A SPECTROFLUOROMETRIC STUDY USING DI-4-ANEPPS OF THE EFFECTS ON MEMBRANE POTENTIAL FROM LASALOCID, CROTOXIN, FOLIC ACID AND α-CHACONINE EXPOSURES

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CHAPTER 1

Introduction and Review of the Literature

Introduction

Overview of Study

Scope and purpose. The scientific discipline, Toxicology, concerns itself with the study of the impact of chemicals, natural or synthetic in origin, on biological structures and processes. Organisms of all types, from bacteria to man, are constantly encountering a wide variety of potentially toxic, or non-toxic, substances and thus, a clear understanding of the interaction of chemical substances with living materials is of paramount importance. Continued efforts by researchers in academic, governmental, and industrial laboratories have made significant progress towards understanding the toxic effects of many compounds. However, the wide range of chemical substances, in existence today, allows for many continued basic and applied toxicology research investigations. There are problems associated with toxicology studies, including: the influence of dosages (or concentrations of exposure); the differing toxicities to different organs, tissues, or cell types; and as is often the case, the slow pace with which these studies produce results.

Although it is unlikely that one particular study or methodology could completely elucidate the toxic actions of a chemical, this research project expands upon the work previously conducted in Dr. James Blankemeyer's laboratory

(Blankemeyer, *et al.*, 1992). This report attempts to further illustrate the suitability of using Di-4-ANEPPS, an electrochromic dye which fluoresces in response to changes in membrane potential, to assess the toxicity of several different compounds. As the maintenance of ionic homeostasis is a fundamental process incumbent upon all cells of all biological tissues, any compound which alters this balance or disrupts a cell's ability to maintain ionic homeostasis could be construed as potentially toxic. The methodology employed throughout the three portions of this research project is capable of detecting a compound's effect on membrane potential and with a few minor modifications, is the same basic toxicology method reported by Blankemeyer, *et al.*, (1992). Although the influx or efflux of ions can significantly affect a cell's normal physiology, structure, and viability, monitoring the changes in membrane potential, alone, can suggest the toxicity of a compound, but cannot definitively decide it.

However, the use of Di-4-ANEPPS and spectrofluorometry is a simple, rapid, and adaptable method that not only evaluates potential toxicity, but provides other benefits as well. As previous work reported by Blankemeyer, *et al.*,(1992) has shown, the procedures are easily performed and offer results within a few minutes to a few hours. Thus, a great many compounds at a wide variety of concentrations can be studied within a short amount of time. Further, as this project used a variety of different biological substances, this methodology can be adapted for use with a variety of cell types or tissues. As a compound can have a toxic effect on one organ or tissue, while not affecting others, the ability of this method to analyze not only different compounds, but different tissues and cell types, is a great benefit. Thus,

recording the changes in membrane potential, using Di-4-ANEPPS and spectrofluorometry, can serve as a valuable tool in toxicology research and can overcome many of the problems relating to different concentrations, different tissues/cell types, and the time involved in producing results. The following chapters are offered in support of this assertion.

The Methodology

Spectrofluorometry

Basic concepts. A variety of substances have the ability to release photons of light upon exposure to light of a certain wavelength. These compounds are called fluorophores and it is this fundamental light absorption and emission property that serves as the basis for this methodology. The energy-level diagram shown in Figure 1 represents the different electronic and vibrational energy levels achieved by a fluorophore exposed to its excitation wavelength of light. The fluorophore absorbs the excitation energy which elevates an electron from the ground state (S_0) up to higher electronically excited states (S_1 and S_2). However, this transition will only occur if the photon energy, $h\nu$, of the excitation wavelength, λ_{ex}, is equal to the energy difference between the ground state and one of the higher excited states. The energy absorbed by the fluorophore is not only used in the elevation of an electron to higher energy levels, but is also transformed into vibrational modes (shown as short horizontal lines on Figure 1).

Additionally, the excited state fluorophore is in contact with surrounding solvent molecules and collisions with solvent molecules transmits some of the absorbed energy to the surroundings. Thus, as the excited molecule contacts surrounding solvent molecules, it gives up energy causing the excited molecule to step down the ladder of vibrational levels. However, most solvent molecules are not capable of accepting the large energy differences between electronic excited states and thus, the fluorophore spontaneously emits the remaining energy as radiation (ie. fluorescent emissions).

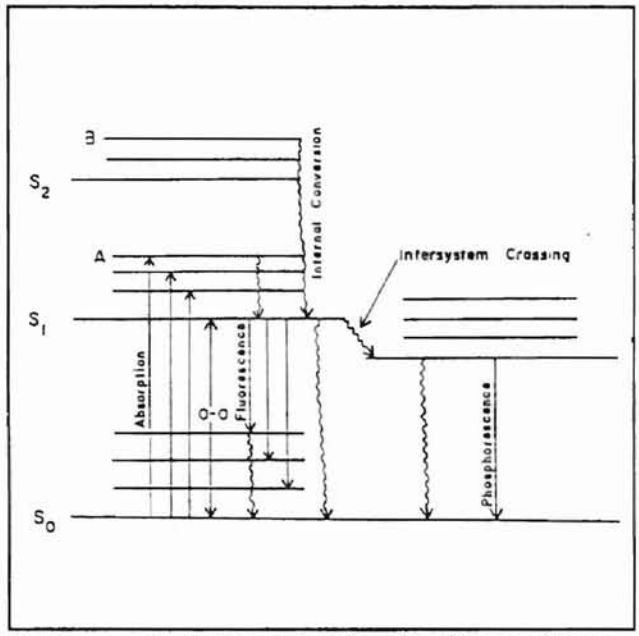

Figure 1: Energy levels of a fluorophore. S_0 is the electronic ground state while higher energy levels are S_1 and S_2. Short, horizontal lines represent different vibrational levels. Straight, vertical lines represent radiative processes, while *wiggly* lines represent non-radiative processes. (Yguerabide and Foster, 1977).

Therefore, the use of fluorescent dyes in biological studies relies on a fundamental energy transition of the dye molecules. The excitation of an electron to higher vibrational and electronic states with the immediate loss of a photon of lower energy, longer wavelength light is a quantum process that is greatly affected by the solvent molecules surrounding the dye. As an example, certain solvents, like water, have widely spaced vibrational levels and may be able to accept the large quanta of vibrational and electronic energy from an excited fluorophore. Thus, water quenches

the fluorescence of the dye molecule by absorbing all of the excited fluorophore's energy in a non-radiative process. Other solvent surroundings, like the phospholipids of a cell membrane, can only absorb a portion of the excited dye's energy with the remainder spontaneously emitted at longer wavelengths. This is the fundamental process at work in the experiments conducted during this study. For a more detailed description of fluorescence, the reader is referred to Atkins (1990) and Yguerabide and Foster (1977).

The electrochromic dye, Di-4-ANEPPS. The fluorophore used in this study is an electrochromic dye, Di-4-ANEPPS, 1-(3-sulfonatopropyl)-4-[b-[2-(di-n-butylamino)-6-naphthyl]vinyl] pyridinium betaine (Figure 2). It is a potentiometric dye that responds to changes in membrane potential (Fluher, *et al.*, 1985). In general, Di-4-ANEPPS increases the magnitude of its fluorescent emissions as a cell depolarizes. Fluorescence intensity decreases as a cell hyperpolarizes. The work reported by Montana, *et al.* (1989) showed that the ratio of fluorescence of Di-4-ANEPPS, excited at two wavelengths, was a reliable measure of membrane potential. Further, the use of ratiometric methods to measure a dye that undergoes a potential-

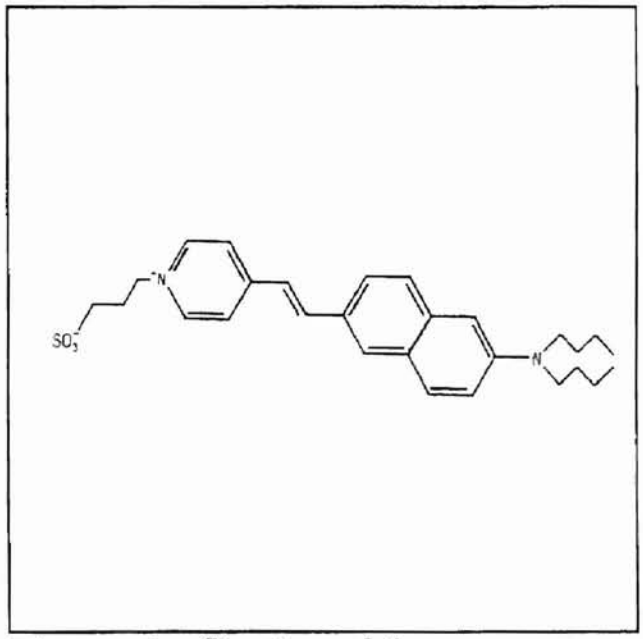

Figure 2: Structure of the electrochromic dye, Di-4-ANEPPS.

dependent spectral shift, like Di-4-ANEPPS, could reliably measure membrane potential without concern for small variations in the level of dye-loading from sample to sample (Montana, *et al.*, 1989). Overall, Di-4-ANEPPS has proven to be a reliable indicator of membrane potential in a variety of biological samples (Blankemeyer, *et al.*, 1992; Loew, *et al.*, 1992).

Furthermore, the lipophilic nature of the dye's elaborate, conjugated hydrocarbon structure makes for easy dye-loading into biological samples. As Pevzner, *et al.* (1993) reported, Di-4-ANEPPS is located in the membrane so that its charged pyridine side of the molecule is near the water (extracellular) interface and the aminonaphthalene side is embedded in the lipid bilayer. Further, they note that it is the polar head of the molecule protruding out of the lipid environment that is responsible for the spectral behaviour of Di-4-ANEPPS. Thus, incubation of a biological sample in an aqueous medium, containing Di-4-ANEPPS, allows for the uniform partitioning of the dye to the lipid environment of cell membranes. Lastly, the hydrocarbon structure of Di-4-ANEPPS not only serves to ensure a consistent distribution of the dye throughout the sample, but may play a role in its potential-dependent fluorescence. An exact mechanism for Di-4-ANEPPS remains unknown.

Introduction to the Three Studies Conducted

Effects of Lasalocid on the Membrane Potential of Chicken Erythrocytes: Use of the Electrochromic Dye, Di-4-ANEPPS.

Use in the chicken and cattle industries. Lasalocid is a polyether antibiotic that has been shown to be active against gram-positive bacteria, mycobacteria,and three *Eimeria* strains (Galitzer and Oehme, 1984; Peeters, *et al.*, 1994). Furthermore, numerous studies have suggested that lasalocid improves feed conversion performance of steers and chickens (Rode, 1987; Rode, *et al.*,1993; Damron, 1994). Because of lasalocid's ability to safeguard against certain microbial infections and its positive effects on certain growth parameters, it has gained widespread use in the broiler chicken and beef cattle industries.

Toxicities at high dosages. Lasalocid feed supplements, at recommended levels, can provide the benefits noted above. However, lasalocid intake that exceeds recommended levels has been associated with neurotoxicity and myotoxicity. Accidental inclusion of lasalocid in broiler chicken feed (Perelman, *et al.*, 1993) and in commercial dog food (Safran, *et al.*, 1993) resulted in leg weakness, ataxia, severe

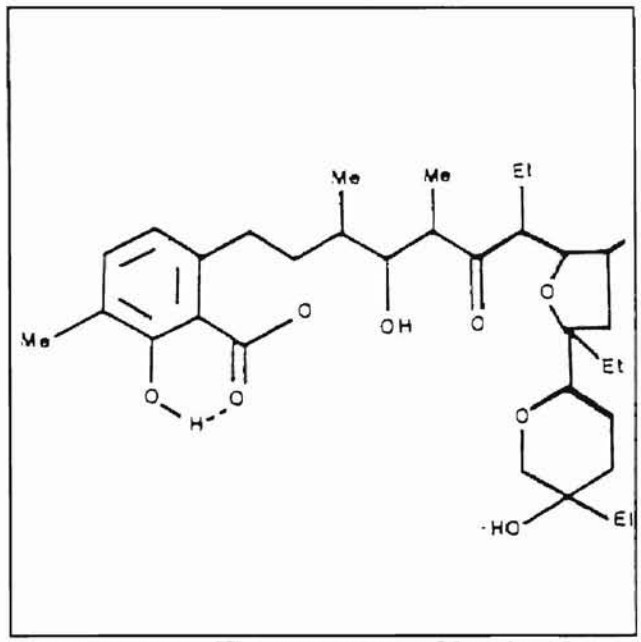

Figure 3: The structure of lasalocid.

muscle damage, and a general lower motor neuron deficit. The exact mechanism for this neuromuscular toxicosis remains unknown.

Membrane transport activity. Lasalocid has been shown to be an effective agent for transporting a variety of compounds across biological membranes. In addition to cations, it can transport ethanolamine, norepinephrine, epinephrine, and isoproterenol (Pressman and de Guzman, 1974). As a result of this transport property, lasalocid has been shown to affect cell physiology (Perragaux and Gabel, 1994), alter mitochondrial calcium flux (Antonio, *et al.*, 1991), and mitochondrial swelling (Mehrotra, *et al.*, 1993).

The lasalocid study, in brief. Given that certain lasalocid exposures have been shown to produce neurotoxic and myotoxic effects in broiler chickens, and given that these effects may be attributable to lasalocid's membrane transport activity, I investigated the effects of various lasalocid concentrations on the membrane potential of avian erythrocytes. By altering the ionic composition of the erythrocyte buffer solution, and recording the changes in Di-4-ANEPPS's fluorescence, I was able to demonstrate that the fluorescence measured was indicative of toxicity. Further, this methodology provided information on the limits of safe lasalocid concentrations versus potentially toxic exposure levels.

By incubating a 1.5 mL sample of Cobb broiler chicken red blood cells with a certain concentration of lasalocid, I demonstrated that this preparation served as a model for studying the transport activity of lasalocid and its effects on membrane potential. Further, chicken erythrocytes are available in large quantities and are

easily drawn from the birds with a minimal degree of harm to the animal. This study of lasalocid was successful in showing the ease with which a spectrofluorometric analysis of membrane potential can serve as a screening tool to predict toxicity.

Evidence That Folic Acid Inhibits the Depolarization of *Xenopus laevis* Embryos Exposed to the Potato Glycoalkaloid, α-Chaconine.

Role of diet in development. Recently, folic acid, a common vitamin, has been shown to protect against the neural tube defects, *spina bifida* and anencephaly (MRC Vitamin Study Research Group, 1991; Wald, 1993; Bower, *et al.*,1993). α-Chaconine, a secondary metabolite synthesized by the potato (*Solanum tuberosum, L.*), may be capable of inducing developmental anomalies, including neural tube defects (Sharma, *et al.*, 1978; Morris and Lee, 1984; Keeler, *et al.*, 1991). Also, epidemiological studies have found the neural tube defects *spina bifida* and anencephaly to be among the most prevalent birth defects, occurring in about 1 in every 1000 births (Elwood and Elwood, 1980; Cragen, *et al.*, 1994). Thus, an unsuitable diet during development can result in the gravest of consequences.

