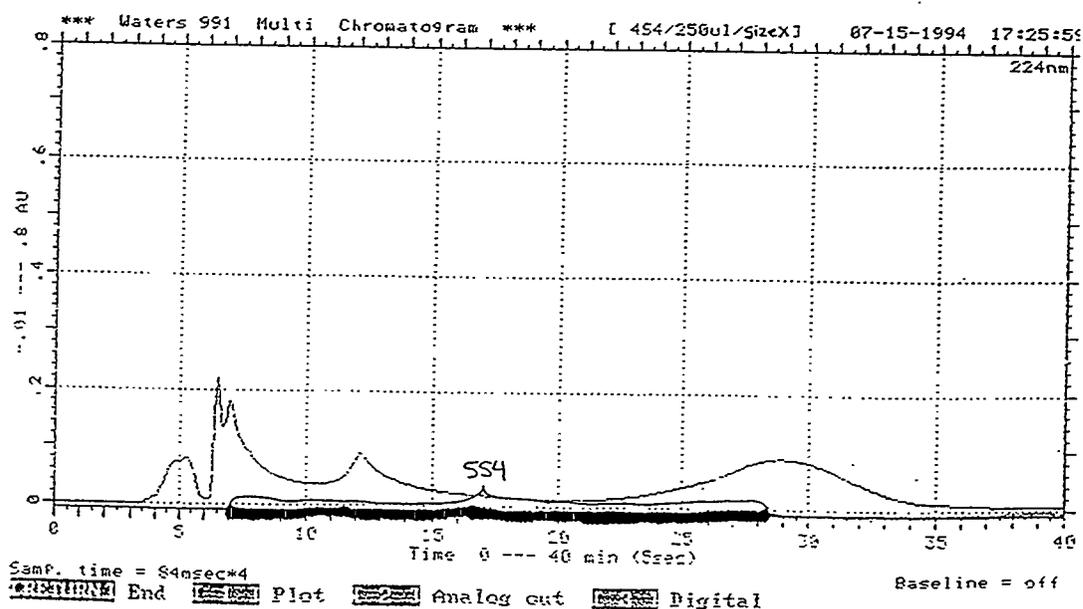


Figure 5. Chromatogram of a TSK-Gel column run.

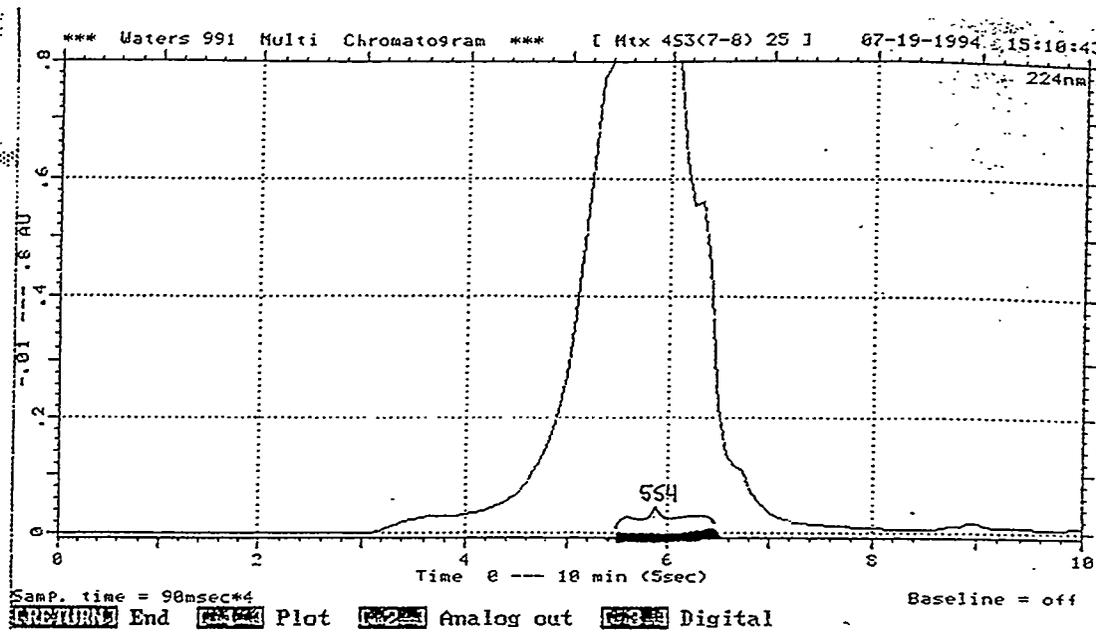


Figure 6. Chromatogram of a TSK-Gel column run.

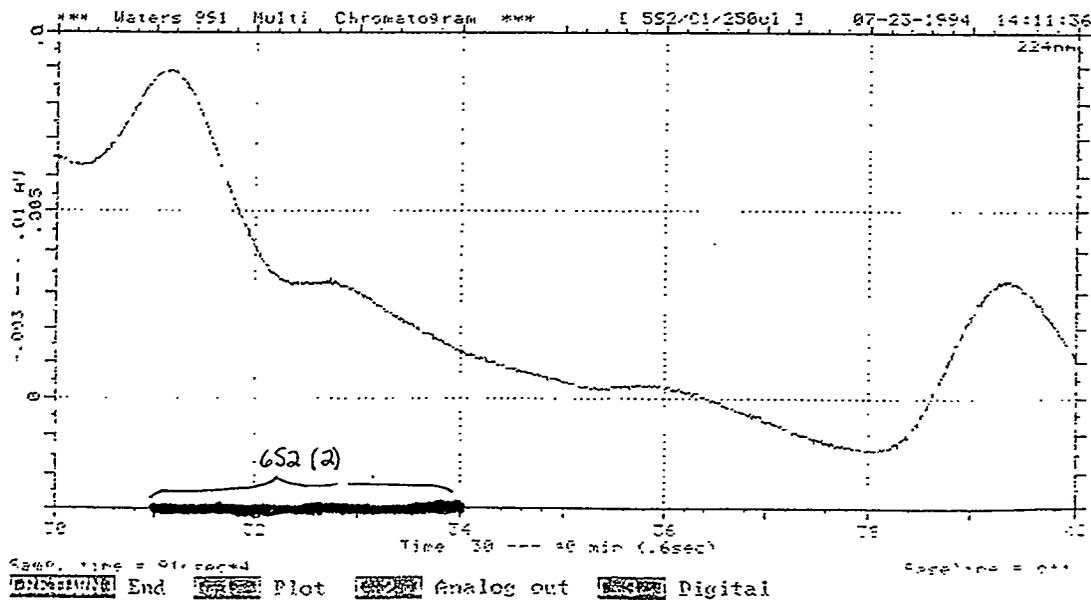


Figure 7. Chromatogram of a C-1 column with buffers run.

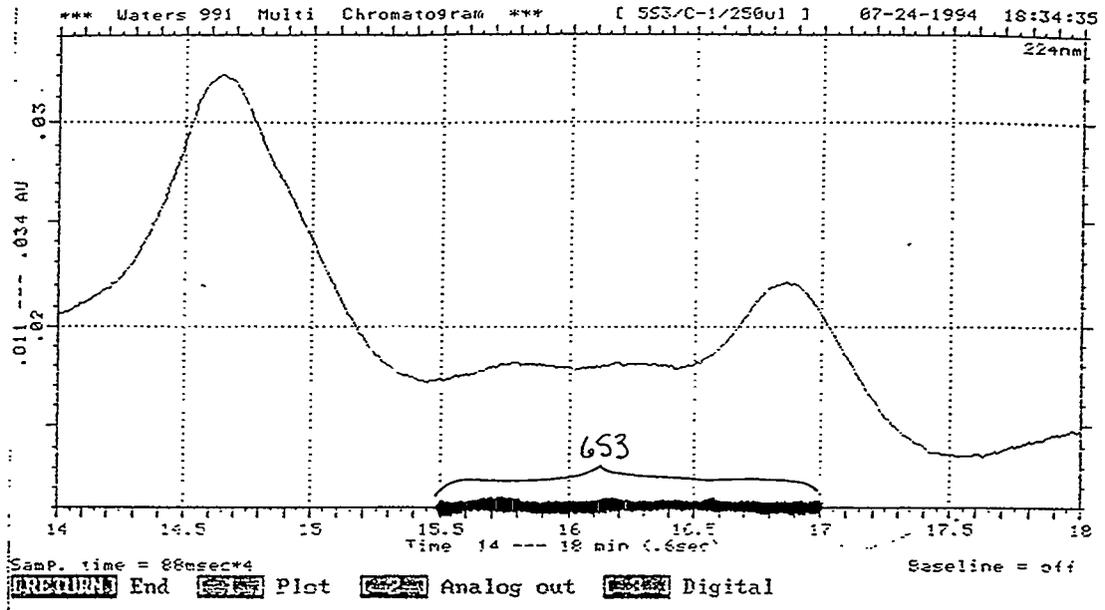


Figure 8. Chromatogram of a C-1 column with buffers run.

Increasing the Treatment of Hypertension through Primary Intervention.

South Carolina is one of the leading states in stroke mortality. Hypertension is one of the major risk factors that lead to strokes. If treated, Hypertension can be controlled and thus reducing the risk of stroke. Unfortunately, many people that are hypertensive do not treat their disease properly due to lack of medical education. Many hypertensives do not realize the problems that may result from leaving the disease untreated.

Scientists do not know exactly what causes hypertension, but there are many factors that put an individual at risk for developing the disease. Low income, lack of education, and minimal access to health care are all major risk factors in the hypertension equation. Other major risk factor are race. African-Americans are more likely to be hypertensive than white Americans.

Taking all of these factors into consideration, I hypothesize that a structural plan can be generated to satisfy the need of primary target at-risk medical care. The specific objectives of this project are to access geographic areas to determine the level of medical representation available. Also to identify areas of medical needs based on race, income and health status. And finally to structure an intervention plan to target areas that are at the in the highest hypertension at-risk category. The methods that I will use to achieve to these objectives are to identify medical coverage by geographic areas. Health in poor areas will also be identified and mapped. Implementation of the intervention strategies will be prioritized based on areas of need.

First of all, each risk category needed to be mapped out to determine degree of risk in each community. Lack of medical education can be seen by looking at the percentage of high school drop outs per county and the percentage of physicians per population of each county. Access to health care can be measured by looking at the percentage of the population is rural, medically uninsured, and infant mortality rate of each county. Economic status is displayed by showing the total population below poverty, on medicare, medicaid, and unemployment. And finally the total and percentage of African Americans in each county was mapped out also.

With all of this information, I created a "risk index" which shows the counties that fall into the high risk category, most frequently. The risk index shows us which communities need a primary intervention program the most.

A primary intervention program will be implemented hopefully next school year. The program has proven successful before. Harleyville-Ridgeville high School was chosen. It is a relatively small rural school with a significant African-American student body. The school administration and the community as a whole were very cooperative and

enthusiastic regarding this project. Furthermore, the school has an active health club with enthusiastic students. Depending on weather conditions and competition, HR draws 300-700 spectators for a home football game. The major activities for the Health Club students involved recruitment of individuals attending the games. Blood pressures were measured for all individuals presenting. A single blood pressure measurement of systolic and fifth phase diastolic were measured and recorded. Participants were provided a record of the measurement. Individuals with measures at or above 140/90 mmHG were advised to see their physician. The results suggest the project was successful with a significant number of measurements taken; literature distributed, and at-risk individuals identified. Several positive results were demonstrated and include: Increase in awareness of high blood pressure focused at the high school. Involvement of community centered around and athletic event. Implementation of a very low cost project. Training high school students in a health related area and encouraging personal career goals. Identification of at-risk hypertension and referral to health care. Motivation of all individuals to be aware of high blood pressure and continue appropriate monitoring of blood pressure. With these types of results the program needs to be implemented in as many schools as possible.

I made a list of the schools that this program would help the most.- In order to get schools to volunteer to participate in the project, I needed the help of a group that had access and influence on schools to volunteer for the project. I met with the members of the Area Health Education Consortium.

The purpose of the S.C SHEC is to provide education, recruitment and retention programs for health care providers by linking the state's academic health science colleges with service agencies and practitioners through community-based health education centers. These centers serve the entire state to improve the delivery of primary health care, especially for under served citizens of the state.

I met with several AHEC coordinators to inform them of the hypertension project. They enthusiastically agreed to help and sent me a list of the schools that were willing to participate in the program. With this list of schools I needed to decide which schools needed the program the most.

First I picked schools that fall in the high risk counties. And then I decided to use the schools that had a high percentage of African American students. Those that were above 70% black were chosen first. I then compiled a list of fifteen schools that have agreed to participate in the program.

In the future, more schools will hopefully participate. It will help save lives and encourage young people to get involved in the health care industry. Programs like this will make and impact on the health of hypertensives, by making them more aware of their

condition. This assessment of South Carolina's counties also points out areas that lack physicians, and status of health care availability among South Carolina's residents.

by Mikel D. Fair
SURAP

**A Comparative Study of the Inner Ear
Structures of Artiodactyls and Early Cetaceans.**

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MUSC Summer Research Program

Introduction

It has been suggested that the order Cetacea (whales and porpoises) are closely related to artiodactyls, even-hoofed ungulate mammals such as the pig and cow. Paleontological and molecular data strongly supports this concept of phylogenetic relationships (Prothero, 1993). In a study of DNA sequences of two mitochondrial ribosomal gene segments of cetaceans, the artiodactyls were found to be closest related to Cetaceans (Milinkovitch et al., 1993; Arnason & Gullberg, 1994). These well accepted studies on the phylogenetic affinities of artiodactyls and cetaceans cause us to conduct a comparative study of the bony structure of the inner ear of these two taxa.

The main goal of the study is to determine the ear structure of the artiodactyls and early whales. The unique structures in whales (but absent in artiodactyls) would represent adaptations to the aquatic lifestyle. Fleischer (1976a) suggests that several specialized inner ear structures are necessary for detection of characteristic frequencies of the cetacean under-water hearing. These structures to be examined include: the size of the spiral ganglionic canal, the narrow width of the secondary bony lamina along with the size of the laminar gap (distance between the primary and secondary bony laminae) and the height ratio of the cochlear spirals. The size of the vestibule and the size of the semicircular canals with their corresponding ampullae will also be compared.

Materials and Methods

The cetacean petrosal studied belongs to an early fossil whale, *Prozeuglodon atrox*. It was collected by Professor Gingerich of The University of Michigan in Zeuglodon Valley in Egypt. The specimen contained deciduous premolars, indicating that the specimen was a juvenile. It is estimated that the geological age of *Prozeuglodon atrox* was about 38 to 40 million years old (Gingerich, 1992). The cetacean specimen was studied and compared with a petrosal of *Sus scrofa* (pig).