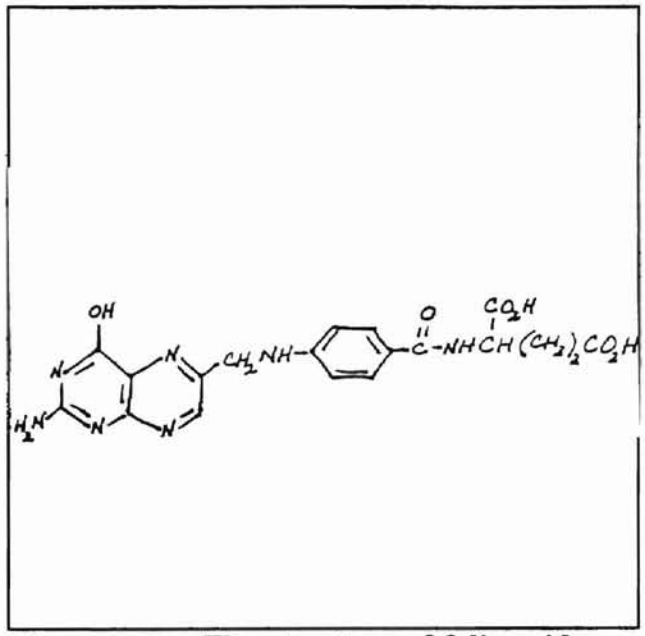

Figure 4: The structure of folic acid.

Prior research with α-chaconine. Work performed by Blankemeyer, *et al.* (1992) has successfully illustrated the effects of α-chaconine on the membrane potential of *Xenopus laevis* embryos. The potato glycoalkaloid was found to strongly depolarizes the embryo cells by more than 1600% relative to negative control groups. This study monitored the changes in fluorescence of Di-4-ANEPPS and served as a foundation for the work related in this thesis. Although it remains unclear as to the exact mechanism whereby α-chaconine can depolarize membranes, some evidence has been offered to suggest a disruption of sodium active transport or some interaction with a sodium channel (Blankemeyer, *et al.*, 1994).

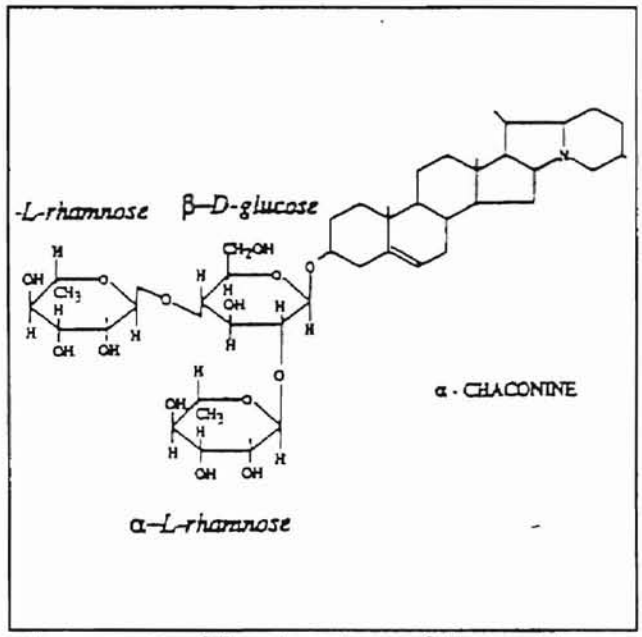

Figure 5: The structure of the potato glycoalkaloid, α-chaconine.

The folic acid/α-chaconine study, in brief. Building on the prior work conducted with α-chaconine, I investigated the effects on membrane potential of various α-chaconine concentrations challenged with various amounts of folic acid. I showed that folic acid was capable of reducing, in a concentration-dependent manner, the strong membrane depolarization caused by the potato glycoalkaloid. As folic acid safeguards against the very neural tube defects that α-chaconine has been shown to cause, the implications to food safety, and human health, warranted this study.

In this study, I used the embryos of the South African Clawed frog (*Xenopus laevis*). These frogs were relatively easy to care for and are capable of producing embryos, numbered in the thousands, with each mating. The large numbers of embryos allowed for numerous experiments and replications. Also, *Xenopus laevis* was the same specie used in the earlier work conducted by Blankemeyer, *et al.*,(1992) and thus allowed for comparison of this study's results with earlier research efforts.

A Study of the Myotoxic Effects of Crotoxin on Murine Skeletal Muscle Isolates Using Microscopy, Spectrofluorometry, Fluorescence Imaging and Creatine Kinase Release

Structure and activity. Crotoxin is the main toxic component of the venom of the South American rattlesnake (*Crotalus durissus terrificus*). It is a dimer of two subunits, A and B, which are noncovalently combined to form the complete venom toxin (Hendon and Fraenkel-Conrat, 1971). When combined, the two subunits' toxicity is greatly increased (Trivedi, *et al*, 1989). The basic protein subunit B has phospholipase A_2 activity and is generally considered to be the more toxic component of crotoxin (Breithaupt, *et al.*, 1974; Aird, *et al.*, 1986; Delot and Bon, 1992). The acidic protein, subunit A, is believed to act as a chaperon to subunit B (Bon, *et al.*, 1979; Aird, *et al.*, 1985).

Toxicities associated with crotoxin. The phospholipase A_2 neurotoxin family of proteins, to which crotoxin belongs, generally affect acetylcholine release at presynaptic sites on the neuromuscular junction (Tu, 1995). However, prior research

has shown crotoxin to have hemolytic and phospholipolytic activity (Jeng, *et al.*, 1978), as well as, myotoxic activity (Gopalakrishnakone, *et al.*, 1984). Although a great deal of research has described the toxic events following crotoxin exposure, there is little known regarding the actual mechanism of action of the toxin. Furthermore, a review of the literature has failed to uncover a study of the effects on murine whole-muscle ionic homeostasis induced by crotoxin.

The crotoxin study, in brief. This study investigated the effects of crotoxin on the membrane potential of murine omohyoid whole-muscle isolates. I incubated the muscle isolates in Di-4-ANEPPS, introduced crotoxin, and monitored the changes in membrane potential using a dual-wavelength spectrofluorometer and a digital imaging system. Fluorescent images illustrated the myotoxic action of crotoxin, while the spectrofluorometer offered quantitative data in support of the fluorescent images. Histopathological effects on myofibers were also studied using light microscopy, and creatine kinase release assays confirmed muscle necrosis induced by crotoxin. Overall, this study provided sufficient evidence that crotoxin is not only a presynaptic neurotoxin, but is capable of acting directly on muscle cells.

Lastly, the work performed during this study not only illustrated the myotoxicity of crotoxin, but also served to demonstrate the adaptablility of this spectrofluorometric method. By incubating the muscle isolates in Di-4-ANEPPS, the tissue samples could then be analyzed using the dual-wavelength spectrofluorometer, or the fluorescence imaging system. Both of these analytic techniques rely on the fluorescent properties of the dye, and when performed together, were successful in

proving the myotoxic effects of crotoxin, both visually and quantitatively. A great part of the success of this study is attributable to the ingenuity and dedication of Dr. Paulo Melo for his preparation of the muscle isolates. The author remains in his debt.

Closing Remarks

The work presented in the following chapters will demonstrate the importance of monitoring changes in cell membrane potentials using spectrofluorometric methods. Further, this report will show that this methodology can be used to provide information on toxic, and non-toxic, concentrations of four different compounds, can be used on a number of different tissues or cell types, can provide results in a relatively short amount of time, and, most importantly, can provide valuable predictions of the toxic effects of the test compound. Thus, a great many of the problems associated with toxicology research are remedied using this spectrofluorometric method. Given the severity of the toxic effects caused by lasalocid, α-chaconine, and crotoxin, the importance of the three studies presented in this report becomes clear.

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Effects of Lasalocid on the Membrane Potential of Chicken Erythrocytes: Use of the Electrochromic Dye, Di-4-ANEPPS

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ABSTRACT

Erythrocytes from male, Cobb broiler chickens and the electrochromic dye, Di-4-ANEPPS, were used to test the effects of various doses of the coccidiostat, lasalocid, on membrane potential. Further, by comparing the changes in membrane potential of the erythrocytes in complete buffer solution with results using selective ion-depleted buffer solutions, we were able to illustrate that lasalocid exhibited ion selectivity in membrane transport activity. Lasalocid exposures of 0.001 μM, 0.010 μM, 0.100 μM, 1 μM, and 10 μM failed to alter the membrane potential in a statistically significant manner, while 100 μM exposures strongly depolarized the cells in all experiments. Results using sodium-free, magnesium-free, and calcium-free buffer solutions indicated that lasalocid has a strong preference for divalent cations over monovalent cations in its membrane transport activity. Chicken erythrocytes were also exposed to a 40 μM concentration of the potassium ionophore, valinomycin, which resulted in a significant depolarization of the cells. Comparisons between the different ion deplete buffer solutions found that valinomycin in the sodium-free buffer was the only experiment to significantly differ from the other buffer types. This study suggests that this spectrofluorometric assay of avian erythrocytes may serve as a reliable and non-lethal model for investigating the effects of different compounds on cell membrane potential and ion transport affinities.

Keywords: lasalocid, valinomycin, Di-4-ANEPPS, membrane potential, erythrocytes, chickens.

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INTRODUCTION

Lasalocid is a polyether, ionophorus antibiotic from the soil microbe, *Streptomyces lasaliensis*, and has been shown to be active against gram-positive bacteria and mycobacteria (Galitzer and Oehme, 1984). It is a coccidiostat that has been shown to be particularly active against three coccidia strains: *Eimeria acervulina, Eimeria maxima, and Eimeria tenella* (Peeters *et al.*, 1994). Because it is an effective coccidiostat, lasalocid has been used extensively in beef cattle and broiler chicken production. Additionally, numerous studies have suggested that lasalocid improves feed conversion performance of steers and chickens (Rode, 1987; Rode, *et al.*, 1993; Damron, 1994).

Recently, lasalocid has been associated with neurotoxicity and myotoxicity. Perelman *et al.* (1993) described the accidental inclusion of 115ppm and 150ppm lasalocid sodium into the feed of breeder broiler chickens which resulted in leg weakness, ataxia, reduced egg production, and sharp decreases in fertility and hatchability. Histological examination showed severe muscle damage to affected chickens. Another accidental addition of lasalocid to commercial dog food was found to be the cause of neuromuscular toxicosis in 10 dogs; all dogs exhibited a generalized lower motor neuron deficit (Safran *et al.*, 1993). However, a review of the literature failed to uncover the exact mechanism for this neuromuscular toxicosis.

Studies on the membrane transport capabilities of lasalocid have shown that in addition to cations, it can complex with and transport ethanolamine,

norepinephrine, epinephrine and isoproterenol (Pressman and de Guzman, 1974; Pressman and de Guzman, 1975). Because lasalocid is very efficient in transporting substances across biological membranes, the compound can have significant effects on cellular physiology (Borrel, *et al*, 1994; Filvaroff, *et al*, 1994; Perragaux and Gabel, 1994). Furthermore, lasalocid treatments have been shown to cause alterations in mitochondrial calcium flux and swelling of mitochondria (Antonio *et al.*, 1991; Mehrotra *et al.*, 1993).

In this study, spectrofluorometry and the electrochromic dye, Di-4-ANEPPS, were used to study the effects of lasalocid on the membrane potential of Cobb broiler chicken erythrocytes. Pevzner *et al.* (1993) found that Di-4-ANEPPS partially embeds itself into the lipid bilayer of biological membranes causing the polar head of the molecule, responsible for the dye's spectral properties, to protrude out of the membrane. Once embedded, Di-4-ANEPPS changes the intensity of its fluorescent emissions in response to changes in membrane potential. (Montana *et al.*, 1989). Previous work using Di-4-ANEPPS to evaluate the effects of toxicants on membrane potential (Blankemeyer, *et al.*, 1992; Blankemeyer, *et al.*, 1993; Stringer and Blankemeyer, 1993; U.S. Patent #5416005) led us to investigate the effect of lasalocid on avian erythrocytes.

A review of the literature failed to uncover the use of chicken erythrocytes in a spectrofluorometric study of membrane potential, of this kind. These cells can serve as an adequate model for this study as they are nucleated, possess mitochondria, synthesize protein and RNA, depend on oxygen for their metabolic

energy, and respond to hormonal stimulation (McManus and Schmidt, 1978). Also, they are readily available in vast quantities affording numerous replications of experimental points of data and are easily drawn from the chicks with a minimal degree of harm or discomfort to the animal.

MATERIALS and METHODS

Preparation of buffer and dye solutions. Buffer solutions for the isolated erythrocytes were prepared fresh on the day of the experiment. The buffer solutions were prepared using Millipore water in acid-rinsed glassware. The target osmolality and pH of these solutions was between 290-300 mOsm and 7.0-8.0, respectively. The complete buffer solution contained ions at approximately the same concentrations found in chicken serum. The complete buffer contained: 7.312 g/L NaCl; 3.402 g/L Na Acetate; 0.345 g/L $CaCl_2$, 0.181 g/L $MgSO_4$; and 0.343 g/L KCl, which resulted in the following values for the ions: 150 mEq/L sodium; 4.6 mEq/L potassium; 4.7 mEq/L calcium; 130 mEq/L chloride; 1.5 mEq/L magnesium. Sodium-free buffer was prepared by substituting lithium for sodium in the solution. Calcium-free buffer was prepared by deleting $CaCl_2$ and adjusting the KCl and NaCl content of the buffer to maintain the desired osmolality. Magnesium-free buffer was prepared by deleting $MgSO_4$ and adjusting the KCl and NaCl content of the buffer to maintain the desired osmolality. A 1 mg/mL stock solution of Di-4-ANEPPS (Molecular Probes, Inc., Eugene, OR) was prepared by dissolving 5 mg of the dye in 5 mL of absolute ethanol.

Preparation of lasalocid and valinomycin solutions. A 2 mM stock solution of lasalocid was prepared in DMSO and diluted in appropriate buffer types. The final concentrations of lasalocid in the buffer solutions was 0.002 μM, 0.020 μM, 0.200 μM, 2.00 μM, 20.0 μM, 100 μM, 140 μM and 200 μM. The final concentration of DMSO in these lasalocid solutions was less than 5%. A 1 mg/mL stock solution of valinomycin was prepared by dissolving valinomycin in DMSO.

Blood collection. Using heparinized syringes, blood was collected from the brachial veins of male, Cobb broiler chickens ranging in ages from 9-12 weeks. The erythrocytes were separated and isolated by an adapted method described by Lee

(1974). Briefly, the heparinized blood was centrifuged at 4°C at 50x g for 10 minutes. The supernatant, containing lymphocytes, was removed and discarded. The pellet was washed three times and resuspended in a buffer solution and recentrifuged as above. The supernatant from this centrifugation was again discarded and the erythrocytes were resuspended in buffer to yeild a 1:1 ratio of cells to buffer. Stained blood smears of this type of solution were examined microscopically and contained only erythrocytes. The erythrocyte solutions were maintained at 10°C until assayed.

Sample preparation. The erythrocyte solutions were first diluted 1:1000 by pipetting a 0.10 mL aliquot of the cell solution into a 100 mL calibrated, volumetric flask and diluting up to the mark with buffer solution. A stock of erythrocytes was diluted in each of the four buffer types: complete, sodium-free, magnesium-free, and calcium-free. A minimum of three, 4 mL plastic cuvets were each filled with 1.5 mL of the cell suspension and 1.5 mL of the test solution. This triplet was prepared for each lasalocid solution. The 50% dilution in each cuvet resulted in final exposure concentrations of 0.001 μM, 0.010 μM, 0.100 μM, 1 μM, 10 μM, 50 μM, 70 μM and 100 μM. Negative control groups consisted of 1.5 mL of the erythrocyte suspension and 1.5 mL of the buffer solution alone.

Calibration of erythrocytes. The spectrofluorometer (Photon Technologies International, Inc., South Brunswick, NJ) was configured to continuously scan for 250 seconds; recording five points of data per second. Excitation wavelengths were set at 480 nm and 570 nm with fluorescence emissions recorded at 620 nm. 1.5 mL of erythrocyte suspension was placed in a plastic cuvet along with 1.5 mL of a given buffer type. 10 μL of the Di-4-ANEPPS stock was added to each cuvet, covered and inverted 3-5 times, and allowed to incubate for 30 minutes at room temperature. A cuvet was loaded into the spectrofluorometer and scanned from 0 seconds to 60 seconds to serve as a negative control trace. At about the 60-65 second mark, 150 μL of pure DMSO was added to the cuvet (to control for the DMSO used in the valinomycin stock solution) and fluorescence measurements continued. At about the 120-125 second mark, 150 μL of the valinomycin stock was added to the cuvet and fluorescence measurements continued to the 180 second mark. The process was repeated for the remaining cuvets and all cuvets were stirred constantly throughout the scanning procedure.

Membrane potential measurements. 10 μL of the Di-4-ANEPPS stock was added to each cuvet, containing 1.5 mL of erythrocyte suspension and 1.5 mL of a test solution. The cuvets were covered, gently inverted three to five times and allowed to incubate, at room temperature, for 30 minutes. At the close of the incubation period, each cuvet was scanned using a PTI DeltaScan dual-wavelength spectrofluorometer (Photon Technologies International, Inc., South Brunswick, NJ) with dual excitation wavelengths set at 480 nm and 570 nm. Photons of fluorescent emission at 620 nm were captured by a photomultiplier tube at 90 degrees to the incident light. Emission photons/sec counts were saved onto disk for later analysis.

Analysis of data. The fluorescent emission data were normalized by dividing by the mean of the negative control groups and recorded as a percentage of negative control fluorescence. The negative control groups were arbitrarily set at 100%. All values are reported as the mean percentage of negative control fluorescence $\pm$ SEM. Curve fitting of these data, by nonlinear regression, was performed using the Inplot program (Graphpad, San Diego, CA). A SAS program provided ANOVA and LSD analyses of experimental data.

RESULTS AND DISCUSSION

Figure 1 shows the results of exposing the erythrocytes to 40 μM valinomycin in DMSO in the four different buffer types. As DMSO was used as the solvent for valinomycin, cells were first exposed to pure DMSO (Fig. 1A). Statistical analysis revealed that the DMSO did not significantly alter the membrane potential from the negative control groups. Subsequent exposure to the potassium ionophore,valinomycin, (Fig. 1B) did increase the fluorescence of the erythrocytes, relative to the negative control groups ($p < 0.05$). ANOVA analysis ruled out any effect from the different buffer types and confirmed the change in membrane potential to be attributable to the valinomycin treatment ($p < 0.0001$). Comparisons between the different ion-deplete buffer solutions showed that valinomycin in the sodium-free buffer was the only experiment to significantly differ from the other buffer types ($p < 0.05$). This illustrates valinomycin's preference for monovalent cations over divalent cations.

Figure 2A shows the results from experiments involving the complete buffer solution (five replicates for five different birds) and the sodium-free buffer solution (five replicates for four different birds). Fluorescence from erythrocytes incubated in lasalocid concentrations ranging from 0.001 μM to 0.100 μM are not

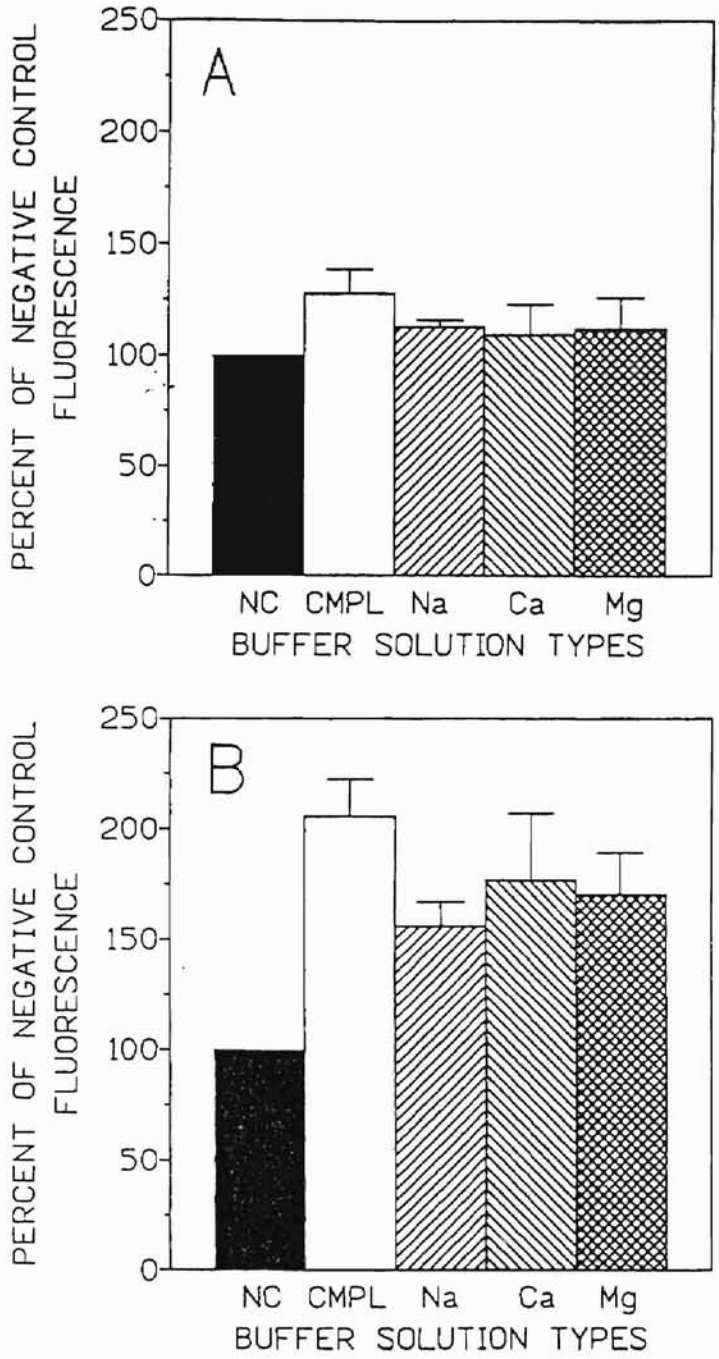

Fig. 1A. Effects on membrane potential of avian erythrocytes, in various buffer solutions, exposed to a 150 uL aliquot of pure DMSO The fluorescence from erythrocytes in plain buffer solution was recorded, continuously, for 60 sec. to serve as a negative control. The DMSO was then added and fluorescence recordings continued for the next 60 sec. Fig. 1B. Lastly, a 150 uL aliquot of the 2 mM valinomycin stock was added to the cuvet and scaning continued for 60 sec. All values are shown as the mean $\pm$ S.E.M. for 3 replicates for 3 different birds. NC= negative control, CMPL= complete buffer; Na= sodium-free buffer; Ca= calcium-free buffer; and Mg= magnesium-free buffer.

shown because they were not significantly different from negative control values, arbitrarily set at 100%. Although the 1 μM and 10 μM treatments did not deviate from the negative control groups, the 50 μM, 70 μM, and 100 μM lasalocid treatments were found to significantly depart from the negative control groups in the complete buffer ($p < 0.05$), while the 70 μM and 100 μM exposures were significantly different in the sodium-free buffer studies ($p < 0.05$).

Figure 2B illustrates the results of experiments involving the calcium-free buffer solution (3-5 replicates for 3-5 different birds) and Figure 2C shows the results of the magnesium-free buffer solution (3-5 replicates for 4-5 different birds). Again, the 0.001 μM to 10 μM treatments were not significantly different from negative controls and the 0.001 μM to 0.100 μM points are not shown. Unlike the complete buffer and sodium-free buffer solutions, the 100 μM lasalocid concentration was the only exposure to cause a statistically significant change in membrane potential relative to the negative control groups ($p < 0.05$). However, the maximum fluorescence, for the 100 μM treatment decreased from 356% in the complete buffer to 256% and 261% in the magnesium-free and calcium-free buffer solutions, respectively. The largest change in fluorescence from a 100 μM lasalocid concentration in the sodium-free buffer solutions was an intermediate value of 317%.

The data from these experiments are in agreement as to the activity of lasalocid in membrane transport. The complete buffer solution study showed the greatest change in membrane potential because all requisite monovalent and

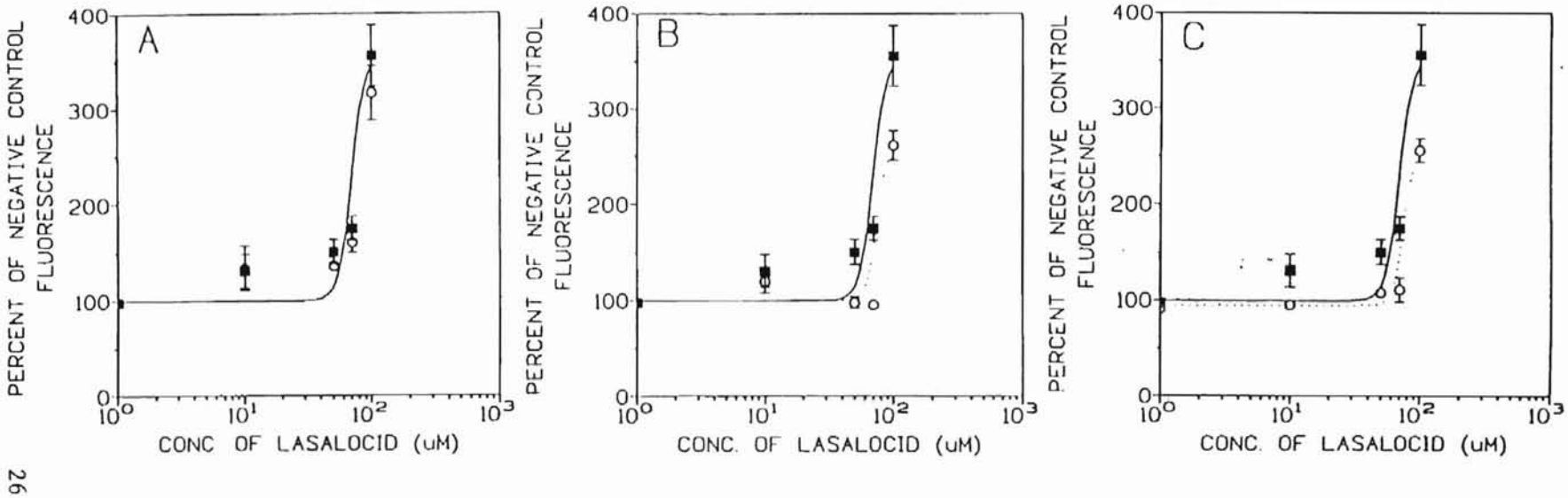

Fig. 2A. Effects of a range of lasalocid treatments on the membrane potential of erythrocytes in complete buffer solution (solid line) and a sodium-free buffer solution (dashed line). The 0.001uM to 0.100 uM treatments did not significantly affect the membrane potential and are not shown. The complete buffer solution results serve as a positive control group. Data are shown as the mean ± S.E.M.

Fig. 2B. Effects of a range of lasalocid treatments on the membrane potential of erythrocytes in calcium-free buffer solution (dashed line). The 0.001 uM to 0.100 uM treatments did not significantly effect the membrane potential and are not shown. The complete buffer solution results (solid line) serve as a positive control and are the same points as in Fig. 2A. Data are shown as the mean ± S.E.M.

Fig. 2C. Effects of a range of lasalocid treatments on the membrane potential of erythrocytes in magnesium-free buffer solution (dashed line). The 0.001 uM to 0.100 uM treatments did not significantly effect the membrane potential and are not shown. The complete buffer solution results (solid line) serve as a positive control and are the same points as in Fig. 2A. Data are shown as the mean ± S.E.M.

divalent cations are readily available to the ionophore. Lasalocid demonstrates its ion-selectivity by the sharp reduction in membrane potential for the divalent cation-free solutions, as well as, the close agreement between the magnesium-free and calcium-free buffer solution measurements. When either of these cations is removed from solution, lasalocid complexes with monovalent cations, reducing the magnitude of depolarization. Alternatively, when sodium is removed from solution, the ionophore has sufficient numbers of divalent cations remaining for transmembrane movement, and the degree of membrane potential change approaches that of the positive control values (i.e. the complete buffer solution).

Further, the results of this project are in accord with observations made by other investigators as to lasalocid's transport of divalent cations, particularly, calcium (Pressman and Oehme, 1974; Fahim, 1992; Satoh, *et al*, 1992). Excessive dosing of lasalocid, resulting in increased intracellular calcium levels, has been implicated as a causative factor in the development of neuropathic lesions in broiler chickens (Gregory, *et al.*, 1995). Safran, *et al.* (1993) reported neurotoxic effects in murine fetal cerebral cell cultures exposed to 1 μM and 2 μM lasalocid concentrations. Lower concentrations (ie. 0.2 μM), however, failed to cause any neuronal damage. In this study, we selected lasalocid exposures which encompassed the recommended therapeutic values and in the case of the 100 μM treatment, exceeded recommended levels (Stipkovits and Juhasz, 1987). The only treatment which significantly altered the membrane potential, the 100 μM concentration, is close to those levels which resulted in the neuromuscular

pathology reported by Perelman, *et al.* (1993).