Each specimen was studied by using two major techniques, serial grinding (Croft, 1950), and computer graphic reconstruction (Otten, 1987). Serial grinding is a technique which involves using a Croft parallel grinder and producing drawings and photographs of each section from the bone. The initial steps include soaking the specimen in a red alizarin stain to highlight important internal ear structures, and then soaking it in glue to protect it from crumbling. The specimen is then mounted in a plaster cylinder, and then four holes are drilled into the cylinder. The holes serve as reference points which will be used in the computer reconstruction.

The next step involves placing the cylinder inside the grinder and grinding the specimen in 50 to 100 um increments. After each grinding, a new surface of the specimen is exposed. The inner ear structures exposed on the surface are drawn using a camera lucida and then photographed twice (with two different cameras) to insure against possible problems in camera operation. Once all important structures are exposed, the serial drawings are then used for reconstruction.

Prior to making the graphic model, the drawings must be sorted. A section for every 200 um will be selected. Drawings of contours selected for digitization are then color coded for easier recognition. Reference holes and structure contours are also matched up. Next the contours of the inner ear structures are digitized with the use of a tablet and the MacReco (Otten, 1987) program. When digitization is complete, the program is carried out and a graphic model of the specimen is produced. The model possesses different shades of colors to represent different areas and structures in the inner ear. The model is then used, along with the drawings and photographs, to answer pertinent questions related to the study.

Discussion

It must be stated before discussion begins that more time is needed to analyze the serial section data on the ear structures of each specimen before quantitative data can be made available. The following presents a preliminary discussion of each structure with

functional importance and a rough comparison between inner ear structure of artiodactyls and whales.

Cochlear structure

The spiral ganglionic canal runs through the inner part of the cochlea. This canal contains the cell bodies of the spiral ganglionic chain that synapses with the cochlear hair cells (Williams et al. 1989; Fleischer 1976 a,b; Ketten 1992; Luo & Marsh, in review). These ganglionic cells relay the signals from the cochlear hair cells to the brain for interpretation and processing. The larger the spiral ganglionic canal, the greater number of ganglionic cells present. With more ganglionic cells present, the ear has a greater resolution for hearing. A measurement cannot be given at this time.

The laminar gap is the distance between the primary and secondary lamina. It approximates the width of the basilar membrane (Wever et al a,b,c; 1971; Fleischer, 1976 a,b; Geisler & Luo, in review). The laminar gap is very important in determining the frequency range in Cetaceans. The smaller the gap in the basal cochlear turn, the higher the hearing frequencies. The larger the gap in the basal cochlear turn, the greater ability to hear low frequencies. The laminar gap of *Prozeuglodon atrox* appears to be wider than in modern mysticete species. Since mysticetes do not have the high frequencies for echolocating and are capable only of low frequency hearing, we interpret that this early whale could only hear low frequencies.

The width of the secondary bony lamina that supports the basilar membrane is very significant. "Fleischer (1976b) suggests that the basilar support in the basal turn is stronger in the Odontoceti, (toothed whales) than in the Mysticeti, and that mysticetes have greater cochlear height to diameter ratios than odontocetes." (Ketten, 1992). The secondary bony lamina of the early cetacean specimen is thinner than those of other later cetacean. It can be seen from the graphic model that the imprint groove for the secondary lamina exists for about 1/2 of the basal cochlear turn.

When comparing the cochlear spirals of the two specimens studied, it is obvious that the pig cochlea is much taller than *Prozeuglodon atrox*. The cochlea of the pig has 3 1/2 turns while the cetacean specimen has 2 1/2 turns. The basal spiral of *Prozeuglodon atrox* appears to have a greater diameter than that of the pig. When accurate measurements are made, it will be determined that the specimen with the smaller height to diameter ratio will be able to detect higher frequencies.

Vestibule

The vestibule is responsible for static equilibrium and detecting movement. Inside the vestibule are two parts known as the utricle and the saccule. The utricle is continuous with the semicircular canals, while the saccule is continuous with the cochlear duct. Both have a sensory epithelium known as the macula which contains receptor cells that monitor the position of the head when stationary (static equilibrium), and monitor straight line changes in speed and direction of head movements (linear acceleration). The macula have sensory hair cells that synapse with the vestibular nerve which corresponds with the brain.

The vestibule of the pig has a spherical shape which is a general condition ("plesiomorphy") shared by all terrestrial mammals. The early whale *Prozeuglodon atrox* also has a spherical vestibule. Similar spherical vestibules are also found in other later mysticete whales but not in toothed whales (Luo & Eastman, in press).

Semicircular canals

The semicircular canals function for the detection of angular acceleration. Three semicircular canals are present, the anterior, posterior and lateral. Each canal has a membranous duct with a corresponding ampulla. The ampulla contain a small crest called the crista ampullaris which contains receptor cells for detecting angular movement.

The artiodactyl ungulate is found to have well developed semicircular canals and ampullae, while the Cetaceans are much smaller and less developed. The observation is obtained by the 3-D models produced by computer from the serial sections. When compared to the inner ear in mammals, the vestibule and associated semicircular canals are twisted medially in the cetacean (Geisler and Luo in review).

When final measurements are produced, it will be more clear how different or similar the ear structures of the two taxa are. It is anticipated that the data will generate new information for estimating the hearing frequency range of early whales and for inferring on the evolutionary relationships between the Artiodactyls and Cetaceans.

Acknowledgements:

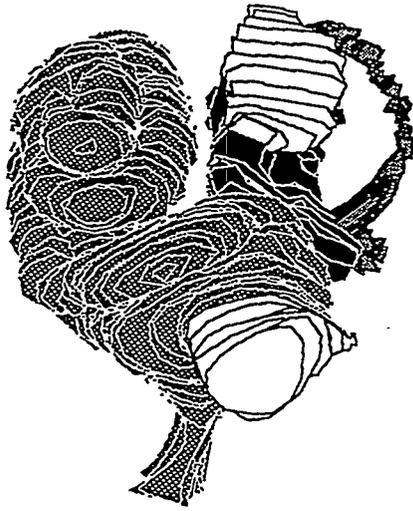
We would like to thank Dr. Philip D. Gingerich of the The University of Michigan for generously providing the *Prozeuglodon atrox* specimen, and also Jonathan Geisler for providing the artiodactyl pig specimen. Appreciation is also expressed to The Medical University of South Carolina for funding the Summer Undergraduate Reseach Program and to Dr. Henry Martin III, program coodinator. I would personally like to thank Dr. Zhexi Luo for allowing me to assist him in this project and for making this a complete learning experience.

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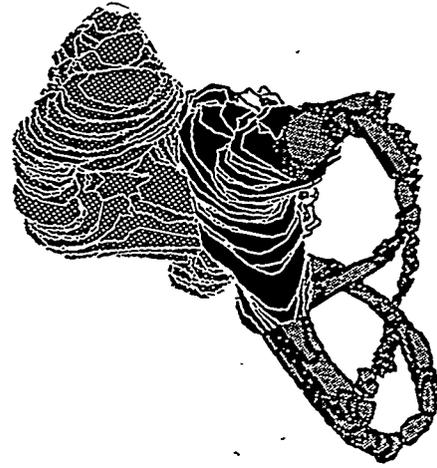
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3-D Models of Specimens



Prozeuglodon atrox



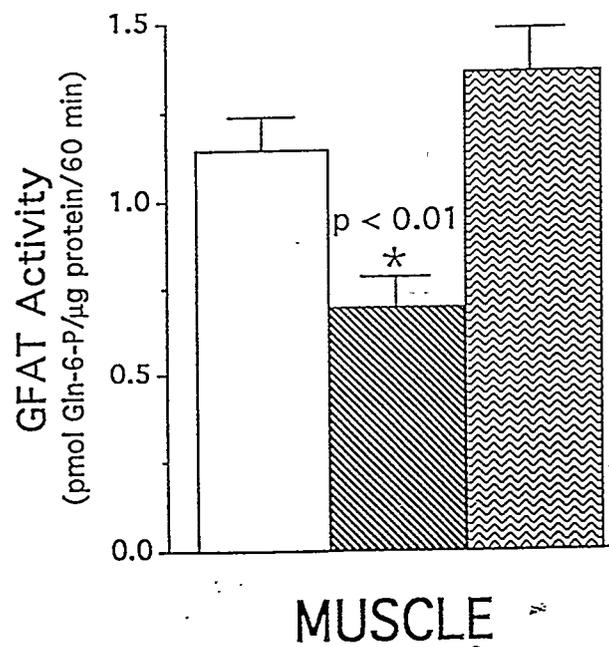
Sus scrofa

**"The Effects of Diabetes on the Activity of the Enzyme Glutamine:
Fructose-6-Phosphate Aminotransferase"**

By: Sylvia P. Nelson
Department of Endocrinology
c/o Dr. Maria G. Buse
August 05, 1994

Abstract

Hexosamine synthetic pathway (HexNSP) controls the supply of essential substrates for glycoprotein synthesis. In vitro studies suggest that increased flux of glucose via the hexosamine synthetic pathway may play a role in glucose induced insulin resistance of glucose transport. Glutamine: fructose-6-phosphate aminotransferase (GFAT) controls flux into the hexosamine synthetic pathway; the major products are UDP-N-acetylhexosamines (UDP.HexNAc= UDP.GlcNAc = UDP.GalNAc). I examined whether diabetes (~7 days post intravenous streptozotocin, and genetically linked) affects the activity of glutamine: fructose-6-phosphate in rat and mouse skeletal muscle in vivo. Nucleotide linked HexNAc were analyzed by high pressure liquid chromatography (HPLC) in deproteinized hind limb muscle extracts.



- Control
- ▨ Diabetic
- ▩ Diabetic + Insulin Tx

Introduction

The hexosamine synthetic pathway controls the supply of essential substrates for glycoprotein synthesis. Glucose enters the hexosamine synthetic pathway as glucosamine-6-phosphate. Recent in vitro studies suggest that increased glucose flux through HexNSP may play a role in the insulin resistance of glucose transport and glycogen synthesis associated with hyperglycemia.

Insulin resistance is a salient feature of type II, non-insulin dependent diabetes mellitus (NIDDM), and is also associated with uncontrolled type I insulin dependent diabetes (IDDM). In type II, the propensity to develop insulin resistance is thought to reflect genetic defects. While in type I it is acquired and reversible with insulin therapy. Insulin resistance is defined as a subnormal biological response to a given concentration of insulin; it may reflect decreased insulin sensitivity, and in most conditions, a decrease in the maximal insulin response. Skeletal muscle is the major site of insulin resistance in type I and type II diabetes. The term, insulin resistance, describes the actions of insulin on glucose homeostasis. For example; patients with diabetes mellitus who require insulin continue to have hyperglycemia despite large doses of exogenous insulin, or patients who are severely insulin resistant can only maintain near normal blood glucose levels through marked elevations of endogenous insulin secretions.

The first rate limiting step for glucose entry into the HexNSP is catalyzed by the enzyme glutamine: fructose-6-phosphate aminotransferase (GFAT), which uses the amido group of glutamine to convert fructose-6-phosphate into glucosamine-6-phosphate. Glucosamine-6-phosphate is then acetylated to N-acetyl-glucosamine-6-phosphate and is eventually converted to SA's, gangliosides, glycoproteins,

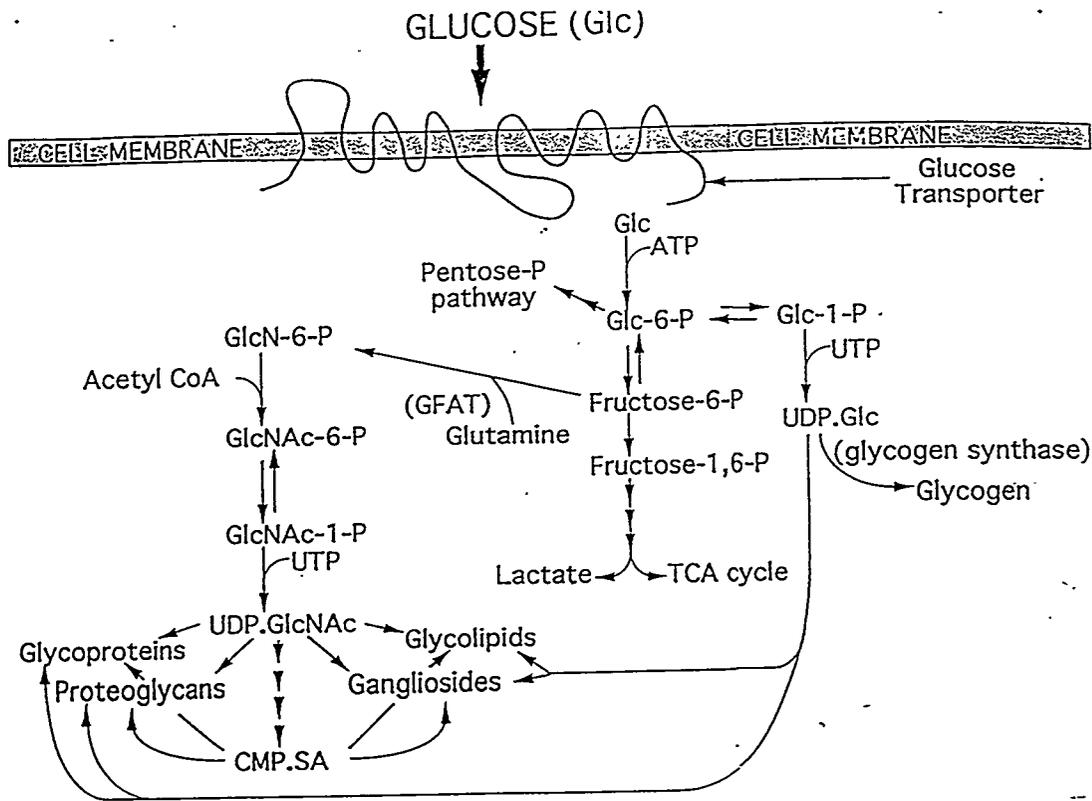


Figure 1: Diagram of glucose metabolism in skeletal muscle and the entry of glucose into the hexosamine synthetic pathway.

Glucose flux through this pathway is regulated by glutamine:fructose-6-phosphate amidotransferase (GFAT). GlcN = Glucosamine, GlcNAc = N-acetyl glucosamine, SA = sialic acid.

This figure does not show that UDP.glucose (UDP.Glc) is at equilibrium ($\sim 3:1$) with UDP.galactose (UDP.Gal) and UDP.GlcNAc with UDP.GalNAc ($\sim 3:1$).

glycolipids, and proteoglycans.