Confirmation of these earlier findings, regarding the membrane transport of calcium by lasalocid, lends validation to this particular methodology and encourages its further development. Isolated avian erythrocytes may be an adequate model for lasalocid toxicosis. This avian erythrocyte spectrofluorometry assay can provide quick, reliable, and easily reproducible membrane transport data for various concentrations of toxicants.

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Evidence That Folic Acid Inhibits the Depolarization of *Xenopus laevis* Embryos Exposed to the Potato Glycoalkaloid, α-Chaconine

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Evidence That Folic Acid Inhibits the Depolarization of *Xenopus laevis* Blastulas Exposed to the Potato Glycoalkaloid, α-Chaconine. Burns, C.F., Blankemeyer, J., Stringer, B.K., and Friedman, M. (1995). *J. Agric. Food Chem.*

Prior research has suggested a role for folic acid in the prevention of certain neural tube defects and has implicated the potato glycoalkaloid, α-chaconine, as a possible causitive agent in these same developmental anomalies. We investigated the interaction of these two compounds by exposing *Xenopus laevis* blastulas, concomittantly, to various combinations of these two compounds and monitoring the changes in membrane potential of the blastulas. We recorded the fluorescence of the electrochromic dye, Di-4-ANEPPS, sensitive to membrane potential changes, using a dual-wavelength fluorescence spectrophotometer. Results of this work indicate that the strong, concentration-dependent, depolarization caused by α-chaconine is inhibited with increasing folic acid exposures, in a statistically significant manner. 20 mg/L and 50 mg/L folic acid challenges returned blastula membrane potentials nearly to those of negative control values. A concentration-response curve for folic acid, alone, reveals no statistically significant departure from negative control values of blastula membrane potential.

Keywords: Folic acid, α-chaconine, Di-4-ANEPPS, *Xenopus laevis*, membranes

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INTRODUCTION

Folic acid is a vitamin that has received much study recently. Recent reports suggest that folic acid can produce a degree of protection against the neural tube defects, *spina bifida* and anencephaly (Wald, 1993; Bower, *et al.*, 1993). Previous work conducted in this lab showed that the potato glycoalkaloid, α-chaconine, strongly depolarized the membrane potential of *Xenopus laevis* embryos (Blankemeyer, *et al.*, 1992; Blankemeyer, *et al.*, 1994; Rayburn, *et al.*, 1994). The compound, α-chaconine, may be capable of inducing developmental anomalies including neural tube defects as some studies have suggested (Friedman, *et al.*, 1991; Friedman, *et al.*, 1992; Keeler, *et al.*, 1991; Morris and Lee, 1984; Sharma, *et al.*, 1978). Owing to the fact that folic acid safeguards against the very neural tube defects that α-chaconine has been shown to cause, we felt that the implications to food safety, and human health, warranted this investigation.

The common domesticated potato (*Solanum tuberosum L.*) synthesizes a group of secondary metabolites, glycoalkaloids, that include α-chaconine. It is estimated that about 95% of the total glycoalkaloid content of the tuber is composed of α-chaconine and the related, α-solanine (Jadhav, *et al.*, 1981). Dao and Friedman (1994) found that fresh White Rose potatoes contained 0.66 mg/100g fresh weight of α-chaconine. After exposure to fluorescent light for 20 days, there was a 300% increase in glycoalkaloid levels. The exposure to light and the storage conditions of the potatoes was found to increase glycoalkaloid levels in

a study by Griffiths, *et al.* (1994); and work performed by Hellenas, *et al.* (1995) found the average glycoalkaloid content from eight different varietes ranged from 51 to 221 mg/kg fresh weight.

The potato glycoalkaloid content in commercial potato products is of importance since α-chaconine may be a developmental toxicant. Considerable interest has been paid to potato glycoalkaloids since the early 1970's when speculation as to their teratogenicity first began. Mun, *et al.* (1974) noted neural tube closure abnormalities in chickens exposed to glycoalkaloids and Jelinek, *et al.* (1976) exposed chickens to blighted potato extracts resulting in caudal development problems. Work performed on hamsters produced 64 abnormal offspring in 26 of 113 matings (Keeler, *et al.*, 1976; Keeler, *et al.*, 1978). Neural tube defects were also noted by Renwick, *et al.* (1984) upon exposure to extracts from the sprouts of the British cultivar, *Arran Pilot.*

The developmental disorders associated with α-chaconine are difficult to quantify as many times the conceptus is either resorped or spontaneously aborted. However, anencephaly and *spina bifida* are thought to occur in the United States in about 1 in 1,000 births (Elwood and Elwood, 1980) with other estimates of 0.9 to 1.3 per 1,000 births (Cragen, *et al.*, 1994). There is a wealth of epidemiologic evidence linking a lack of suitable folate in the diet with incidences of neural tube defects (Campbell, *et al.*, 1986; Seller, 1987; Slattery and Janerich, 1991). Many studies have shown that the intake of 0.8 mg/day to 4 mg/day of folic acid may reduce the liklihood of a neural tube development problem (MRC Vitamin Study

Research Group, 1991; Czeizel and Susanszky, 1992). Trotz, *et al.* (1987) reported that the rates of exencephaly, induced by valproic acid exposures, in NMRI mice were decreased by 50% upon challenge with folinic acid.

In our work, we challenged the potato glycoalkaloid, α-chaconine, with various concentrations of folic acid. Using the blastulas of *Xenopus laevis* frogs, we followed the changes in membrane potential of the embryos upon exposure to these test compound mixtures. Di-4-ANEPPS is an electrochromic dye that embedds itself into the membrane leaflet of cells (Pevzner, *et al.*, 1993) and changes the intensity of its fluorescent emissions in response to changes in membrane potential (Fluher, *et al.*, 1985; Montana, *et al.*, 1989). Thus, by loading the dye into the embryos' membranes, incubating them in the test solutions, and recording the changes in fluorescence of the electrochromic dye with a spectrofluorometer, we were able to show that folic acid does ameliorate the toxicity of the teratogenic potato glycoalkaloid, α-chaconine.

MATERIALS AND METHODS

Test materials. FETAX solution was composed of: 10.8 mM NaCl; 1.2 mM NaHCO3; 0.58 mM MgSO4; 0.44mM CaSO4; 0.4 mM KCl; and 0.14 mM CaCl2 with a final pH of 8.0. All FETAX salts were purchased from Sigma (St. Louis, MO). α-Chaconine was purchased from Sigma (St. Louis, MO), purified to 99% by HPLC, and dissolved into FETAX solution as described previously (Friedman, et al., 1991) for a final stock concentration of 10 mg/L at a pH of 6.5. Likewise, folic acid (Sigma, St. Louis, MO) was dissolved into FETAX solution at a stock concentration of 100 mg/L and a pH of 6.5. Di-4-ANEPPS (Molecular Probes, Inc., Eugene, OR) was dissolved in absolute ethanol at 1 mg/mL.

Frog breeding and embryo preparation. Albino *Xenopus laevis* frogs were purchased from Xenopus I (Ann Arbor, MI). Breeding pairs were conditioned for one month to six weeks prior to usage. Approximately 12 hours prior to each experiment, the frogs were injected with 400-600 IU of Human Chorionic Gonadotropin (Sigma, St. Louis, MO) and placed in a covered, quiet, false-bottom breeding cage over-night. Following successful amplexus, embryos were collected and their jelly coats were removed using 2% (w/v) L-Cysteine adjusted to pH 8.1. Embryos were seperated into viable and non-viable groups. Mid-to-late balstula stage to neurala stage embryos were selected under a dissecting microscope. Groups of 65 viable embryos were gathered into covered plastic petri dishes.

Sample preparation. Each concentration of glycoalkaloid, 0.0 mg/L, 2.5 mg/L, 7.5 mg/L, and 10 mg/L, was challenged with 0.0 mg/L, 2.5 mg/L, 10 mg/L, 20 mg/L, and 50 mg/L of folic acid. Each mixture was tested with at least three groups of embryos. Each set of experimental embryos was compared against a set of negative control embryos (3-5 groups) which were exposed only to 20 μL of Di-4-ANEPPS and FETAX solution. For the experimental groups, the mixtures of test compounds and 20 μL of Di-4-ANEPPS were added to each petri dish. Both sets of petri dishes, experimental groups and the negative control groups, were covered with aluminum foil and allowed to incubate at room temperature, undisturbed, for 30 minutes.

Membrane potential measurements. At the end of the incubation period, the embryos were drawn into a glass pipette and placed, individually, into a 3 mm quartz glass microcuvet, which had been filled with FETAX solution. The embryos sank to the bottom of the cuvet and stacked themselves vertically inside. Any excess solution was removed from the top of the cuvet by a second pipette attached to a vacuum. The cuvet was placed within the sample chamber of a PTI DeltaScan (Photon Technologies International, Inc., South Brunswick, NJ), a dual-wavelength spectrofluorometer, and illuminated by 470 nm and 580 nm excitation energy. With slits set at 2 nm, the number of photons of 620 nm light emitted at right angles to the incident light were recorded for 20 seconds by a dedicated computer and stored on disk.

Analysis of data. The emission data was fit by linear regression and a mean number of emission photons was recorded. The mean number of photons emitted for the experimental groups was divided by the negative control groups' average value and recorded as a percent of negative control fluorescence. Curve fitting of this data, by nonlinear regression, was performed using the Inplot program (Graphpad, San Diego, CA). A SAS program provided ANOVA and LSD analyses of experimental data.

RESULTS

The information found in this project reveals that folic acid has the capability of inhibiting the strong depolarization of *Xenopus laevis* embryo membranes in a concentration-dependent manner. As illustrated in Figure 1, α-chaconine, alone, strongly depolarizes the frog blastulas in a concentration-dependent manner. The marked increase in the numbers of photons emitted at 620 nm upon exposure to a range of α-chaconine concentrations was found to be a statistically significant difference ($p < 0.05$) from the negative control data. Treatment of the embryos with only folic acid, ranging upto 80 mg/L, did not significantly alter photon emission data ($p < 0.05$).

A statistical analysis of the data shown in Figure 1 found that the 10 mg/L, 25 mg/L, and 50 mg/L exposures were ruled to be significantly different at the $p < 0.05$ level. The Inplot program (GraphPad, San Diego) was used to fit the data with a sigmoid model ($R^2 = 0.995$) and from this an EC_{50} value of 6.607 mg/L was found.

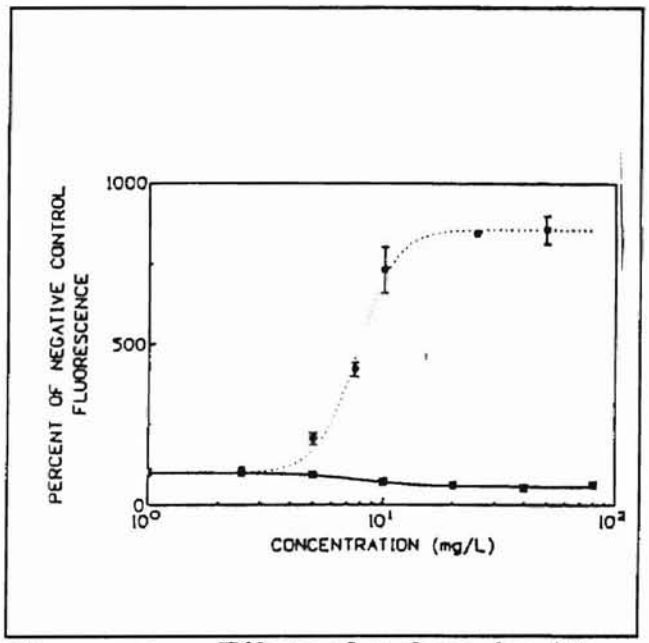

Figure 1: Effects of α-chaconine (●) and folic acid (■) on the fluorescence of Di-4-ANEPPS. Data are the mean ± SEM for four replicates and those marked "*" were found to significantly deviate from negative control groups, arbitrarily set at 100%.

Figure 2 shows the results of a series of experiments involving smaller doses of folic acid challenged against the same α-chaconine range shown in Figure 1 (noted as the 0 mg/L Folic Acid curve on Figure 2). These results show the reduction in α-chaconine's effect with increasing concentrations of folic acid. Statistical analysis, used to compare the experimental data points from each challenge with the positive control values from the α-chaconine concentration-response curve, found that concentrations of folic acid above 10 mg/L significantly reduced the effect of the teratogenic potato

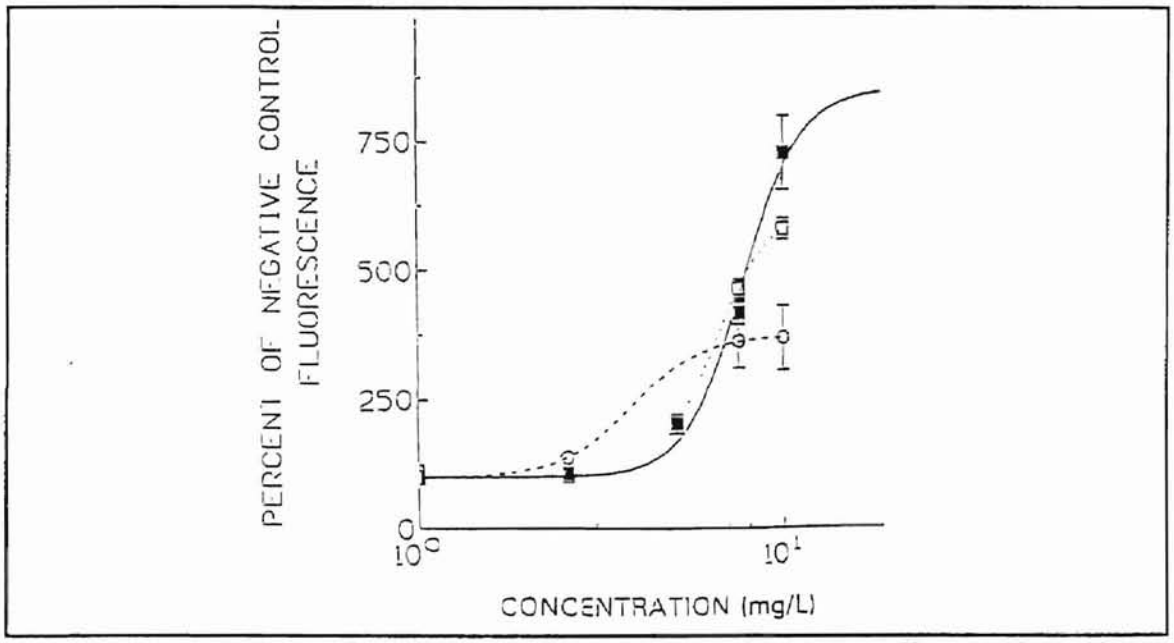

Figure 2: Effects of small folic acid challenges (□= 2.5 mg/L;○ = 10 mg/L) on the α-chaconine (■) induced fluorescence of Di-4-ANEPPS. Data are shown as the mean ± SEM for four replicates and those data marked "*" were found to be significantly different ($p<0.05$) from the positive control data (0 mg/L Folic Acid).

glycoalkaloid ($p < 0.05$). Folic acid doses below this threshold failed to produced any prophylactic effect. This observation is further shown in Figure 3, which illustrates the larger folic acid challenges of 20 mg/L and 50 mg/L.

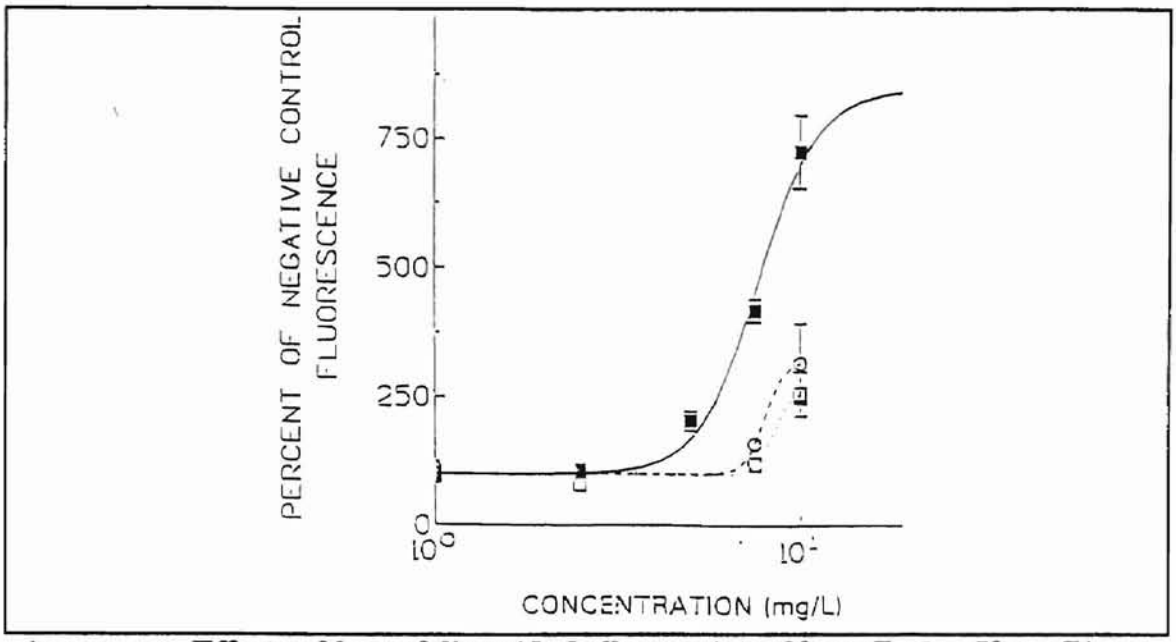

Figure 3: Effects of large folic acid challenges (□ = 20 mg/L;○ = 50 mg/L) on the α-chaconine (■) induced fluorescence of Di-4-ANEPPS. Data are shown as the mean ± SEM for four replicates. Those data marked "*" were found to be significantly different ($p<0.05$) from the positive control data (0 mg/L Folic Acid).

DISCUSSION

This report illustrates the ability of the potato glycoalkaloid, α-chaconine, to strongly depolarize the cells of the developing *Xenopus laevis* embryo. Additionally, increasing concentrations of folic acid are capable of negating this sharp depolarization and return membrane potential values closer to those of the negative control groups. An examination of the data relating the folic acid and α-chaconine challenges seems to suggest that folic acid exposures in excess of 10 mg/L elicit the greatest degree of prophylaxis against α-chaconine's effects. At folic acid concentrations of 20 mg/L and 50 mg/L, the inhibition of α-chaconine's depolarization is nearly certain and complete.

Although this study focused on changes in cell membrane potential, neither the exact mechanism of action nor the ions involved in these membrane potential changes has been elucidated. Previous work has presented evidence to suggest that the depolarization seen upon exposure to α-chaconine may be the result of either a direct, or indirect, inactivation of sodium active transporters or sodium channels (Blankemeyer, 1994). The implication from this work being that exposure to α-chaconine results in increased intracellular sodium levels and concommitant membrane depolarization. Research reported by Strum (1979) offered findings that the removal of sodium from the incubation medium of rat jejunum everted sacs reduced mucosa-to-serosa folic acid transport by 62% and 77% at pH 6. His investigation also found that ouabain pretreatment of the serosal side of the jejunal sacs reduced folic acid transport by 64% at pH 6, relative to negative control groups. These findings may provide the explanation for the membrane potential changes seen upon folic acid challenge of α-chaconine, however, further investigation is warranted.

The membrane potential sensitivity of the dye, Di-4-ANEPPS, is capable of following the total effects on cellular ionic homeostasis, however, other fluorescent dyes must be employed to monitor specific ionic events. This spectrofluorometric method, developed by Dr. James Blankemeyer, is one which is adaptable to these needs, rapid in its performance, and simple in its enactment. The future research efforts of our laboratory will focus on the possible role of sodium, or other ions, in folic acid's amelioration of α-chaconine's membrane depolarization effects. It

is hoped that the results of these experiments, and those pending, will provide some insights into the mechanism of action of the potato glycoalkaloids and offer additional information on the relationship of diet and developmental toxicity.

ACKNOWLEDGMENT

Portions of the work performed in this study are covered by the United States Patent #5416005 "A Method for Rapid Toxicity Testing of a Liquid Sample", held by Dr. James Blankemeyer.

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A Study of the Myotoxic Effects of Crotoxin on Murine Skeletal Muscle Isolates Using Microscopy, Spectrofluorometry, Fluorescence Imaging and Creatine Kinase Release

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A Study of the Myotoxic Effects of Crotoxin on Murine Skeletal Muscle Isolates Using Microscopy, Spectrofluorometry, Fluorescence Imaging, and Creatine Kinase Release. Burns, C.F., Melo, P.A., Ownby, C.L., and Blankemeyer, J.T. (1996) *Toxicon*.

To study the myotoxicity of crotoxin (CTX), we used an electrochromic fluorescent dye, Di-4-ANEPPS, to measure the changes in membrane potential (MP) of murine omohyoid muscle isolates over 20 min. Omohyoid isolates were pre-loaded with 1 μM Di-4-ANEPPS, exposed to various CTX treatments, and analyzed using either a dual-wavelength spectrofluorometer or digital imaging. Spectrofluorometry studies indicated that 1, 5, and 10 μg/mL exposures of CTX depolarized the muscles within 4 min. The 10 μg/mL treatment showed the greatest MP change of 22% relative to negative controls. Depolarization by valinomycin (1 μM in saline containing 100 mM KCl) served as a positive control, while exposure to a saline sham solution served as a negative control. Digital imaging studies exposed the same muscle preparations to 10 μg/mL CTX and captured fluorescent images every 1 or 2 minutes. Within minutes of CTX exposure, images revealed muscle contractions, localized areas of increased fluorescence, derangement of myofibrils, and differing sensitivity to CTX along neighboring muscle bundles. Light microscopy results confirmed this variable disruption of muscle fibril integrity and differing fibril-sensitivity to CTX. Lastly, we measured the increase of creatine kinase (CK) release above the basal level. CTX (10 μg/mL) caused an increase of CK release from a basal level of 0.32 $\pm$ 0.09 U/g/hr to 5.27 $\pm$ 0.53 U/g/hr after 10 minutes. CK release rates increased to 10.93 $\pm$ 1.12 U/g/hr and 19.51 $\pm$ 3.39 U/g/hr after 20 and 30 minutes, respectively, indicating muscle necrosis.

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INTRODUCTION

Crotoxin is the main toxic component from the venom of *Crotalus durissus terrificus* (South American rattlesnake) and has been the subject of many investigations. Structurally, crotoxin is composed of two subunits, A and B, which combine, noncovalently, to form the complete venom toxin (Hendon and Fraenkel-Conrat, 1971). When examined alone, the two subunits elicit little to no toxic effects, however, when combined the toxicity is greatly augmented (Trivedi, *et al.*, 1989). Prior research has indicated that subunit B is a basic protein, exhibits phospholipase A_2 activity, is mildly neurotoxic (Breithaupt, *et al.*, 1974; Bon and Delot, 1992; Aird, *et al.*, 1986), and myotoxic (Gopalakrishnakone, *et al.*, 1984). The acidic protein, subunit A, is believed to serve the role of a chaperon to subunit B, more so, than as a toxic component of the venom complex, as it fails to exhibit any enzymatic or toxic activity (Bon, *et al.*, 1979; Aird, *et al.*, 1985; Aird, *et al.*, 1990).

Owing to the phospholipase A_2 activity of crotoxin's B subunit, the venom component belongs to a family of snake venoms called the phospholipase A_2 neurotoxins, or β-neurotoxins (Bon, *et al.*, 1994). In addition to neurotoxicity, this group of compounds has been shown to cause cardiotoxicity (Lee, *et al.*, 1977; Fletcher, *et al.*, 1982; Chang, *et al.*, 1983), anticoagulation activity (Ouyang and Teng, 1972; Huang, *et al.*, 1984), and myotoxicity (Mebs and Ownby, 1990). Crotoxin has been shown to be potentially neurotoxic by affecting acetylcholine

release at presynaptic sites on the neuromuscular junction, additionally, the venom component can have postsynaptic effects as well (reviewed by Tu, 1995). In addition to its neurotoxicity, research has shown crotoxin to be both hemolytic and phospholipolytic (Jeng, *et al.*, 1978), and myotoxic (Gopalakrishnakone, *et al.*, 1984).

Although a great amount of research has detailed the toxicities associated with the snake venom phopholipases A_2 (reviewed by Kini and Evans, 1989), there remains a great amount of uncertainty as to the mechanism of action of these compounds, including crotoxin. Information as to the activities of these toxic phospholipases A_2 at the cellular and molecular levels is slowly accumulating (Ng and Howard, 1980; Schmidt and Betz, 1989; Gutierrez, *et al.*, 1990; Faiz, *et al.*, 1995). Work focusing on the involvement of extracellular and/or intracellular ions in the mechanism of venom induced myotoxicity is offered by Helmke and Howard (1986). While Johnson and Ownby (1994), studying a myotoxic component of *Agkistrodon contortrix laticinctus* venom, suggest the involvement of sodium, not calcium, ions in the initiation of pathological changes, in murine *biceps femoris* preparations. However, a review of the literature has failed to uncover a study of the effects on murine whole-muscle ionic homeostasis induced by crotoxin.

In this study, spectrofluoromety and the electrochromic dye, Di-4-ANEPPS, were used to study the effects of crotoxin on the membrane potential of murine omohyoid isolates. Pevzner, *et al.* (1993) found that Di-4-ANEPPS partially

embeds itself into the lipid bilayer of biological membranes causing the polar head of the molecule, responsible for the dye's spectral properties, to protrude out of the membrane. Once embedded, Di-4-ANEPPS changes the intensity of its fluorescent emissions in response to changes in membrane potential (Montana, *et al.*, 1989). Previous work using Di-4-ANEPPS to evaluate the effects of toxicants on membrane potential (Blankemeyer, *et al.*, 1992; Blankemeyer, *et al.*, 1993; Stringer and Blankemeyer, 1993; U.S. Patent #5416005) led us to investigate the effect of crotoxin on the membrane potential of murine whole-muscle isolates.

The use of fluorescent dyes and spectrofluorometric techniques has become an increasingly common method in the study of various toxicants. Kao, *et al.* (1989) examined intracellular calcium changes in fibroblasts and lymphocytes using fluo-3, while, cytosolic free sodium levels, determined by fluorescence ratio imaging, were reported by Harootunian, *et al.* (1989). Ionic homeostasis was monitored, using fluorescence imaging, in individual myocytes by Satoh, *et al.* (1994) and Okamota, *et al.* (1995). Recent improvements in the techniques of fluorescence imaging have resulted in increased sensitivity and improved image resolution. This technique has been able to reveal increasingly subtle temporal and spatial cellular events (Carrington, *et al.*, 1995; Tsugorka, *et al.*, 1995; Nelson, *et al.*, 1995).

In this report, we detail the results of exposing murine omohyoid whole-muscle isolates to the rattlesnake venom component, crotoxin, over a 10 to 30 minute period. The fluorescence of the electrochromic dye, Di-4-ANEPPS,

recorded using both spectrofluorometry and digital imaging offers insights into the early myotoxic events following crotoxin exposure. Myotoxicity was determined *in vitro* by recording increases in creatine kinase release rates (Melo and Suarez-Kurtz, 1988; Melo, *et al.*, 1994), image capture of Di-4-ANEPPS fluorescence, and light microscopy investigations.

MATERIALS AND METHODS

Materials. Crotoxin was purified from the venom of the rattlesnake, *Crotalus durissus terrificus* (Instituto Butantan, S. Paulo, S.P., Brasil) by chromatography on Sephadex G-75 and DEAE-Sephadex A-50 columns as described previously (Seki, *et al.*, 1980; Aird and Kaiser, 1985). A fresh stock of crotoxin was prepared for each experiment by dissolving the purified crotoxin in physiological saline solution at a concentration of 10 μg/mL. Valinomycin (Sigma, St. Louis, MO) was dissolved in pure dimethylsulfoxide at a final concentration of 1 mg/mL. A stock solution of Di-4-ANEPPS (Molecular Probes, Inc., Eugene, OR) was made up as 1 mg/mL in absolute ethanol. The physiological saline solution had the following composition: 135 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; 1 mM $MgCl_2$; 15 mM $NaHCO_3$; 1 mM NaH_2PO_4; and 11 mM glucose. All physiological saline solution salts were purchased from Sigma (St. Louis, MO). Saline solution was equilibrated with 95% 0_2 / 5% CO_2 and maintained at a final pH of 7.38 $\pm$ 0.02.

Tissue preparation. The omohyoid muscle was dissected from adult, female CD-1 mice (Charles River Laboratories, Inc., Wilmington, MA) weighting 24-32 g. Caution was exercised to avoid mechanically damaging the tissue during the dissection. Muscle isolates were transferred to either a plastic petri dish (for imaging studies or creatine kinase release studies) or a 4 mL plastic cuvet tube (for spectrofluorometry studies) and bathed in physiological saline solution (described below). An aliquot of Di-4-ANEPPS was introduced to the saline media, 30 minutes prior to each analysis, at a final concentration of 1 μM, and samples were allowed to incubate, undisturbed, at room temperature (24-26^0 C) for at least 30 minutes. For the positive control and negative control trials, the isolates were allowed to incubate as above. Once the dye-loading was complete, the dye-containing saline solution was removed using a 5 mL syringe and replaced with plain saline solution for analysis by spectrofluorometry or fluorescence imaging.