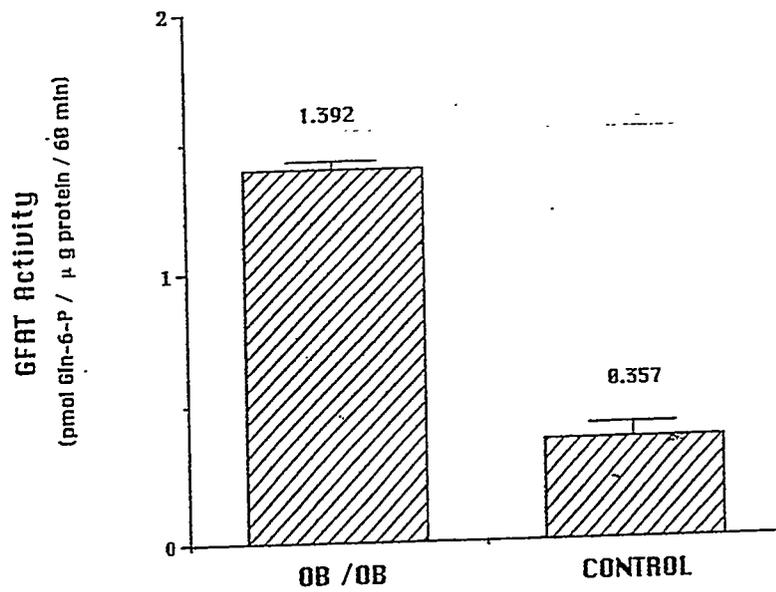
Specific Aims

Increased flux of glucose through the hexosamine synthetic pathway may result in the accumulation of metabolites which alter protein processing (glycosylation). Previous experiments suggest that GFAT activity is altered in the muscle of diabetic rats. This is thought to be caused by changes in the kinetics of the enzyme. An increase in the K_m of this enzyme would suggest that there is post-translation modification of the GFAT enzyme. Muscle extracts were taken to be analyzed by high pressure liquid chromatography to find out if the GFAT activity was indeed altered. From these results a graph was composed and from this graph the V_{max} and the K_m were determined. (Discussed later in detail).

Methods

Specifically, the first step was to find out if the GFAT activity was indeed altered in the muscle of diabetic rats. For this particular experiment two different models of rats were used. One model was the ob/ob mouse. This particular mouse carries the genes for obesity, one from the mother and one from the father. Once the mouse grows it becomes obese and as a result of the obesity it develops diabetes. This is very similar to the human type II diabetes. This was of developing diabetes is considered to be genetically linked. The second model that was used was a male Wistar rat. Diabetes was induced in this particular species by injecting them with streptozotocin. Streptozotocin is a fungal toxin that destroys the beta cells in the islets

GFAT Activity in OB/OB Mice



of Langer Han found in the pancreas. These are the cells which produce insulin. Thus, after being destroyed the pancreas no longer produces insulin and the blood sugar level goes sky high, then the rat has become diabetic. This is induced diabetes. 4-7 days after the streptozotocin injection the rats are sacrificed for analysis.

Both the ob/ob mouse and the Wistar rat were anesthetized with Metofane, an inhalation anesthetic. The hind limb muscles were removed and frozen in liquid nitrogen. The frozen muscles were then powdered and homogenized in 5 vol. of 25 mM HEPES, pH 7.5, 4°C, 5mM EDTA, 10mM KCl, 5mM glucose-6-phosphate, and 1mM PMSF. The glucose-6-phosphate used in the GFAT homogenization buffer stabilizes the GFAT enzyme.

Following homogenization the mixture was centrifuged at 60,000 x g for 15 min. in 4°C. The supernatants were removed and centrifuged at 100,00 x g for 60 min in 4°C. Centrifuging was necessary to remove the heavy unwanted solid material and isolate the aqueous portion of the cell, the cytosol, which contains the GFAT enzyme. Sephadex G-25 columns were prepared 15 min. prior to the end of the 60 min. centrifuge. The sephadex G-25 was pre-equilibrated with 25mM potassium phosphate, pH 7.5, 1mM EDTA, and 50mM KCl (Assay Buffer). The columns are used to remove the small molecules also found in the cytosol. G-25 represents the molecular mass cut off. Any molecule larger than 25,000 Daltons will pass through the column. This is another way of isolating the GFAT enzyme, it is ~67,000 Daltons. The sephadex G-25 columns were centrifuged at 500 x g for 2 min. at 4°C.

The aliquots of the filtered extract were incubated with 50 ul of different

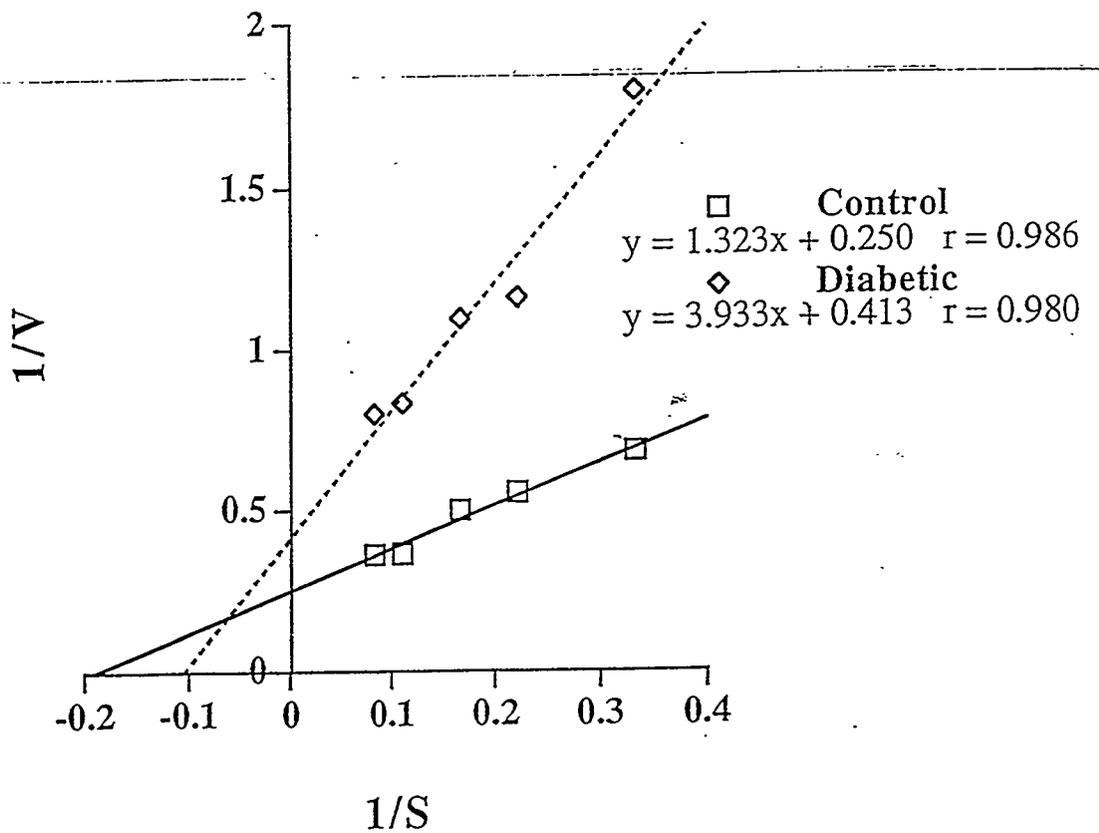
concentrations of fructose-6-phosphate (2.0mM to 12.0mM) and 50ul of 12mM glutamine in assay buffer for 60 min. at 37°C. The fructose-6-phosphate and glutamine are the substrates of this reaction. In the presence of glutamine: fructose-6-phosphate aminotransferase act with the amido group of the glutamine to convert fructose-6-phosphate to glucosamine-6-phosphate. The reaction was stopped with 150ul of 0.33mM PCA. This reagent stops the reaction by denaturing the enzyme protein. The samples were centrifuged for 10 min. at 4°C. The top aqueous layer was removed and derivatized with o-phthalaldehyde at pH 9.8, and neutralized with .1M sodium phosphate, then filtered over 0.45um filters. Glucoasamine-6-phosphate was quantified by fluorescence detection on a Beckman Ultra sphere ODS 5um C₁₈ HPLC column (4.6 x 250mm) eluted with a linear gradient from 90% 17mM sodium phosphate, pH 7.2 (A), 5% 2-propanol (B), and 5% acetonitrile (C), to 76% 19.7mM A, 12% B, and 12% C over 6 min., then back to the original concentrations over 4 min.

Results

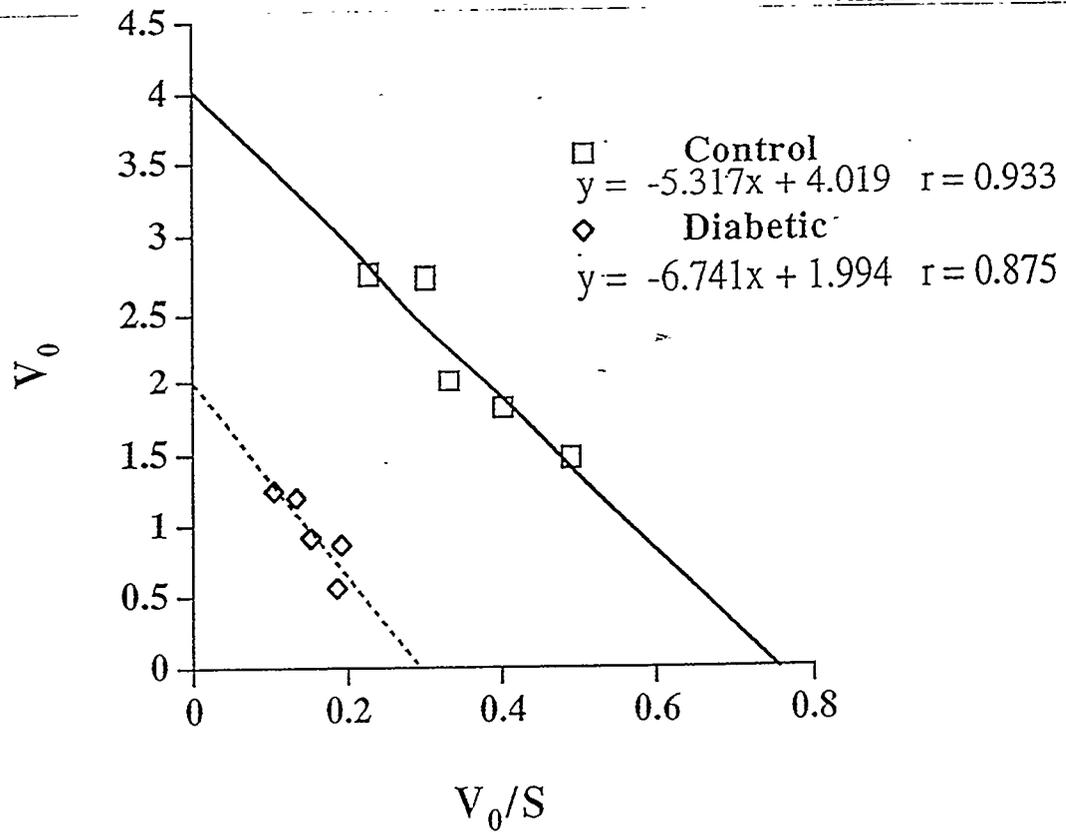
The results were obtained from the analysis of the HPLC. The HPLC calculates the amount of product, glucosamine-6-phosphate, produced in pmol. By dividing the area of the peaks by the protein concentration found in the muscle extracts we were able to determine the velocity of the enzyme. Once this was determined we the calculate $1/v$ for each of the different substrate concentrations ranging from 2.0mM to 12.0mM. we also calculated $1/S$. We combined these numbers to make a Lineweaver Burke plot. On the Y-axis we plotted $1/v$ and on the X-axis we plotted $1/s$. this type of plot is also known as a double reciprocal plot. The y-intercept is $1/V_{max}$ and the x-intercept is $1/K_m$. The slope of the line is K_m/V_{max} . The V_{max} is when the enzyme is

	1	2	3	4	5	6
		Control	Diabetic		Control	Diabetic
1	Set 3 7-27	Vmax (pmol/ug/60 min.)			Km (mm)	
2	1/v vs. 1/s	4	2.42		5.18	9.56
3	v vs. v/s	4.02	1.99		5.32	6.74
4						
5	Set 1 8-1					
6	1/v vs 1/s	4.63	2.05		10.6	21.3
7	v vs. v/s	3.34	1.48		6.46	14
8						
9	Set 2 8-2					
10	1/v vs. 1/s	4.26	1.32		5.92	12.46
11	v vs. v/s	3.28	1.13		3.61	9.65