Digital imaging studies. Petri dishes were placed on the stage of a Nikon Labophot microscope (Nikon, Japan) with a 20X objective lens. The microscope was attached to a Hitachi CCTV Camera #HV-17AU (Hitachi Denshi, Ltd., Japan) interfaced with a dedicated 486 image processing computer system. Once the isolate field was in focus, an image was recorded and stored on disk under normal, visible light. Images were captured using the DT2853 Frame Grabber software (Data Translation, Inc., Marlboro, MA). The light source was then changed to the 470 nm excitation light and another image was captured and stored on disk as a fluorescent negative control image. The normal physiological saline solution was replaced, entirely, with one containing crotoxin at a given concentration and images were captured at 2 minute intervals for up to 20 minutes from the time the crotoxin was first introduced. All images were stored on cassette tape storage for later analysis, using the image processing software, IRIStutor (Data Translation, Inc., Marlboro, MA).

Membrane potential measurements. Once the cuvets were loaded into the spectrofluorometer, at the appropriate time, the saline solution was removed by syringe and replaced with saline solution containing crotoxin at a given concentration. For the positive control studies, at the completion of dye-loading, a saline solution containing valinomycin at a final concentration of 1 μM was introduced. Later, during the scanning, this saline solution was exchanged for a saline solution containing 100 mM KCl and scanning continued for 10 minutes. For the negative control studies, the muscle was suspended in a saline sham solution for the entire 10 minute analysis time.

Cuvets were loaded into a PTI DeltaScan dual-wavelength spectrofluorometer (Photon Technologies International, Inc., South Brunswick, NJ) with dual excitation wavelengths set at 480 nm and 570 nm. Photons of fluorescent emission at 620 nm were captured by interposing an Oriel 1/8 M monochrometer at 90 degrees to the incident light. Emission photons/second counts were saved onto disk for later analysis. Scans were conducted at 1 minute intervals for 10 minutes and then 2 minute intervals for the next 10 minutes, following exposure to crotoxin. A scan was taken before each trial, to serve as a negative control reference. Alternatively, scans were conducted continuously for 12 minutes, with the first two minutes serving as a negative control region and the test material introduced at about the 2 minute mark. Continuous scanning could not be conducted for 20 minutes owing to computer memory limitations.

Creatine kinase release measurements. *In vitro* CK release experiments were modified from Melo, *et al.*, 1994. Briefly, omohyoid muscle isolates, mounted in petri dishes (see below) were superfused continuously with physiological saline solution at a rate of 3 mL/min. At 10 min. intervals, the perfusion solution was collected and replaced with fresh media. The collected samples were stored at 4°C until their CK activity was determined using a diagnostic kit purchased from Sigma (St.Louis, MO). The CK activity was expressed as international units, where 1 U is the amount that

catalyzes the transformation of 1 μmole of substrate at 25°C. The rate of CK release from the isolated muscles is expressed as enzyme units released into the medium per gram of muscle per hour of collection (U/g/hr). The basal release rate refers to the enzyme loss from the muscles into the saline at the beginning of the experiment, after the preparations had been mounted in the sample collecting units for at least 1 hour. In all experiments, crotoxin was added to the bath medium at time zero after collecting three negative control samples.

After collecting samples for CK assay, the muscles were removed from the petri dishes, seperated from tendons and connective tissue, dried with absorbent filter paper, and weighed. In all experiments, the right and left omohyoid muscle pairs from the same animal were used. One muscle was treated with crotoxin while the contralateral muscle remained bathed in physiological saline solution to serve as a negative control. Each experimental protocol was repeated at least three times.

Light microscopy examinations. Myotoxicity induced by crotoxin was also investigated with light microcopy. Muscles used in the CK release studies were fixed for 2-3 hours in 1.6% glutaraldehyde in 0.16% sodium cacodylate buffer (pH 7.4). The muscles were then washed three times in 0.08 M sodium cacodylate buffer containing 0.18 M sucrose and fixed again for 1 hour in 1.0% aqueous OsO_4. The tissue was dehydrated in ascending concentration of acetone (30-100%) and embedded in Polybed 812 resin. Thick sections (1 μM) for light microscopic examination were obtained using a Sorval MT 5000 ultramicrotome and stained with Mallory's toluidine blue-azure II dye. Additionally, on three separate muscles, the sarcolemmal permeability or damage was examined using the indicator dye, trypan blue. The muscles were washed three times with fresh saline and incubated for 30 minutes in 0.04% trypan blue in physiological saline solution. After the incubation, the muscles were washed with saline and examined with a dissecting microscope to determine if the trypan blue diffused into the muscle cells. The presence of typan blue inside of muscle cells indicates damaged sarcolemmal membranes.

Analysis of data. The emission data was fit by linear regression and a mean number of emission photons was recorded. The mean number of photons emitted for the experimental groups was normalized by dividing by the mean of the negative control groups and recorded as a percentage of negative control fluorescence. All values are reported as the mean percentage of negative control fluorescence $\pm$ SEM. Graphing of this data, was performed using the Inplot program (Graphpad, San Diego, CA). A SAS program provided ANOVA and LSD analyses of experimental data.

The CK release data are expressed as the mean $\pm$ S.E.M.. Data were graphed using the Sigmaplot program and experimental means were compared against control values using the Student t-Test. In all statistical analyses, data were judged significant at the 0.05 level.

Cuvet and petri dish preparation. For the digital imaging studies and the creatine kinase release studies, the isolated omohyoid muscle was mounted, horizontally, across a small plastic petri dish. The muscle was fixed at the ends to a semicircular wire seated along the inside, bottom edge of the petri dish. To avoid any interferences from any mechanical damage resulting from the attachment of the muscle ends to the wire, the attachment points were sealed in inert silicone gel, thus leaving only the middle and central portions of the muscle exposed to the bath medium.

Similarly, for the spectrofluorometric analysis, two small holes were drilled into the side of a 4 mL plastic cuvet. Fine suture threads were tied to either end of the muscle and threaded through the holes in the cuvet, thus, suspending the muscle, vertically, within the cuvet tube. These attachment points were sealed using inert dental wax and the cuvet was filled with bath medium. In both preparations, care was taken not to mechanically injure the muscle tissue, nor stretch the muscle between attachment points.

RESULTS

Digital imaging studies.

Figure 1 shows some of the fluorescence images captured during one of the digital imaging studies. Figure 1A shows the fluorescent negative control image wherein the muscle is oriented such that myofibers run from the lower-left up to the upper-right side of the field. A small dark spot appears on the muscle, in the lower-right side of the field, and is used as a landmark throughout this series of images. The loading of the Di-4-ANEPPS appears uniformly distributed

Figure 1 (*Following pages*): **FLUORESCENCE IMAGING DATA.** A murine omohyoid muscle isolate was mounted across a plastic petri dish, as in **Materials and Methods**. The isolate was allowed to incubate for 30 minutes at 24-26°C in a saline bathing solution containing 1 μM Di-4-ANEPPS. The saline solution and Di-4-ANEPPS were replaced by plain saline solution; the petri dish was placed upon the stage of a Nikon Labophot microscope with a 20X objective; and the preparation was illuminated with 470 nm excitation light. The fluorescent images were captured using the DT2853 Frame Grabber software.

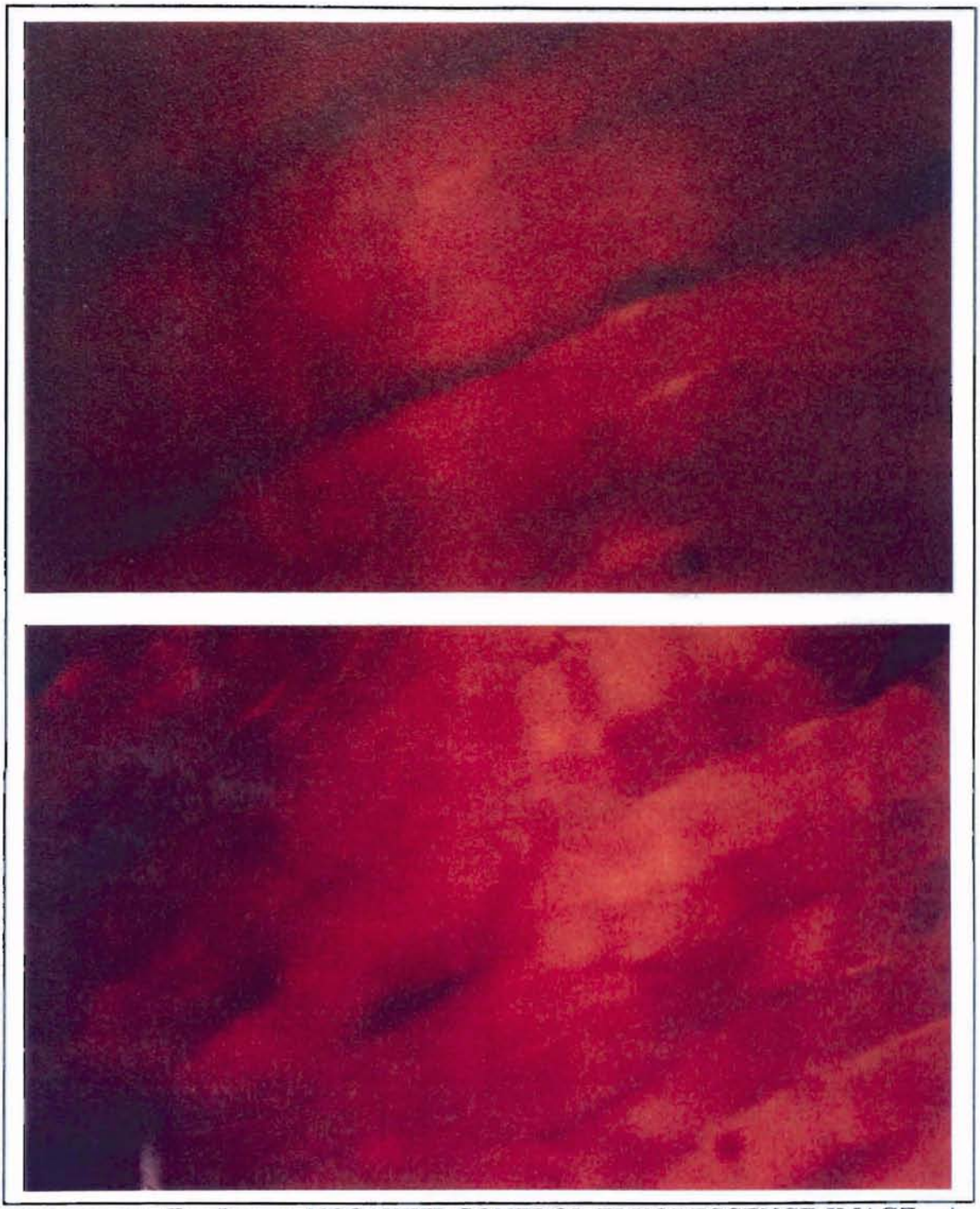

Figure 1A ***Top Image*****: NEGATIVE CONTROL FLUORESCENCE IMAGE.** A murine omohyoid muscle prior to exposure to crotoxin (10 μg/mL). **Figure 1B** ***Bottom Image*****: FLUORESCENT IMAGE FOLLOWING EXPOSURE TO CROTOXIN.** The same muscle as in **Figure 1A** about 2 minutes after exposure to crotoxin (10 μg/mL).

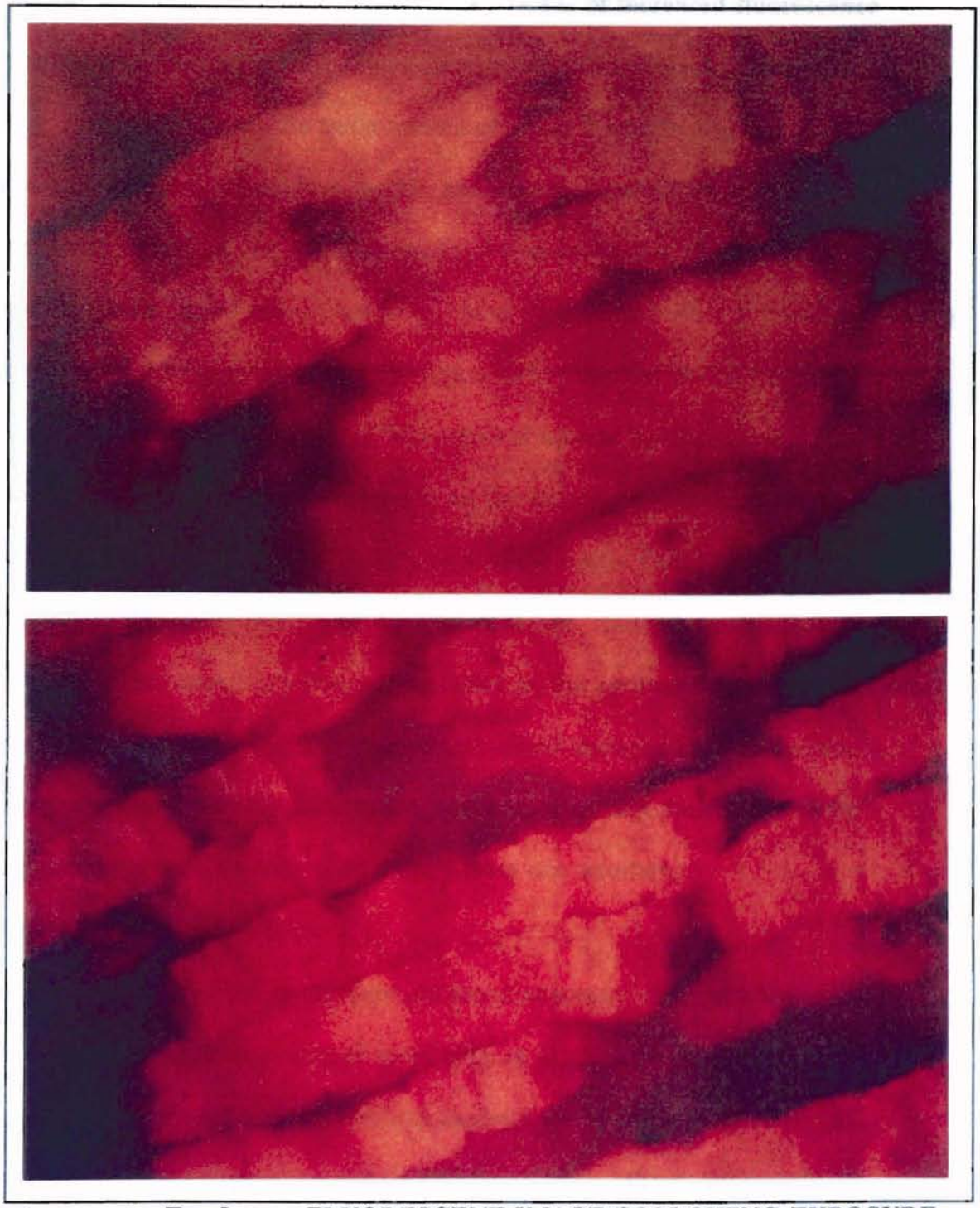

Figure 1C Top Image: FLUORESCENT IMAGE FOLLOWING EXPOSURE TO CROTOXIN. Same omohyoid muscle as in Figure 1A about 5 minutes after exposure to crotoxin (10 μg/mL). **Figure 1D Bottom Image:** Same omohyoid muscle as in Figure 1A about 10 minutes after exposure to crotoxin (10 μg/mL).

throughout the muscle while the lack of any areas of increased fluorescence intensity, indicates no breach of muscle integrity nor mechanical damage.