Lineweaver Burke - Plot
Set 3 7-27

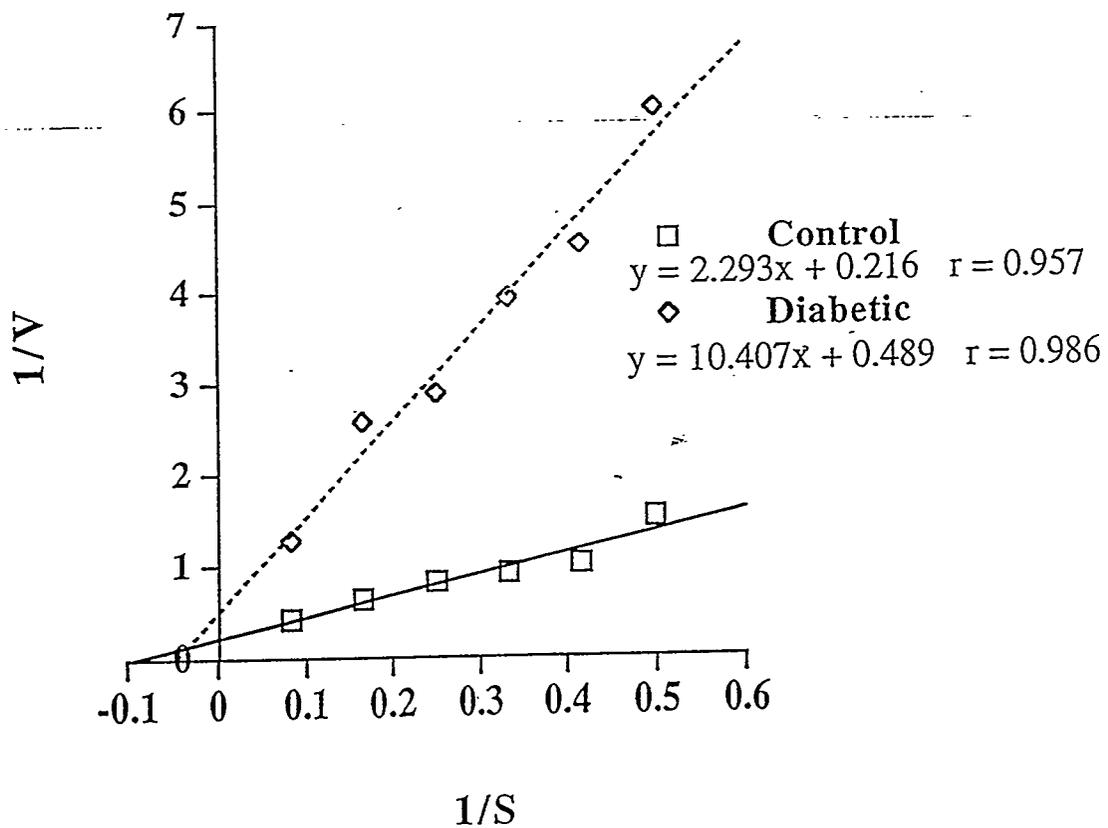


Eadie - Hofstee Plot
Set 3 7-27

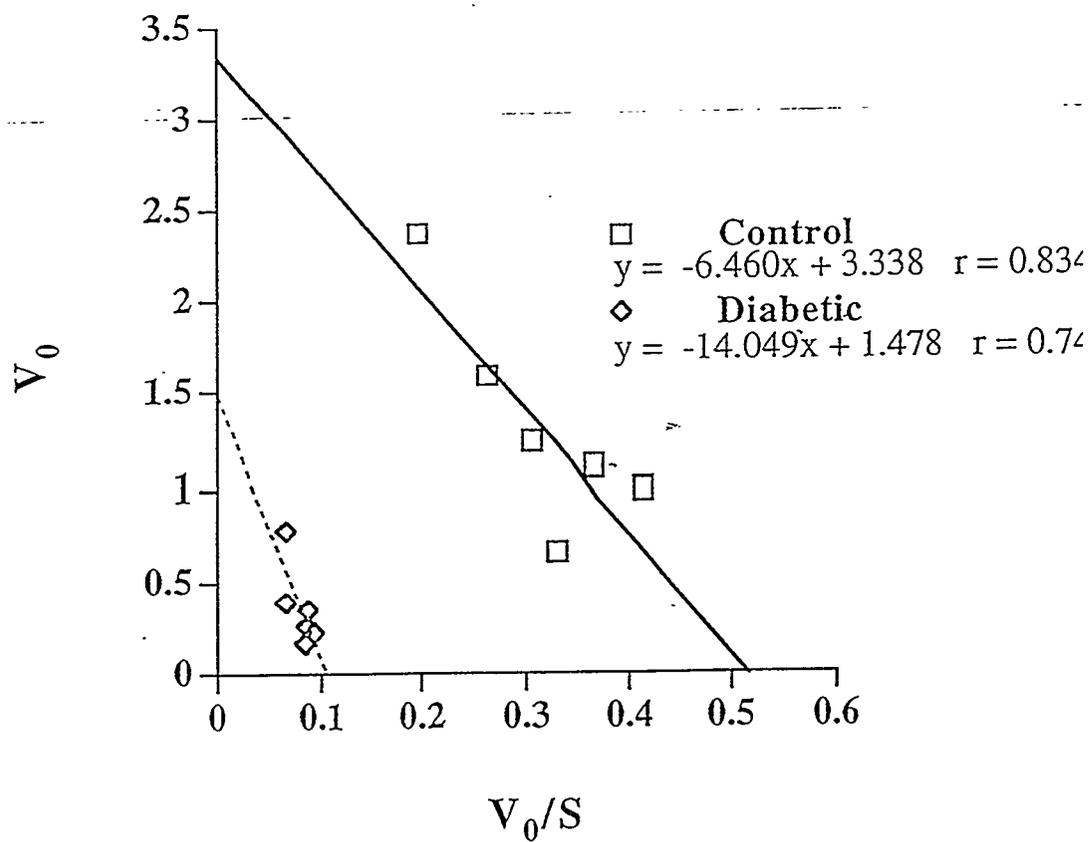


Lineweaver - Burke Plot

Set 1 8-1

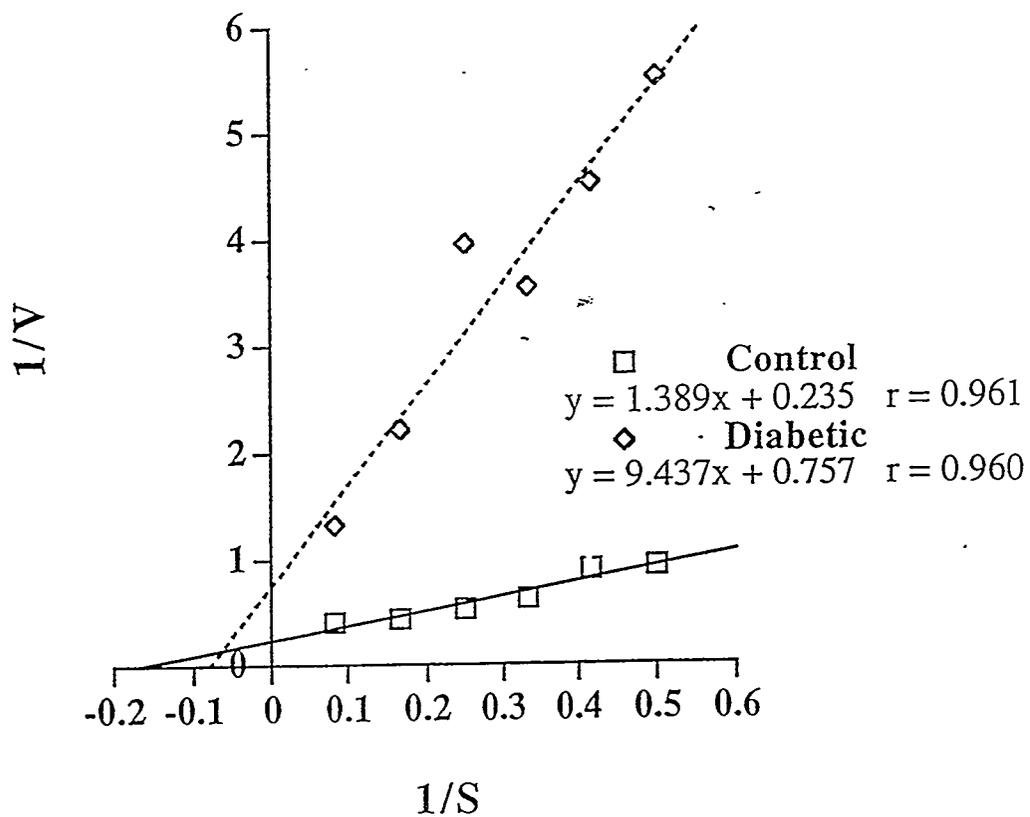


Eadie-Hofstee Plot
Set 1 8-1



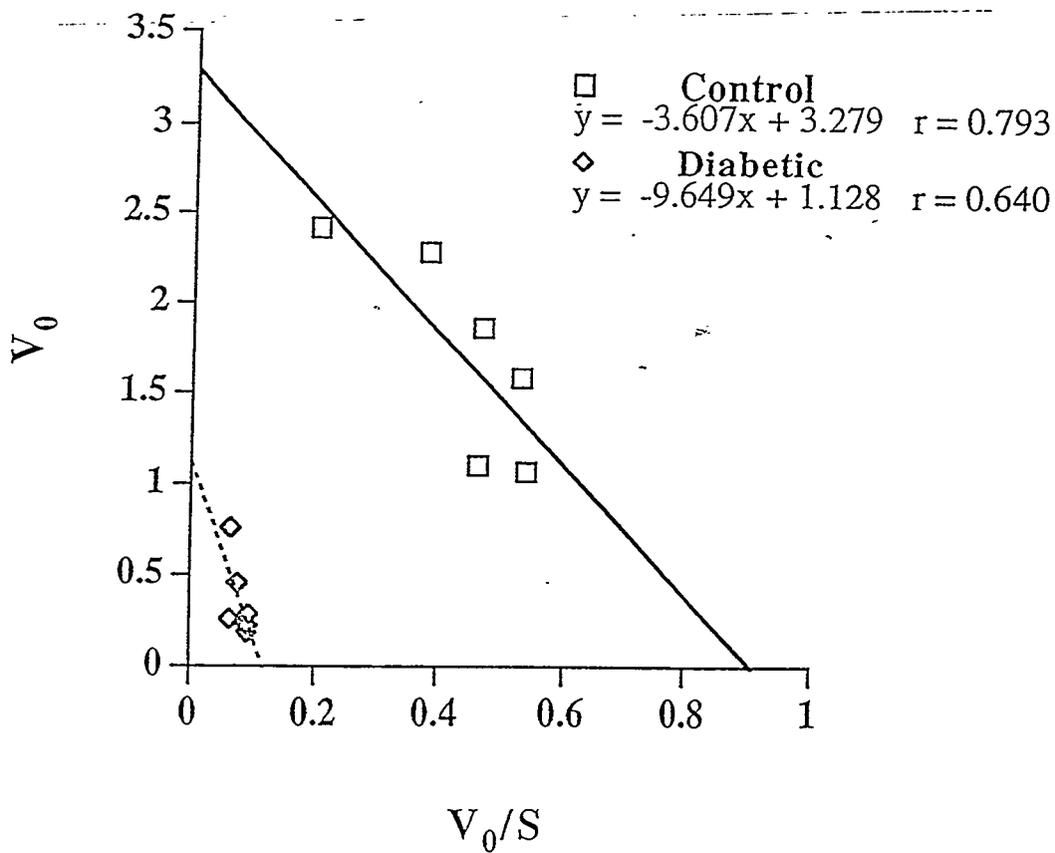
Lineweaver-Burke Plot

Set 2 8-1



Eadie-Hofstee Plot

Set 2 8-1



saturated, the maximal initial velocity is reached. The K_m is the Michaelis-Menton constant where K_m is equal to the substrate concentration at which the initial reaction velocity is half maximal. The second plot that we used was a Eadie-Hofstee plot. It magnifies the departure from linearity that might not be seen in a double reciprocal plot. On this particular graph the y-intercept is the V_{max} and the x-intercept is V_{max}/K_m . The slope is equal to the negative K_m .

Conclusions

There are yet several samples yet to be run in the lab, but 3-5 days of diabetes decreases the activity of the enzyme GFAT in muscle. This represents a decrease in V_{max} and very likely also an increase in K_m . This could represent non-competitive inhibition by a "purified" molecule, or possibly a conformational change in the protein. We think the first possibility is less likely since the Sephadex G-25 purification eliminates most of the small molecules. Experiments in our laboratory are in progress to measure the mRNA levels and enzyme protein levels by immunoblotting. This should tell us whether or not the decrease in V_{max} reflects a modification of the protein.

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**AN ANALYTICAL ASSESSMENT OF
POPULATION REACTION TO
ENVIRONMENTAL HEALTH HAZARDS**

Beth Stasiukaitis
Mentor: Dr. Dan Lackland

Summer 1994
SURAP

The Savannah River Site (SRS), being a nuclear production facility, has created concern for the communities in the surrounding areas. Risk perception is the study of how and why people's views of risk differ. After completing the Perceived Risk Survey (PRS) in 1993, it was found that some people express their concerns by contacting a public official. Thus, the Legislative Environmental Health Survey (LEHS) was created. This survey asked the legislators of Georgia and South Carolina to respond to questions concerning various environmental concerns. The questions reflected how the legislators viewed their constituencies' concerns. These two surveys were compared to find differences in legislators' and public views.

My hypothesis was that population perceptions to environmental hazards are associated with geographical areas proximal to the SRS. I thought that people living close to the SRS would have different risk perceptions than those living farther away from the SRS.

With this project, I expected to analyze population survey data (from the PRS) to identify determinants of reaction. The survey of Georgia and South Carolina legislators was to be implemented and the responses from it analyzed. The third objective of my project was to geo-code areas proximal to the SRS.

The methodology included an analysis of responses to a population survey (the PRS) completed in 1993. The survey was developed to identify potential predictors of community action, e.g. race and proximity to the SRS. Following the analysis, a survey concerning environmental issues (the LEHS) was mailed to all legislators in Georgia and South Carolina. The results of the legislators' attitudes were then analyzed. Finally, the reactions and attitudes regarding the SRS were assessed.

The study areas for the PRS included three geographic areas around the SRS. These areas were selected by their proximity to the SRS. They included counties in both Georgia and South Carolina which had similar demographic characteristics. For assessment, a sample was selected and evaluated using a random digit dialing telephone survey methodology developed by Waksberg in 1978.

The LEHS surveyed legislators throughout Georgia and South Carolina. The surveys were mailed to the legislators with a letter asking them to complete the surveys and return them. The questions asked about their constituency's concern with several environmental issues. Issues

covered included the SRS, pollution, hazardous waste, and public contact with legislators. The results are summarized below.

As shown in Figure 1, there was great concern for the general environment. Percentages for both the legislators and the public were high. Of the Georgia legislators that responded, 94% said their constituency was concerned or very concerned about the general environment. In SC, 92% of those legislators that responded felt that way. The PRS showed that 90% of the public in Georgia and 87% of the public in SC believe the environment is more contaminated today than ever before. Percentages for Georgia in both questions were slightly higher.

The LEHS showed that legislators from Georgia and South Carolina overestimate their constituents' concern for air pollution as seen in Figure 2. For Georgia, 82% of the legislators that responded said their district was concerned or very concerned about air pollution. In South Carolina, it was 85%. Yet, in the PRS, only 48% of the public surveyed in Georgia claimed they felt there was harmful air pollution in their community. In South Carolina, 47% of the public felt this way.

Although most of the legislators of Georgia and South Carolina felt their districts were concerned about water pollution, the public was considerably less concerned. In Georgia, 92% of the legislators felt their constituency was concerned or very concerned about water pollution. However, only 34% of the public surveyed in Georgia responded that they felt there was harmful water pollution in their area (see Figure 3). In South Carolina, 95% of the legislators saw their constituents as concerned or very concerned. The PRS showed that just 38% of those surveyed felt there was water pollution in their community. South Carolina's percentages were slightly higher for both questions.

For South Carolina, there was a big difference in the legislators' view of concern and the public's concern about nuclear related issues, shown by Figure 4. Of the legislators that responded, 56% felt their constituency was concerned or very concerned about nuclear power; but only 11% of the South Carolina public that was surveyed felt there was harmful radiation pollution in their area. The difference between percentages for Georgia was not as diverse. The LEHS showed that 29% of the Georgia legislators felt that their district was concerned or very concerned about nuclear power. The PRS showed that 21% of the Georgia public surveyed felt their community contained harmful radiation pollution.

Oddly, 100% of the Georgia legislators that responded said they were not contacted any during the week concerning the SRS (see Figure 5). In South Carolina, 74% of the legislators said they were not contacted about SRS concerns. These high percentages would reflect low public concern. Only 19% of the Georgia public surveyed felt the SRS had a negative impact on the state and 17% of the public in South Carolina felt that way.

People have expressed their concerns in many ways, one of those being through a letter to a public official. As seen in Figure 6, the LEHS showed that 95% of the Georgia legislators that responded received less than 10 letters a month concerning environmental issues. The remaining 5% percent said they receive between 11 and 20 letters a month. Figure 7 shows that of the public surveyed in Georgia, 10% said that they had written a letter to a public official. For South Carolina, 86% of the legislators receive less than 10 letters a month. Receiving between 11 and 20 letters a month were 5% of the legislators and 10% said they receive more than 20 letters a month. The PRS reports that 8% of those surveyed in South Carolina have written a letter to a public official.

Differences in race and income were noted in the PRS concerning letters written. White respondents had 11% who said they had written letters whereas, only 3% of blacks had. Also, those whose income was less than \$25,000 were less likely to write letters than those with an income greater than \$25,000 (5% versus 11%).

Phone calls were another way people voiced their concerns. Figure 8 shows that 47% of Georgia legislators who responded to the LEHS receive between 1 and 5 phone calls a week about environmental concerns and 3% of them said they were contacted by phone between 6 and 10 times a week. South Carolina legislators were contacted a little more frequently; 51% of them received between 1 and 5 phone calls a week about environmental concerns. There were 7% who are called between 6 and 10 times a week and 7% are contacted by phone more than 11 times a week.

Environmental groups are an additional way for people to show their concern. Of the legislators, 31% from Georgia and 31% from South Carolina felt the environmental groups in their constituency were organized or well organized, as seen in Figure 9. The PRS reported that 15% of the Georgia public surveyed had joined or contributed to a special interest group or organization. In South Carolina, 18% had joined or contributed.

There were a few limitations to this project. First, the PRS was not intended to be analyzed by state. The respondents were selected according to their proximity to the SRS. Also, the LEHS had a low response rate. Only 81 of the 406 surveys (20%) sent out were returned. The percentages calculated from a low response rate might not be an accurate representation.

Another limitation was that the LEHS surveys were not marked according to districts, making it difficult to examine community versus legislative district perception. This last limitation supports the process of geo-coding. Geo-coding is the process of classifying household addresses into census tracts and compiling the recorded information into a database. A database containing individual addresses within each county would permit a detailed mapping technique for such things as perceived risk, environmental concerns, and cancer occurrence. The present survey could only be analyzed by state, thus limiting detailed analysis.

In conclusion, little difference was observed between responses from the public concerning environmental issues for Georgia and South Carolina. The LEHS responses exhibited that Georgia and South Carolina legislators appear to overestimate their constituents' concerns about the environment. Finally, a database consisting of geo-coded addresses would be beneficial for future risk perception or health studies by permitting detailed geographical analysis of data.

CONCERN ABOUT THE GENERAL ENVIRONMENT

legislators (LEHS) versus public (PRS)

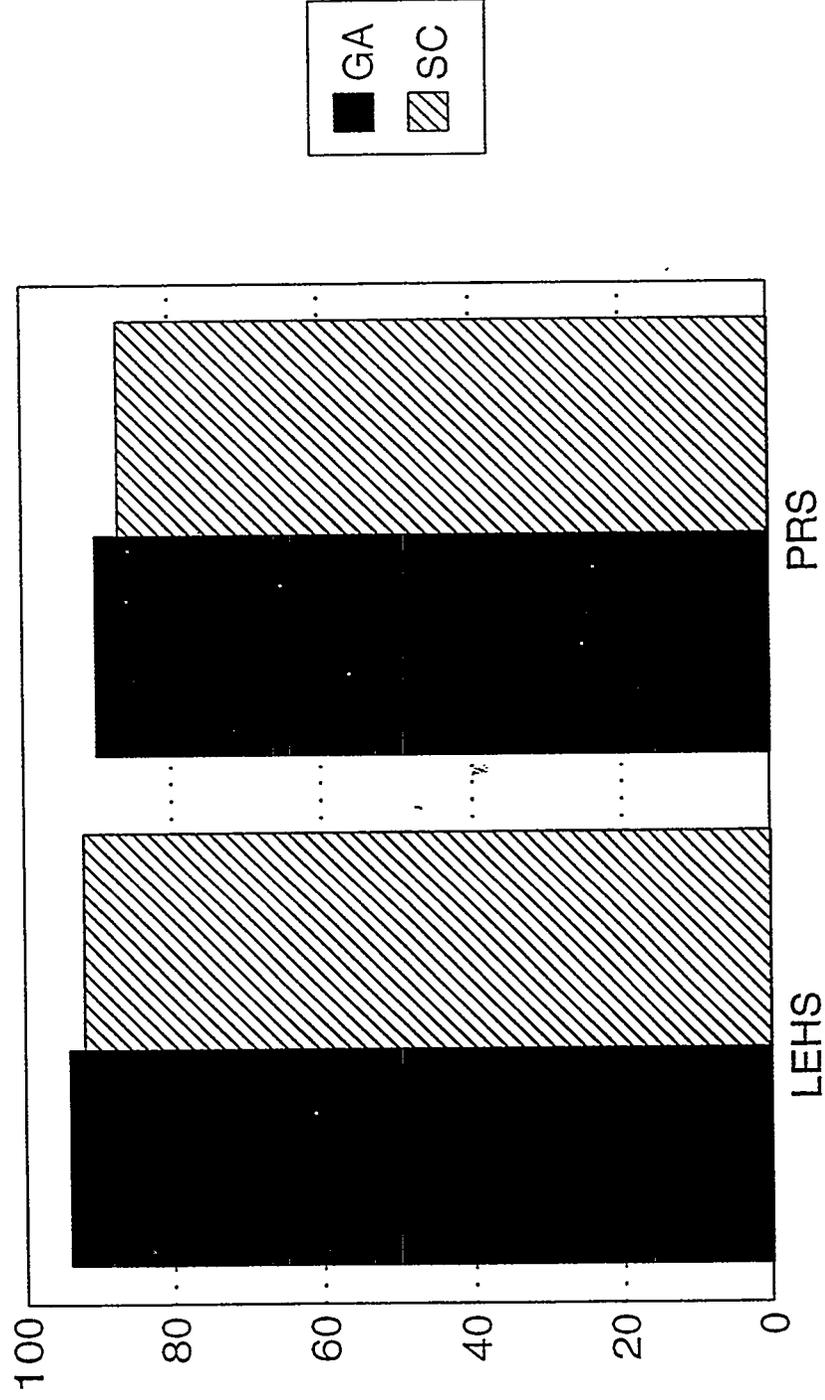


Figure 1

CONCERN ABOUT AIR POLLUTION

legislators (LEHS) versus public (PRS)

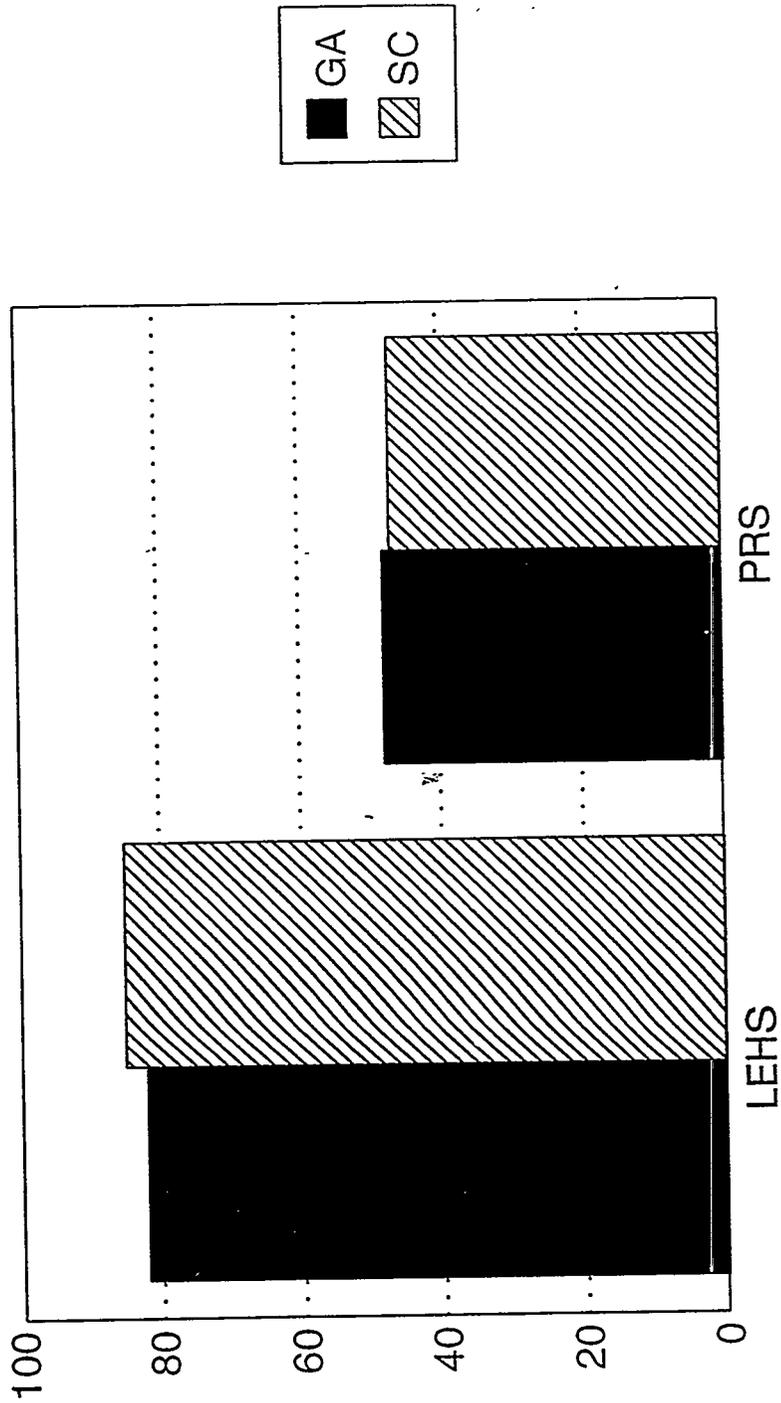


Figure 2

CONCERN ABOUT WATER POLLUTION

Legislators (LEHS) versus public (PRS)

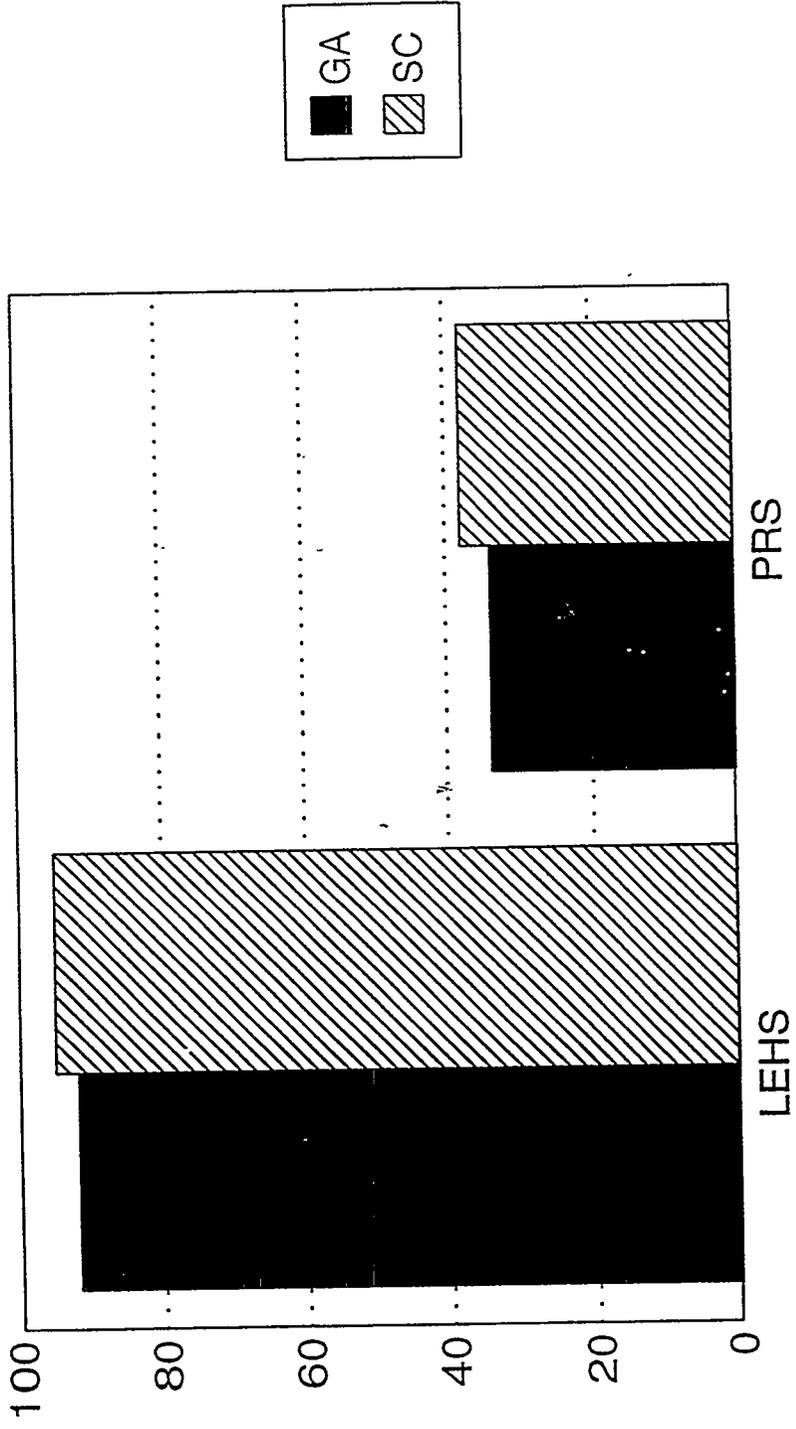


Figure 3

CONCERNS ABOUT NUCLEAR RELATED ISSUES

legislators (LEHS) versus public (PRS)