Figure 1B shows the same muscle at about 2 minutes following a 10 μg/mL crotoxin exposure. The whole muscle has begun contracting, the myofibers have become slightly disorganized, and localized regions (within the center of the field) show increasing fluorescence intensity. Increasing fluorescence is consistent with a depolarization of membranes. Very thin areas of depolarization are seen, particularly, along the line where two adjacent muscle bundles meet. Figure 1C shows this continued effect of crotoxin on the omohyoid muscle after five minutes. At the ten minute mark (Figure 1D), almost the entire field shows increased fluorescent emissions relative to the control image. Indeed, some areas appear as very bright "hot-spots"(see arrows) indicative of the greatest degree of membrane depolarization. The smooth, consistent myofiber arrangement has become disorganized and convoluted with the appearance of very dark areas where neighboring myofibers have pulled away from one another.

Light microscopy examination.

Muscles exposed to crotoxin produced structural changes which can be observed at the light microscopic level (Figure 2). Longitudinal sections, of the muscles (from the CK release studies) exposed for 30 minutes to 10 μg/mL of crotoxin, revealed damage to certain myofibers while others, apparently, remained unaffected. Examination of damaged fibers suggested a pattern of necrosis similar to the progression of myopathy reported by Johnson and Ownby (1994) for ACL

Figure 2: LIGHT MICROGRAPH OF A LONGITUDINAL SECTION OF A MURINE OMOHYOID MUSCLE AFTER EXPOSURE TO CROTOXIN. Omohyoid muscle after 30 minutes of exposure to crotoxin (10 μg/mL). Muscle was fixed in 1.6% glutaraldehyde in 0.16% sodium cacodylate buffer; washed repeatedly in 0.08M sodium cacodylate buffer; fixed again in 1.0% aqueous OsO_4. The tissue was dehydrated in acetone (30-100%); embedded in Polybed 812 resin; and 1 μm sections were stained with Mallory's toluidine blue-azure II dye.

myotoxin. Omohyoid muscle samples, in this study (Figure 2), presented with large, clear vacuoles, regions of disorganized myofibrils, and finally, areas of densely clumped myofibrils. Additionally, longitudinal sections showed myofibers which contained all, or some, of these structural changes along one fiber, while neighboring fibers appeared undamaged.

Lastly, staining of three separate omohyoid muscles, exposed to 10 μg/mL of crotoxin, with 0.04% trypan blue (data not shown) consistently revealed the

incorporation of the dye into the muscle cells. The presence of the dye within the muscle cells is consistent with damage to the sarcolemma.

Spectrofluorometric studies.

Figure 3 shows the results of exposing the omohyoid muscle isolates to the potassium ionophore, valinomycin, in a saline solution containing 100 mM KCl. Because the dissection procedure, handling of the muscle tissue, or the spectrofluorometric procedure itself could have attributed to changes in the membrane potential, we placed three separate muscle samples in the spectrofluorometer in normal saline for 10 minutes and recorded membrane potential changes. As Figure 3 illustrates, and as statistical analysis confirms, the muscles did not undergo any significant change.

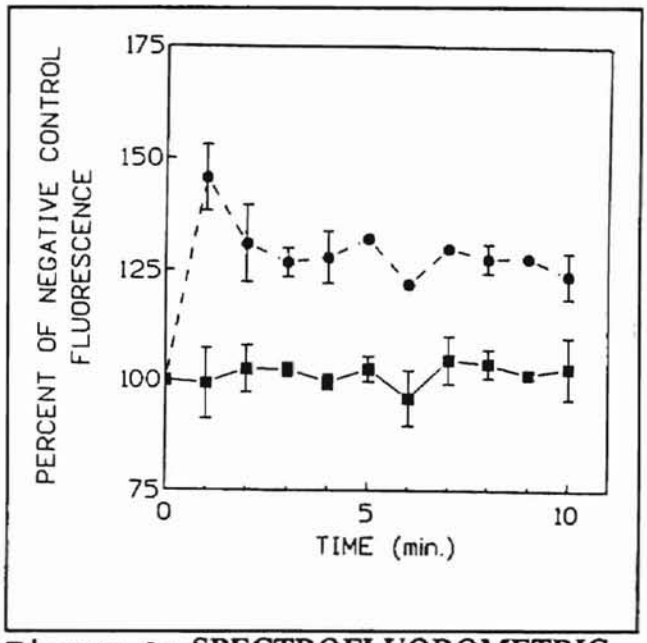

Figure 3: SPECTROFLUOROMETRIC CALIBRATION OF OMOHYOID MUSCLE ISOLATES. 1 μM Di-4-ANEPPS was loaded into murine omohyoid muscle isolates for 30 minutes, whereupon, the bath solution was replaced with a sham saline solution; the isolates were scanned for 10 minutes (■) in a dual-wavelength spectrofluorometer with excitation wavelengths of 480 nm and 570 nm. The sham solution was replaced with a saline solution containing 1 μM valinomycin and the isolates were incubated for about 5 minutes. The solution was replaced with a saline solution containing 100 mM KCl, scanning was immediately begun and continued for 10 minutes (●). Data are shown as the mean $\pm$ SEM for three muscle isolates.

Exposure to valinomycin, however, did strongly depolarize the muscle isolates in a statistically significant fashion ($p < 0.05$), by upwards of 50% over the negative control values arbitrarily set at 100%. Thus, an increase in the fluorescence emissions from Di-4-ANEPPS occurs in response to a depolarization of the murine muscle isolates.

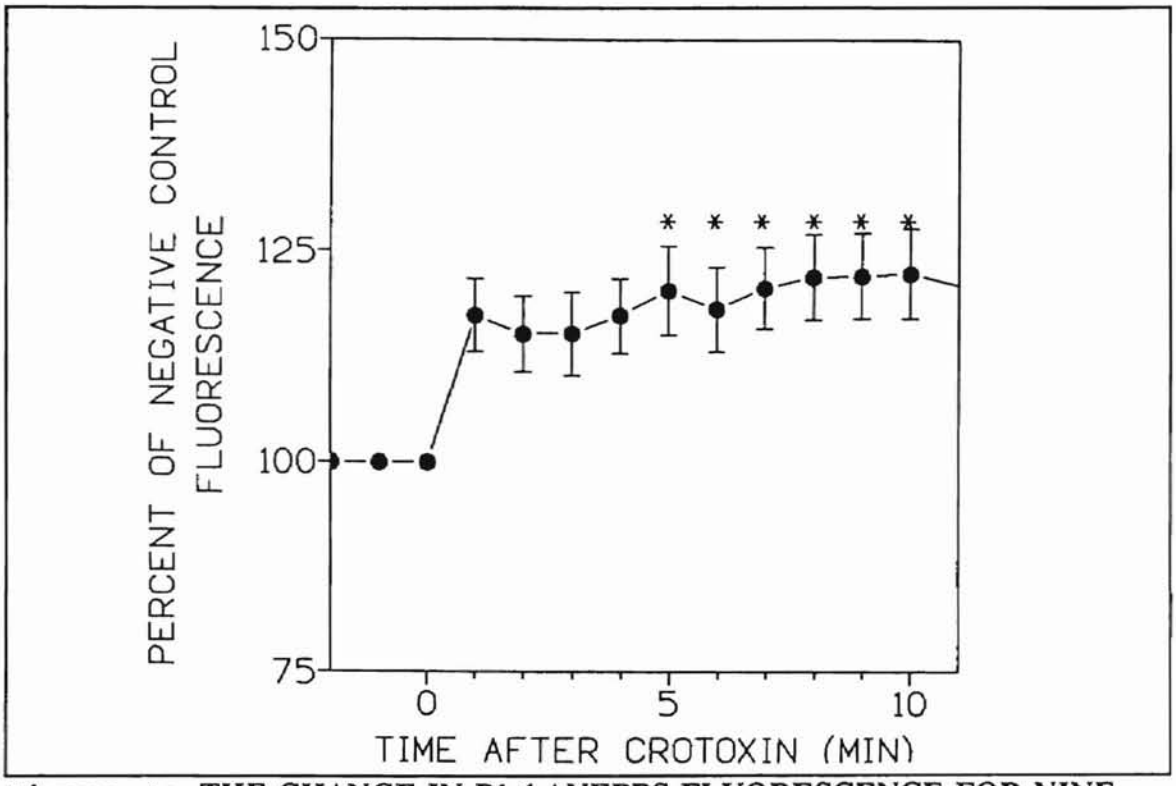

Figure 4: THE CHANGE IN Di-4-ANEPPS FLUORESCENCE FOR NINE MURINE OMOHYOID MUSCLE ISOLATES EXPOSED TO CROTOXIN. Negative control fluorescence was established for each muscle and arbitrarily set at 100%. Crotoxin (10 μg/mL) was introduced at time = 0 minutes. The mean number of photons emitted for the experimental groups was normalized by dividing by the mean of the negative control fluorescence for each muscle. All values are shown as the mean percentage of negative control fluorescence $\pm$ SEM. Marked data "*" were found to significantly deviate from the negative control value of 100% at $p < 0.05$.

Figure 4 shows the results of nine different omohyoid muscle isolates exposed to a 10 μg/mL crotoxin concentration. Although the membrane potential shows a depolarization within one minute of crotoxin exposure, statistical analysis determined only those points beyond the four minute mark to be significantly different from the negative controls ($p < 0.05$). The membrane potential remains at about 22% over negative controls for the remaining 20 minutes and does not return to initial values.

Figure 5 shows the effect on membrane potential of different crotoxin concentrations. The maximum percent change for the 1 μg/mL and 5 μg/mL concentrations were both 115% at time = 10 minutes, and were found to not be significantly different from the negative control value of 100%. Both were considerably less than the 122% (at t = 10 min.) maximum change for the 10 μg/mL crotoxin concentration over the same 10 minute time period.

Creatine kinase release studies

The basal CK release in negative control conditions was not significantly affected during the 30 minute time course of sample collection for ten muscles. At time zero, in the presence of physiological saline, the CK release was 0.32 $\pm$ 0.09 U/g/hr for 10 muscles. Figure 6 shows the changes in CK release rate from eight omohyoid muscles exposed to 10 μg/mL of crotoxin. Crotoxin induced a time-dependent increase in the rate of CK release as evidenced by CK release rates of 5.27 $\pm$ 0.53 U/g/hr (n = 8 muscles); 10.93 $\pm$ 1.12 U/g/hr (n = 8 muscles); and 19.51 $\pm$ 3.39 U/g/hr (n = 8 muscles), at 10 minutes, 20 minutes, and 30

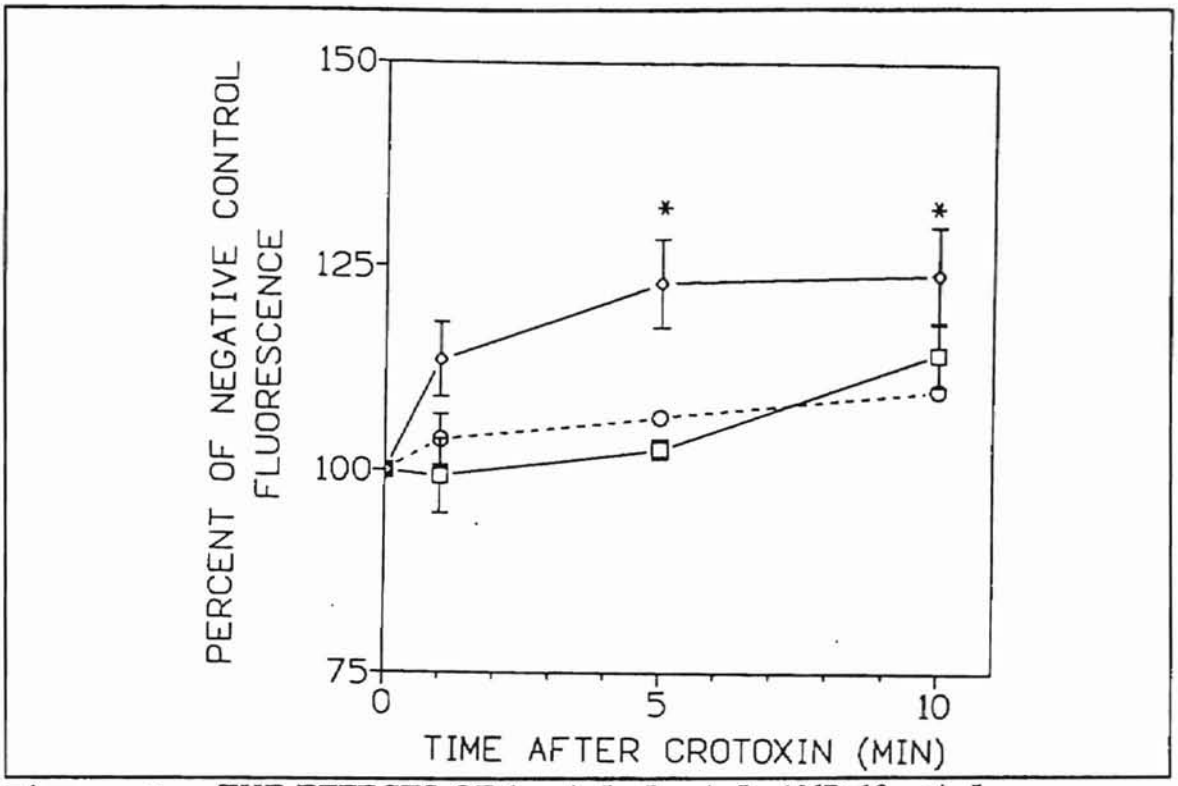

Figure 5: THE EFFECTS OF 1 μg/mL, 5 μg/mL, AND 10 μg/mL CROTOXIN EXPOSURES ON THE MEMBRANE POTENTIAL OF MURINE OMOHYOID MUSCLE ISOLATES. For the 1 μg/mL (□) and 5 μg/mL (○) concentrations, two muscles were scanned in a sham solution as negative controls; the saline solution containing crotoxin was introduced at time zero with scan conducted at 1,5, and 10 minute intervals. The 10 μg/mL concentration data (◇) are the same values shown in Figure 4 (n=9). All data are shown as the mean ± SEM and marked data "*" were found to be significantly different than negative controls ($p < 0.05$).

minutes, respectively. To ensure that our 10 minute sample collection interval was valid, in some experiments, we collected the samples at 5 minutes after crotoxin addition. The rate of CK release at 5 minutes was not significantly different from the negative control muscles (data not shown).