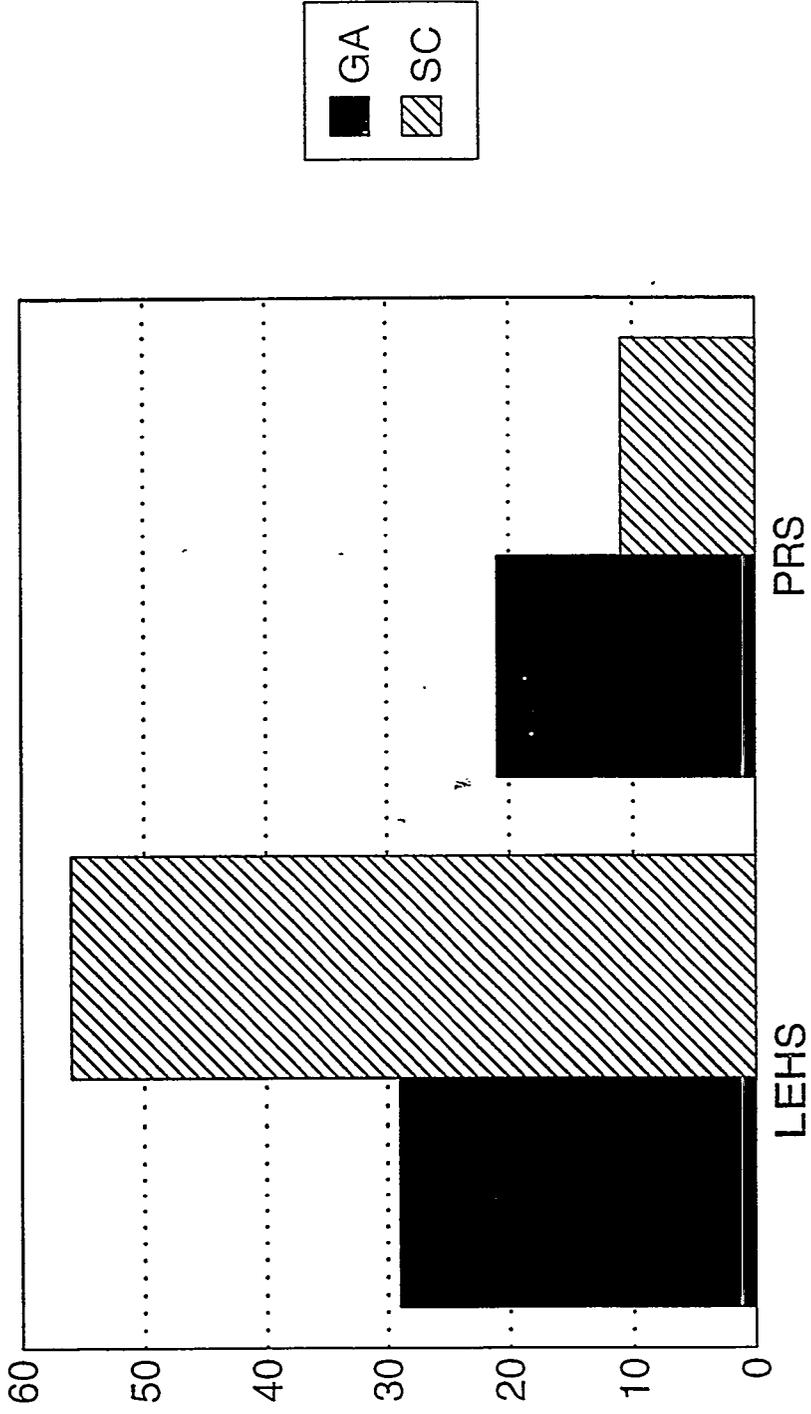
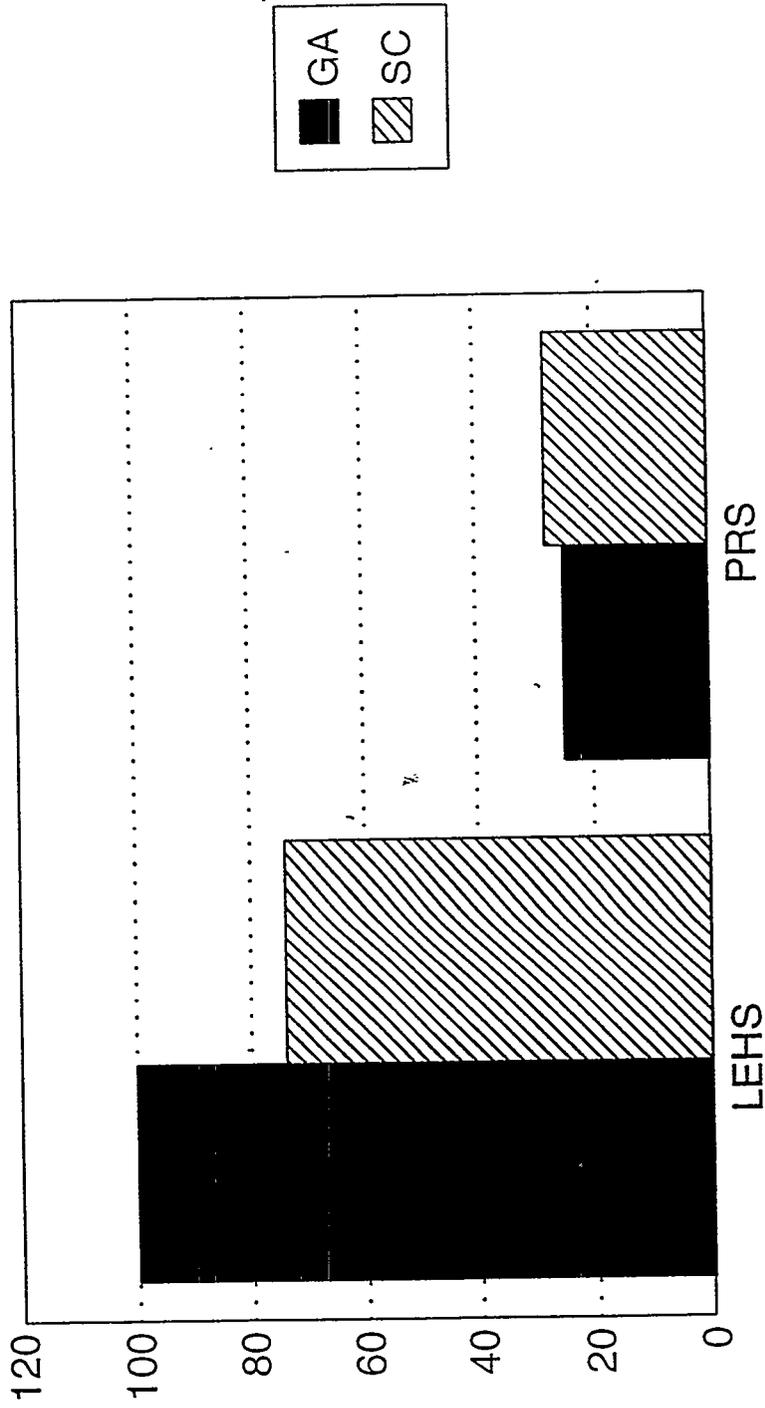


Figure 4

CONCERN ABOUT THE SRS

legislator contact (LEHS) versus public perception (PRS)



LETTERS RECEIVED BY LEGISLATORS

per month

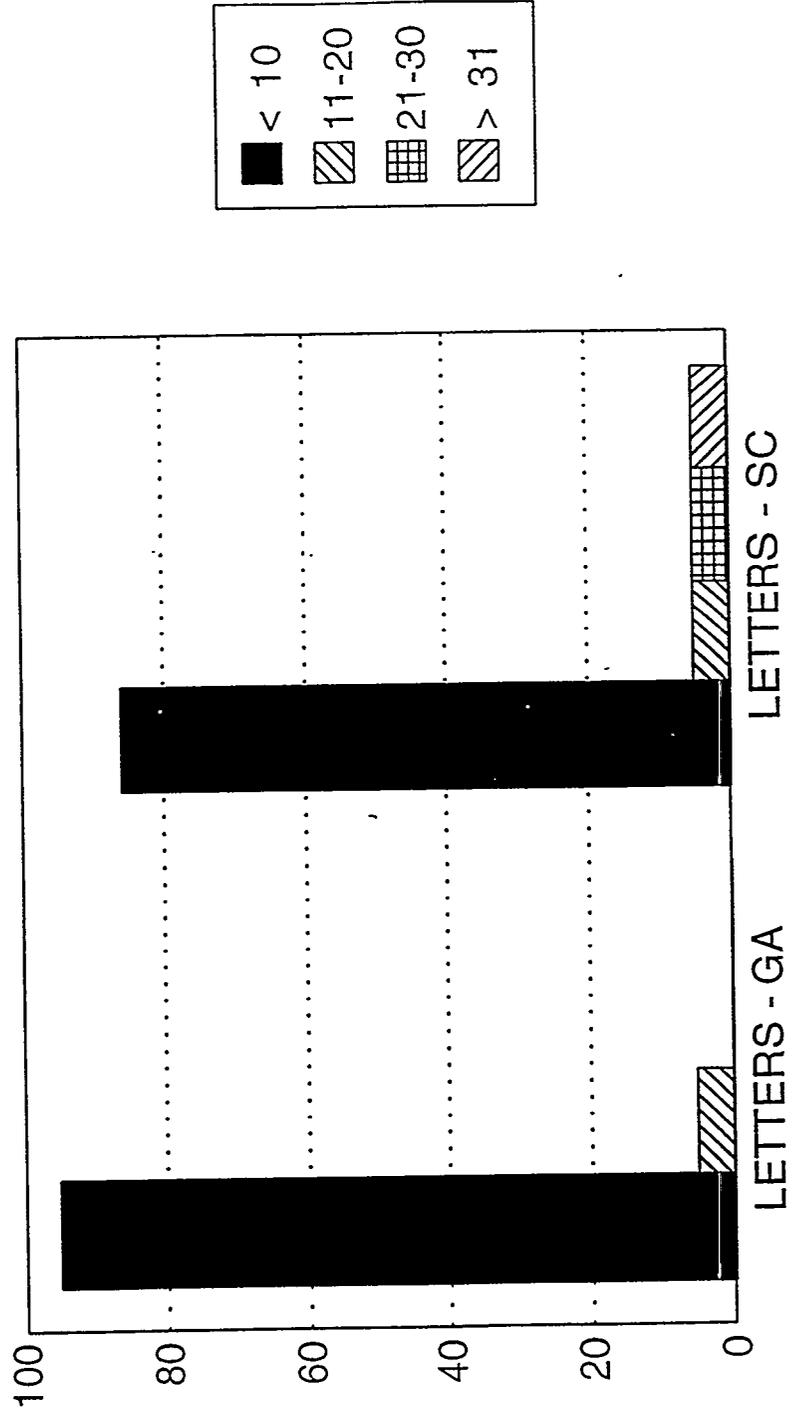


Figure 6

LETTERS WRITTEN BY PUBLIC to a public official

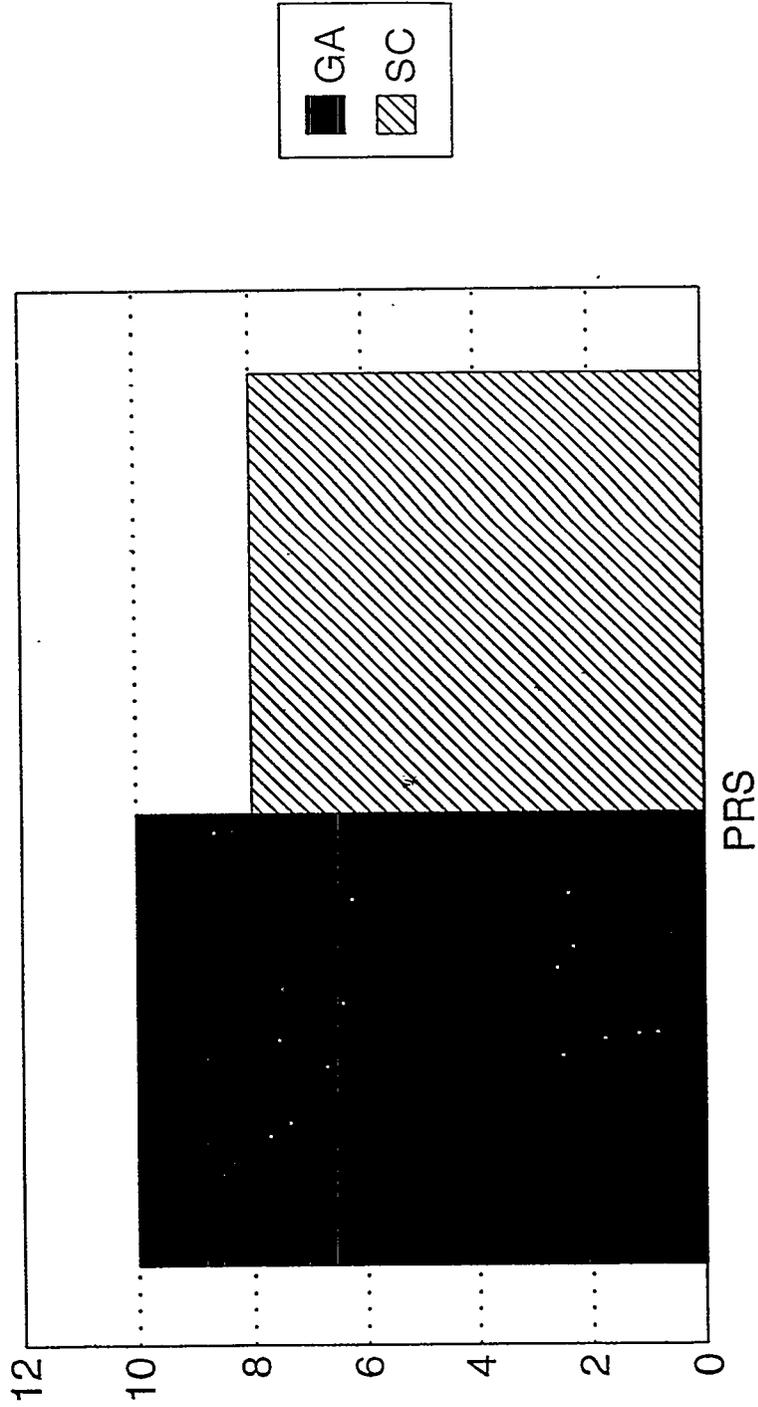


Figure 7

PHONE CALLS RECEIVED BY LEGISLATORS

per week

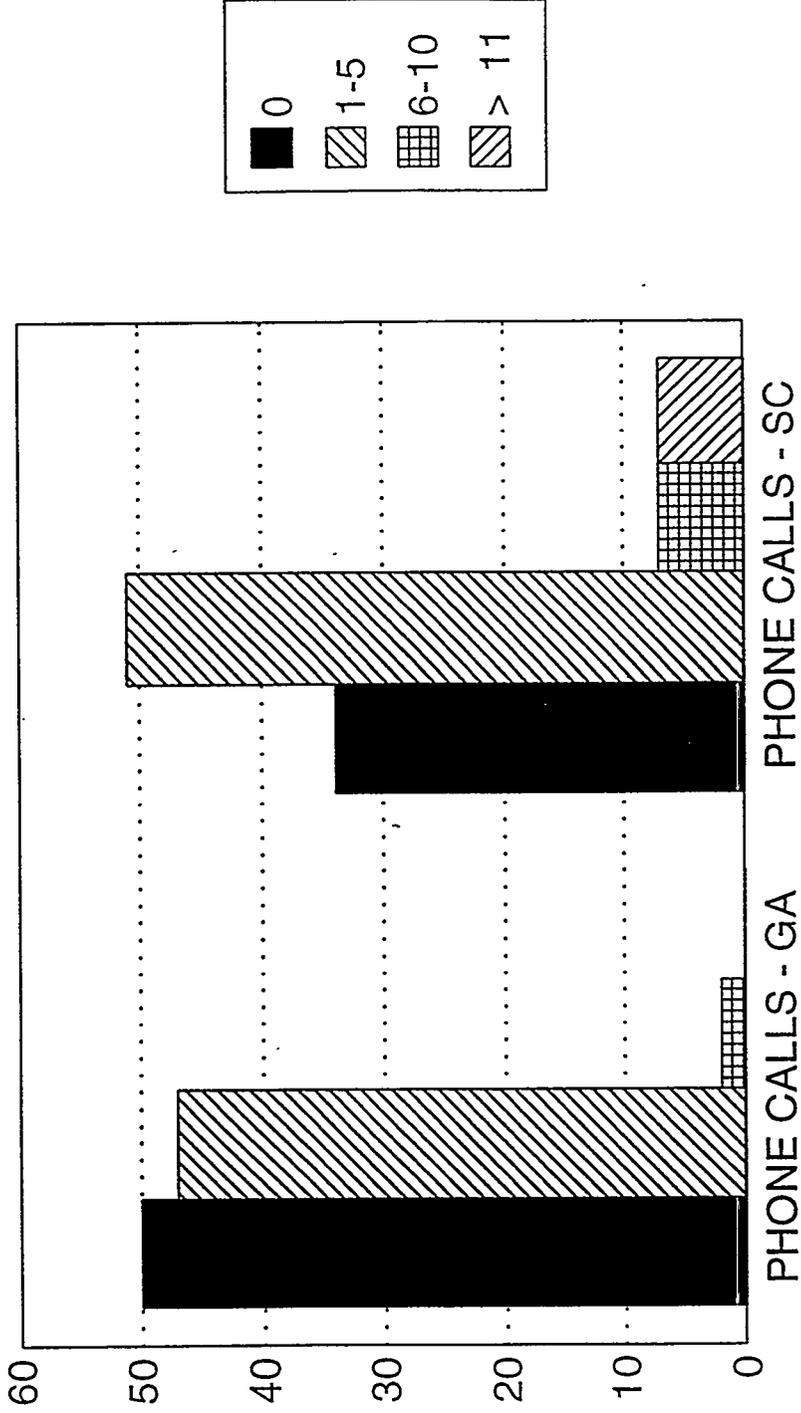
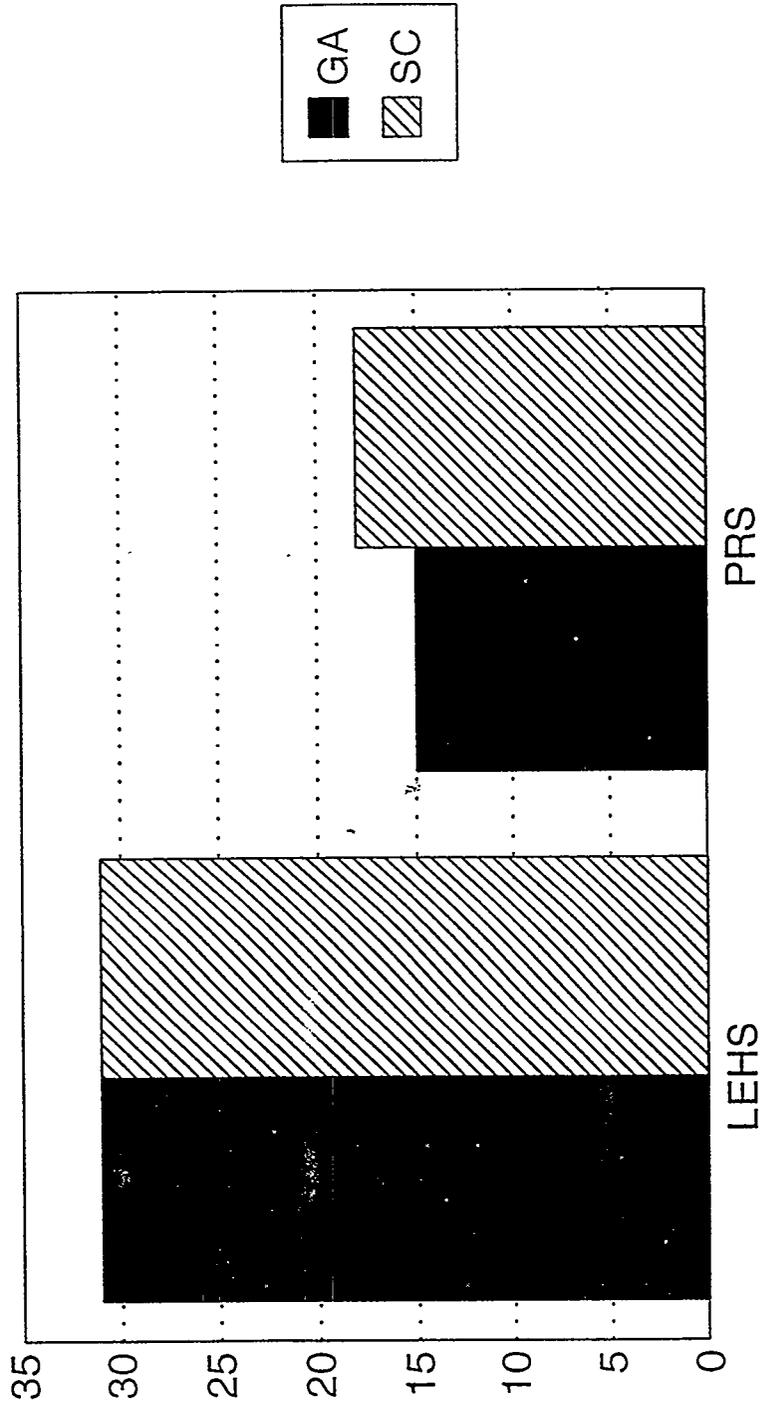


Figure 8

ENVIRONMENTAL GROUPS

legislator perception (LEHS) versus public support (PRS)



**THE NEUROLOGICAL EFFECTS OF BREVETOXIN
ON NEONATAL RATS**

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Yong G. Peng and Thomas B. Taylor

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ABSTRACT

We have investigated the neuroexcitatory and neurodegenerative effects of brevetoxin on neonatal rats. Four studies were done: dose response, northern analysis, immunohistochemistry and neurodegeneration. We found that neonatal rats are much more sensitive to brevetoxin than adult rats. The effectiveness of c-fos as a biomarker is being investigated, because of the high basal expression in young animals. The neurodegeneration, although not available yet, should provide valuable information.

INTRODUCTION

Brevetoxin (PbTx), a marine-biotoxin that has been implicated in several seafood poisoning incidents, is produced by the dinoflagellate Gymnodinium brevis. G. brevis is responsible for the harmful red tides that have been found along the gulf coast of the United States and as far north as North Carolina (Tester 1988).

Intoxication of brevetoxin occurs either by ingestion of the poison through shellfish or by breathing air-borne sea spray that contains the aerosol form of the toxin (Templeton 1989). Symptoms such as disturbances of the gastrointestinal tract and neurological problems following exposure include tingling and numbness of the lips, tongue and throat, severe muscular aches, gastrointestinal cramps and dizziness (Hungerford). Hot-cold temperature reversal, as seen in ciguatera poisoning, is also experienced with brevetoxin intoxication (Hungerford). Choking and gasping for breath occur when the toxin is inhaled. The duration of the poisoning is between 1 hour and 72 hours (Hungerford). Brevetoxin poisoning is not fatal; however, its long-term risks are undefined.

Nine separate forms of brevetoxin have been identified. All of them are heat stable up to 300° C and resistant to organic or aqueous solvents (Hungerford). Brevetoxins are polyether lactones that have two structurally different backbones. An alcohol of brevetoxin (PbTx-3) is used in this project.

Brevetoxin's mode of action on the cell is to activate the sodium channels at binding site 5 and to alter the membrane's properties causing excitability. This binding causes continuous depolarization of the cell. Ciguatoxin acts on the same site as brevetoxin and in several ways, is similar in its properties (Lombet 1987).

Brevetoxin's neurotoxic effects are well documented in the adult animal, but little is known about its effects in neonatal animals. This study will provide some valuable information because neonates may be at a higher risk to brevetoxin due to the lack of the blood-brain barrier at this young age. This information can then be used to assess the risk to human infants.

In this study, we report on the effects of brevetoxin on neonatal rats. A dose response was performed to determine the minimum effective dose that caused symptoms. Next, we attempted to distinguish between reversible and irreversible effects. To test for reversible or neuroexcitatory effects, c-Fos was used as a biomarker. Neonatal rat brains were investigated by northern analysis to determine if brevetoxin induced c-fos mRNA. Immunohistochemical staining of the c-Fos biomarker is reported to show the neuronal pathways in the neonatal rat brain. Finally, permanent neurological effects were determined using a cupric-silver staining method.

PROCEDURES

Experimental Animals

Long Evans Hooded neonatal rats were obtained with a mother from Charles River Laboratory (Wilmington, MA). They were given a constant supply of Harlan Teklad Rodent Diet and water. A 12 hour light:dark cycle was maintained. Rats were used at day 2 (P2) and day 10 (P10). P10 pups correspond to a newborn human infant in the maturity of the cerebral cortex, while P2 pups give a good comparison (Romijn 1991).

Dose Response

Rats were given intraperitoneal injections of PbTx-3 above and below the expected minimum effective dose for their weight. The minimum effective dose for P2 and P10 rats was 0.12 mg/kg and 0.07 mg/kg, respectively. The expected effective dose was 0.5 mg/kg. After each injection, the neonatal rats were observed for one hour. The symptoms and time of death were recorded. Control animals were appropriately injected with sesame oil and 5% ethanol and observed as well.

Northern Analysis

Rats were injected with the previously determined minimum effective dose, anesthetized and sacrificed by decapitation after 30 minutes and 60 minutes. A vehicle was treated with sesame oil and 5% ethanol, and one rat was given no treatment. The brain tissue was quickly dissected and frozen in liquid nitrogen for RNA extraction.

Total RNA was prepared using TRI-reagent, a single-step RNA isolation described by Chomczynski. Equal amounts of total RNA were loaded in each lane of a horizontal 1% agarose formaldehyde gel as described by Morgan et al. The gel was analyzed by constant current electrophoresis at 60 V and then stained with ethidium bromide and viewed under an ultraviolet box. The RNA was transferred to a nylon membrane (Hybond N+, Amersham) overnight using 20X saline-sodium citrate (SSC), and the RNA was cross-linked onto nylon membrane with 1200 microjoules for 30 seconds using UV cross-linker (Stratagene, CA). A 1.75kb SstI/EcoRI insert from pc-fos (mouse)-3 clone (Miller) containing 3 of the 4 exons encoding c-fos was purified from an agarose gel by electroelution and labelled with [³²P] dATP (3000 Ci/mmol, Amersham), using random primers DNA labeling system (BRL, MD) (specific activity 1.0×10^8 - 1.0×10^9 cpm/ug DNA). The membrane was prehybridized for at least 4 hours and then hybridized at 50° C. with the labelled probe for overnight. The membranes were washed at room temperature with 2x SSC twice for 20 minutes each. The membranes were analyzed by autoradiography with intensifier screens on XAR-5 film.

Immunohistochemistry

Immunostaining of the c-Fos biomarker was performed using the method described by Hoffman et al. Pups were given the minimum effective dose of PbTx-3 for 1 hour and then anesthetized with a muscle relaxer (ketamine hydrochloride and acepromazine maleate). Rats were perfused with 4% paraformaldehyde with 2% acrolein at pH 6.8 for 10 minutes. The brain was dissected and put in 25% aqueous sucrose overnight. Forty um frozen sections were cut on a cryostat and attached to gelatin subbed slides. The slides were washed in PBS and treated with 1% (w/v) sodium borohydride for 20 minutes to neutralize the acrolein, and 3% H₂O₂ for 30 minutes to prevent background staining of the red blood cells. The following were added: 1) primary sheep anti-c-fos (Cambridge Research

Biochemicals) in PBS-0.4% Triton X-100 at 1:10,000 for 48 hours; 2) biotinylated anti-sheep IgG (Vector Laboratories, Inc, CA) at 1:600 for 1 hour; 3) 4.5 ul/ml avidin-biotinylated peroxidase complex of vector "Elite" kit for 1 hour (Vector Laboratories Inc, CA); and 4) sodium acetate buffer containing Ni₂SO₄ (25 mg/ml), DAB (3,3'-diaminobenzidine-HCl; 0.2 mg/ml) and H₂O₂ (0.83 ul of a 3% solution/ml) for 10-30 minutes. Slides were mounted on a coverslip and analyzed for specific c-Fos staining.

Neurodegeneration

P2 neonatal rats were injected with a 0.06 mg/kg dose of brevetoxin. The rats were perfused after three days and brain samples sent to Dr. Robert C. Switzer III (Neuroscience Associates in Knoxville, TN) for further analysis. The work will show if any neuronal degeneration has occurred after exposure to brevetoxin (Switzer 1991).

RESULTS/DISCUSSION

We investigated the neurological effects of brevetoxin on neonatal rats in four different areas. The dose response showed a minimum effective dose of 0.12 mg/kg for the P2 rats. This amount is 4 times less than the expected effective dose for the P2 pups. The P10 rats' minimum effective dose was 0.07 mg/kg, which is 7 times less than the expected dose of 0.5 mg/kg. Similar symptoms were seen in both ages, such as head wags, loss of balance, trembling, diarrhea, sweating, convulsions and death by respiratory paralysis. Table 1 gives a complete list of the symptoms for both ages of neonatal rats and for the adult rat. From this data, we concluded that neonatal rats at both ages are much more sensitive to brevetoxin than that of adult animals. In fact, any dose given higher than the determined minimum effective dose killed the rat pups within 15-25 minutes.

The northern analysis yielded some unexpected results. A c-fos mRNA band occurred in the vehicle and no treatment lane, while no bands appeared in the lanes of the 30' or 60' PbTx-3 treated animals. Expectations were mRNA bands in the lanes of the 30' and 60' PbTx-3 treated animals. This data seems to indicate that PbTx-3 inhibited c-fos induction in the 30' and 60' treated animals, however, this experiment must be repeated to verify the results.

The immunohistochemical procedure showed specific staining of c-fos in the nuclei of the cells in the brain. No induction of c-fos

was detected in specific neuronal pathways in rat exposure to brevetoxin, however. The staining was seen evenly throughout the entire brain regions, both in the control animal and the brevetoxin treated animal. This similar staining in both animals is probably due to the high basal expression in neonates because of the growth and developmental activity of the neurons. One reason that induction in specific neuronal pathways by brevetoxin is not seen may be that the basal expression is masking any small effect that brevetoxin may have.

The results of the neurodegenerative study will come later. If neurodegeneration is seen, then this will indicate long-term effects of brevetoxin on the rat pup. This will provide some valuable information.

In summary, this project shows that neonatal rats are at higher risk to brevetoxin than adult rats. We will reevaluate in the future the use of c-fos as an effective biomarker in neonates and we hope to get some definitive results from the cupric-silver staining for neurodegeneration.

ADULT RAT

SYMPTOMS

EFFECTIVE DOSE

respiratory depression	0.5 mg/kg
decreased body temperature	
cardiac arrhythmias	
increased pulse pressures	
ataxia	
depression	
head bobbing	
head tilt	
uncontrolled muscle movements	

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NEONATAL RATS

SYMPTOMS

MINIMUM EFFECTIVE DOSE

excitability upon injection	0.12 mg/kg (P2)
head wags	
loss of balance	0.07 mg/kg (P10)
trembling	
urination/diarrhea	
sweating	
raising hind legs	
irregular walking pattern	
convulsions	
turned white before death	
death (respiratory paralysis)	

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***TRICHLOROETHYLENE TOXICITY IN A HUMAN
HEPATOMA CELL LINE***

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INTRODUCTION

Trichloroethylene (TCE) is an important industrial solvent widely used for adhesives and lubricants. It is also used as a solvent in the textile industry, as a low-temperature heat transfer fluid and in vapor degreasing. It has been used as a component in spot removers and cleaning fluids for rugs (Lloyd et al. 1975). In medicine and veterinary practice, TCE has been used as a general anesthetic in surgical, dental, and obstetrical procedures. Also, it has been used as a disinfectant and detergent for skin, minor wounds and surgical instruments (IARC 1979). The US Environmental Protection Agency has estimated that approximately 60% of the total annual world production of trichloroethylene is released to the environment with annual emission of about 540 million Kg to the atmosphere and 9.1 million Kg to the ocean (Fuller 1976). Trichloroethylene's widespread use has made it a common soil and ground water contaminant at and around chemical waste sites and at Department of Energy sites. It has been shown to cause liver cancer in a particular mouse strain, B6C3F1, mice but not in rats.

Trichloroethylene falls into a class of compounds known as peroxisome proliferators-which can cause an increase in the number of hepatocyte peroxisomes. Peroxisome proliferators are a group of structurally diverse compounds that produce similar changes in experimental animals. These responses occur primarily in hepatocytes and include enlargement of liver, increases in relative content of peroxisomes and SER, and 10-fold or greater induction in fatty acid β -oxidation (Moody et al. 1992).

Hepatocellular carcinogenesis is a property of all peroxisome proliferators tested thus far and, with few exceptions, is not associated with any direct genotoxic action of the compound. The increased production of H_2O_2 which may overwhelm protective enzymes within the hepatocyte and produce indirect genotoxic injury (i.e. the oxidative stress hypothesis), and the propensity of PPs to induce hepatocyte replication have both been argued to contribute to the carcinogenic action of these compounds (Moody et al. 1992).

Peroxisome proliferation is consistently associated with hepatomegaly, which arises from a combination of cellular hypertrophy and hyperplasia. Initial studies on the fine structure of hepatocytes revealed that the increase in hepatocyte size was associated with the predominant increase in peroxisomes and a more modest increase in SER (Moody et al. 1992).

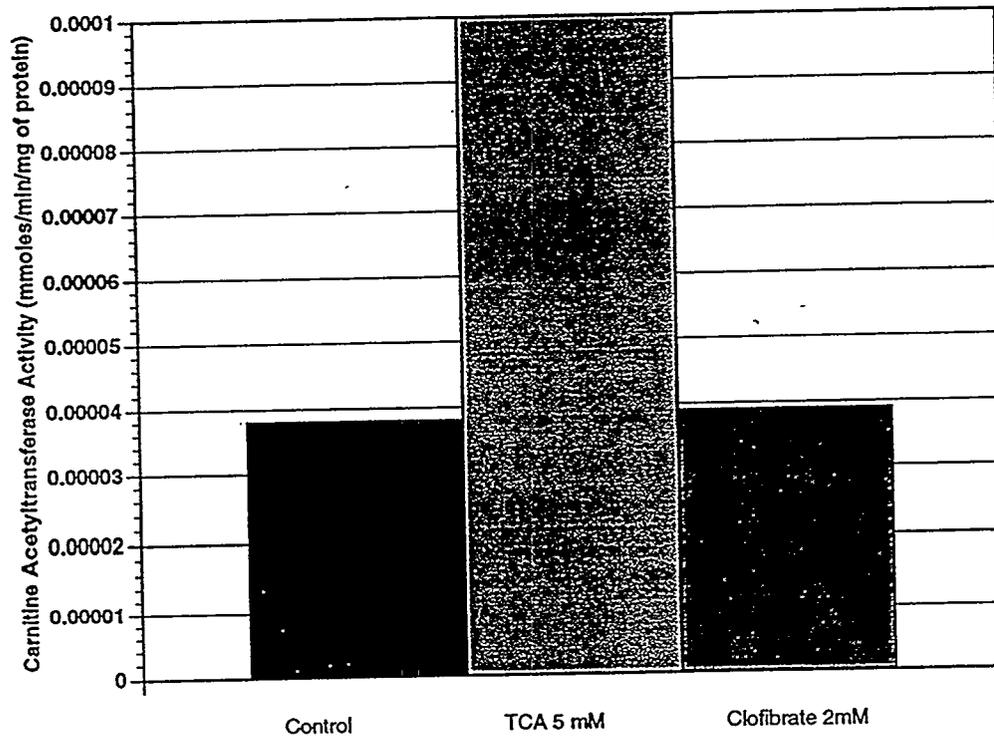
The mechanism(s) involved in the development of tumors after the administration of peroxisome proliferators are not understood; however, the involvement of reactive O_2 species has been postulated. It is

suggested that the imbalanced increases in catalase and peroxisomal H₂O₂-generating oxidase (e.g., cyanide-insensitive palmitoyl CoA oxidation) lead to an increase in the steady-state concentration of intracellular H₂O₂, and this may lead to cytotoxicity and DNA damage, which in turn could result in a mutational event and eventually neoplasia (Bruyninckx et al., 1978; Gifford 1968). Although the main target organ of Trichloroethylene toxicity is the liver, its mode of toxicity is not clear (Spencer and Schaumurg 1985).

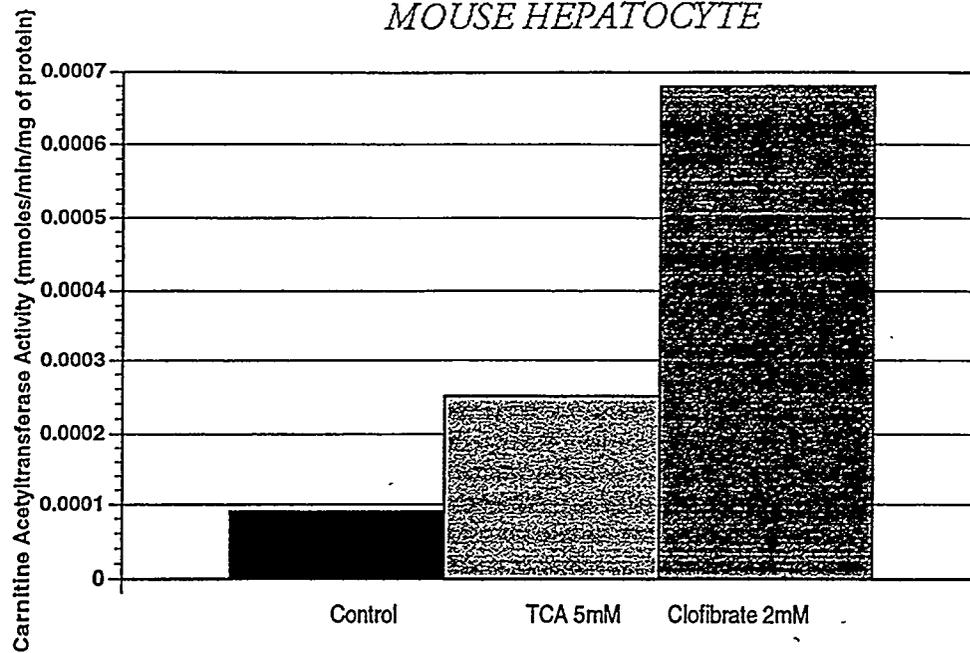
The experiments conducted in this study were designed to determine the usefulness of hepatocyte cultures and a human hepatoma cell line as model systems for assessing human susceptibility to hepatocellular carcinoma due to exposure to trichloroethylene.

The results obtained from these studies will then be analyzed to determine if human cell lines can be used to conduct future experiments of this nature.

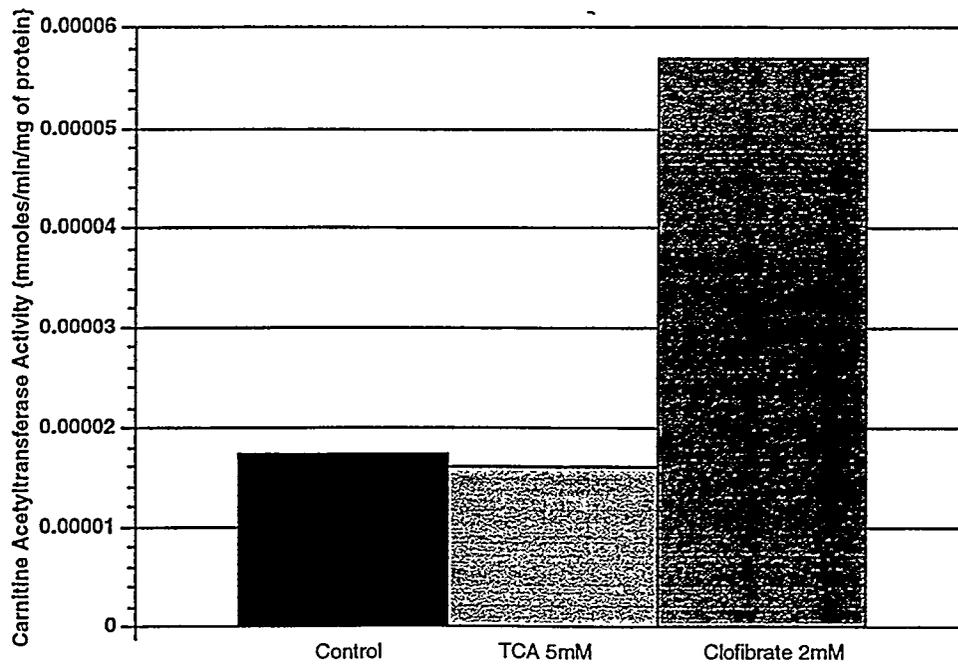
HEP3B HUMAN HEPATOMA CELLS



MOUSE HEPATOCYTE



RAT HEPATOCYTE



RESULTS

Peroxisome proliferation was assessed 48 hours after the addition of 5mM trichloroacetic acid and 2mM clofibrate. In Figure 1 TCA (5mM) treatment resulted in induction of Carnitine Acetyltransferase activity in mouse, but not in rat hepatocytes. In both the mouse and rat hepatocytes treated with 2 mM of clofibrate resulted in induction of carnitine acetyltransferase activity at a much higher rate in both the mouse and rat hepatocytes treated with TCA (5mM). Clofibrate in this experiment was used as a positive test which would be an indication that peroxisome proliferation would take place in the treated hepatocytes

In Figure 2 the Hep3B cells treated with TCA (5mM), exhibited increased carnitine acetyltransferase activity. Although TCA (5mM) induced carnitine acetyltransferase activity in the Hep3B cell, the Hep3B cells treated with Clofibrate (2mM) showed no increase in this enzyme activity when compared to the control.

Cytotoxicities of trichloroacetic acid and clofibrate were evaluated by lactate dehydrogenase release into the medium, TCA 5mM was not cytotoxic in the mouse and rat hepatocytes when compared to the control. However, mouse and rat hepatocytes treated with clofibrate 2mM did exhibit cytotoxicity.

DISCUSSION

The data presented was obtained from isolated liver hepatocyte cells from mouse (B6C3F1), rat (Long Evans) and Hep3B human hepatoma cultured cells. This study was used to assess human susceptibility to hepatocellular carcinoma when exposed to trichloroethylene. In this study TCA (5mM) and clofibrate (2mM) were used to determine if peroxisome proliferation would take place. Peroxisome proliferation is generally associated with hepatocellular carcinoma. In these studies TCA (5mM) did induce peroxisome proliferation in mouse hepatocytes but not in rat hepatocytes. Surprisingly, peroxisome proliferation in response to TCA was high in the Hep3B cell line.

TCA results suggest that human cells can respond to some peroxisome proliferation. However, Hep3B cells are a human derived cell

line thus many of the natural functions of the hepatocytes have been lost. These cells may respond in a different manner to TCA than would normal human hepatocytes.

In conclusion, Trichloroacetic acid at the concentration of 5mM did induce peroxisome proliferation in mouse hepatocytes and Hep3B cells. Further studies at other concentrations are needed to fully determine whether hepatocyte cultures and human hepatoma cells can be used as model systems in assessing TCE's risk to humans.

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