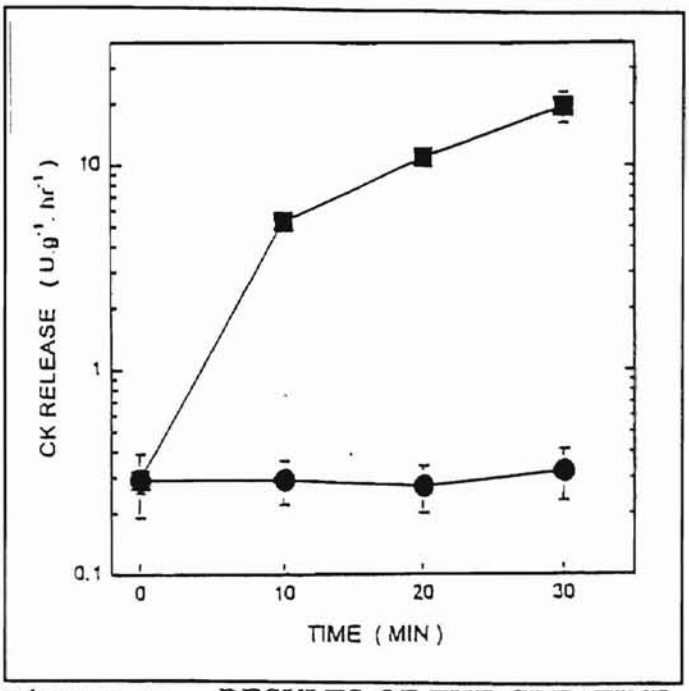

Figure 6: RESULTS OF THE CREATINE KINASE (CK) RELEASE STUDIES. The above results show the time-dependent increase in CK release from murine omohyoid muscle isolates exposed to crotoxin (10 μg/mL). Filled circles are the mean $\pm$ SEM for 10 muscles exposed only to a saline sham solution. Filled squares are the means $\pm$ SEM for 8 muscles exposed to a saline solution containing crotoxin (10 μg/mL).

DISCUSSION

The work presented here was successful in describing some of the early effects of a very complicated protein, crotoxin. Although a great amount of research has been conducted on crotoxin, very little is known regarding its mechanism of action. In this project, crotoxin was found to depolarize mouse omohyoid muscle isolates in a relatively short amount of time. Of particular interest is that the depolarization brought on by crotoxin is significant, repeatable, and steady. At about the four minute mark, 10 μg/mL of crotoxin has already brought about a significant depolarization that steadily continues through the 20 minutes of total exposure time. This concentration was found to produce a maximum membrane depolarization of about 22% relative to negative controls. Lower concentrations of crotoxin, 1 μg/mL and 5 μg/mL, appeared to affect membrane potentials but to a far lesser extent than the 10 μg/mL exposures.

Although the spectrofluorometric studies did not conclusively reveal the exact cause of the muscle depolarization, they did prove beneficial in supporting the findings of the fluorescent digital images. It is unfortunate that no quantifiable fluorescence data could be acquired from the imaging experiments that could be statistically correlated to the spectrofluorometry data. However, as the images revealed, crotoxin did appear to act directly on the muscle fibers, perhaps, in addition to acting on pre-synaptic sites, within a few minutes of exposure. The fluorescent images show areas of increased fluorescence that intensified in a time-dependent manner. These observations were echoed by the time-dependent increases in fluorescence seen in the spectrofluorometry studies.

The digital images also showed the effects of crotoxin on the architecture and morphology of the muscle isolates. The fluorescent images, although not as clear as traditional light microscopy, were capable of showing gross morphological effects of crotoxin. The images revealed muscle fiber contractions, separations between muscle fiber bundles, and an overall loss of muscle fiber organization. The light microscopy examinations confirmed these digital imaging observations and revealed additional histopathological changes. Omohyoid muscles examined by light microscopy contained clear vacuoles, areas of disorganized myofibrils, and areas of clumped myofibrils. The fluorescent images lacked the necessary resolution to detect the crotoxin effects that light microscopy illustrated, however, the technique was capable of generally indicating the myotoxicity that crotoxin induced.

The time-dependent increases in creatine kinase release rates were able to specifically indicate crotoxin-induced myotoxicity. As crotoxin acted on the muscle membranes, over time, the creatine kinase enzyme was increasingly leaked to the surrounding medium. Thus, crotoxin was sufficiently active to degrade the muscle membrane integrity allowing the increased escape of the muscle enzyme. Further, the light microscopy studies seem to support the creatine kinase release findings, as the exact same muscles were used in both investigations.

In closing, this report offers analytic techniques that may be capable of elucidating the mechanism of action of crotoxin as it acts on muscle tissue. The fluorescent images are able to generally indicate, in real-time, both the anatomical and physiological alterations induced by crotoxin. When taken alone, the images provide observations which are limited, however, when supported by the spectrofluorometry, light microscopy, and creatine kinase studies, the fluorescent digital images provide a much clearer picture of crotoxin's myotoxic effects. Experimentation continues towards greater resolution of crotoxin's myotoxicity and mechanism of action.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Lasalocid Depolarizes Avian Erythrocytes

A Review of the Lasalocid Study

Introduction and significance. In this study, I exposed Cobb chicken erythrocytes to various concentrations of lasalocid to evaluate any changes in membrane potential. Since lasalocid is used extensively in the broiler chicken industry, chicken erythrocytes should serve as an appropriate model for studying lasalocid's effects on membrane transport. Avian erythrocytes are relatively easy to obtain and cause a minimal amount of harm to the animals. They also provide a vast quantity of cells from a 5 mL whole-blood sample. Thus, I was able to study a wide range of lasalocid concentrations with numerous replications. Since lasalocid has been implicated in a neuromuscular toxicosis in broiler chickens, a careful study of its effects on membrane transport could provide valuable information relating to this toxicity.

Summary of results. Lasalocid depolarized the membrane potential of chicken erythrocytes in a concentration-dependent manner. In general, 0.001 μM to 10 μM lasalocid did not significantly alter the membrane potential. However, lasalocid concentrations of 50 μM, 70 μM, and 100 μM were found to significantly depolarize ($p < 0.05$) the membranes, relative to the negative control groups, in the ion-complete

buffer solutions, while the 70 μM and 100 μM exposures were significantly different ($p < 0.05$) in the sodium-free buffer solutions. The 100 μM lasalocid exposure was the only concentration to significantly depolarize the avian erythrocyte membrane potential ($p < 0.05$) in the calcium-free and magnesium-free buffer solutions.

The maximum change in fluorescence occurred with the 100 μM lasalocid treatments, however, the magnitude of the change decreased from 366% in the complete buffer solution to 256% and 261% in the magnesium-free and calcium-free buffer solutions, respectively. The maximum fluorescence change was 316% upon exposure to 100 μM lasalocid in a sodium-free buffer.

Conclusions. This study showed that lasalocid causes the depolarization of avian erythrocyte membranes. Concentrations below 10 μM, which includes the recommended therapeutic amounts, failed to significantly affect the membrane potential. Larger concentrations of 50 μM, 70 μM, and 100 μM, in general, resulted in a significant depolarization of chicken red blood cells in most buffer solution types. These concentrations exceed the recommended therapeutic amounts and were the only exposures to cause a change in membrane potential. Thus, this study clearly provided information relating to lasalocid exposure limits. As lasalocid over-dosing has been found to cause serious, toxic, neuromuscular effects in chickens and dogs, the results of this project should prove beneficial to the treatment of animals following lasalocid insult.

Furthermore, by studying the effects of lasalocid on membrane potential, we were capable of showing that this spectrofluorometric method could be used in

assessing the toxicity (or potential toxicity) of compounds. Lasalocid, at certain concentrations, depolarized the erythrocyte membranes. This depolariztion occurred throughout a great many replicates (five measures/ treatment) and between many different birds (3-5 different birds/ experiment). Also, the magnitude of this depolarization depended on the ionic composition of the buffer solution. The ion-complete buffer and sodium-free buffer posted the largest changes in membrane potential, however, the change in membrane potential was less in the divalent ion-depleted buffers (ie. the calcium-free and magnesium-free buffers). Since lasalocid exposures were the same for each experiment, this reduction in membrane potential change may be attributable to the different ionic compositions of the buffer solutions.

Overall, this study was successful in showing how the use of chicken erythrocytes and this spectrofluorometric assay can quickly provide information relating to acceptable exposure concentrations, can be used for different compounds, and most importantly, can be used as a reliable screening tool for potential toxicity. Additionally, using this methodology allows for considerable replication of experiments and poses little harm or damage to the Cobb chickens used in this study. This work presents an important and reliable technique suitable for a variety of toxicology studies.

Folic Acid Inhibits α-Chaconine Induced Membrane Depolarization

A Review of the Folic Acid and α-Chaconine Study

Introduction and significance. Previous research has implicated the potato glycoalkaloid, α-chaconine, in the development of neural tube defects. Further, the intake of the vitamin, folic acid, has been shown to reduce the occurrences of *spina bifida* and anencephaly. I investigated the possible interaction of these two compounds by exposing the embryos of the frog, *Xenopus laevis*, to various concentrations of α-chaconine challenged with various amounts of folic acid. I monitored the changes in membrane potential by recording the magnitude of the fluorescence of the electrochromic dye, Di-4-ANEPPS. Epidemiologic studies have shown these neural tube defects to be one of the most commonly occurring defects. The results of this investigation could be applied to human health. Also, the potato glycoalkaloids are a commonly produced secondary metabolite of the potato (*Solanum tuberosum L.*), and I considered this study to offer valuable food safety information.

Summary of results. Exposure of the embryos to a range of α-chaconine concentrations, alone, produced a statistically significant depolarization in the membrane potential, relative to the negative control groups. α-Chaconine concentrations of 10 mg/L, 25 mg/L, and 50 mg/L were significantly different at the $p < 0.05$ level. However, exposure to folic acid alone, ranging through 80 mg/L, failed to significantly alter the membrane potential measurements.

Increasing folic acid concentrations decreased the magnitude of the

depolarization induced by the potato glycoalkaloid. Statistical analysis, used to compare the experimental data points from each challenge with the positive control values from the α-chaconine concentration-response curve, found that concentrations of folic acid above 10 mg/L significantly reduced the effect of the teratogenic potato glycoalkaloid ($p < 0.05$). The 20 mg/L and 50 mg/L folic acid challenges presented the greatest degree of inhibition of the depolarization caused by 2.5 mg/L, 7.5 mg/L, and 10 mg/L α-chaconine.

Conclusions. The potato glycoalkaloid, α-chaconine, is capable of strongly depolarizing *Xenopus laevis* embryos in a concentration-dependent manner. It has been shown to depolarize membranes by as much as 1600%. Folic acid was able to inhibit that marked depolarization in a concentration-dependent manner. Folic acid at 20 mg/L and 50 mg/L reduced the membrane depolarization of a 10 mg/L α-chaconine exposure from about 750% of negative control fluorescence to values as low as 250% of negative control fluorescence. Unfortunately, the 5 mg/L folic acid trials were too inconsistent to produce any reliable data, however, with folic acid concentrations as low as 2.5 mg/L, an inhibition of α-chaconine's activity is suggested. Inhibition was clearly significant with at least 10 mg/L folic acid.

By using the electrochromic dye, Di-4-ANEPPS, and spectrofluorometry we were able to illustrate the potentially toxic effects on ionic homeostasis caused by α-chaconine. Additionally, this study revealed that folic acid exhibits some prophylactic activity against the strong depolarization induced by the potato glycoalkaloid. As the results of these experiments show, the prophylaxis shown by

folic acid against *spina bifida* and anencephaly may relate to its ability to somehow restore ionic homeostasis or interfere with the α-chaconine's mechanism of action. However, the exact details of the interaction between these two compounds remains unclear and necessitates further investigation.

Despite the lack of an exact mechanism of action describing this interaction, this study was capable of illustrating the utility of this methodology in toxicology research. As with the use of avian red blood cells in the lasalocid study, using the embryos of the *Xenopus laevis* frog provided a large amount of material with which to conduct experiments; resulted in only minor discomfort for the animals; and produced results within a matter of hours. Although these experiments could not explain the workings of folic acid nor α-chaconine as they affect membrane potential, they were successful in evaluating the potential toxicity of α-chaconine and the potential prophylaxis of folic acid. The results of these experiments, and those pending, will provide some insights into the mechanism of action of the potato glycoalkaloids and offer additional information on the relationship of diet and developmental toxicity.

Crotoxin Exhibits Myotoxic Effects on Murine Skeletal Muscle Isolates

A Review of the Crotoxin Study

Introduction and significance. Crotoxin is the main toxic component of the venom of *Crotalus durissus terrificus* (South American Rattlesnake) and is a protein composed of two, noncovalently bound subunits, A and B. Subunit B is a basic protein, that exhibits phospholipase A_2 activity, and is mildly toxic; while, subunit A

is an acidic protein believed to serve the role of a chaperon to subunit A. Although capable of a wide variety of toxicities, crotoxin is considered, primarily, to be a pre-synaptic neurotoxin. However, crotoxin may be myotoxic, in addition to neurotoxic, and may be capable of acting directly on muscle tissue. In an effort to investigate this possibility, I used a variety of techniques and assays to evaluate the existence, or degree, of muscle damage, induced by crotoxin, in murine omohyoid muscle isolates. Further, there is a lack of information describing the mechanism of action of crotoxin and there is no citation in the literature that details a study of the effects on murine whole-muscle ionic homeostasis induced by crotoxin. Thus, this work may provide valuable insights into the workings of crotoxin and that the uniqueness of this approach towards gaining these insights would make this a study of singular value.

Summary of results. This study employed four different techniques to investigate the myotoxic effects of crotoxin: fluorescence imaging; spectrofluorometry; light microscopy; and a creatine kinase release assay. Fluorescent images showed areas of increased fluorescence from Di-4-ANEPPS during 20 minutes of exposure to crotoxin (10 μg/mL), however, we were unable to obtain exact quantitative data. Also, images revealed contractions of myofibers, and the once smooth, consistent myofiber morphology became disorganized and convoluted. Neighboring myofibers were seen to separate from one another.

Light microscopy studies provided increased histopathological details, which the fluorescent images could not provide. Light micrographs, of longitudinal sections of omohyoid muscles exposed to crotoxin (10 μg/mL) for 30 minutes, showed large,

clear vacuoles, regions of disorganized myofibrils, and finally, areas of densely clumped myofibrils. Additionally, the micrographs showed myofibers which contained all, or some, of these structural changes along one fiber, while neighboring fibers appeared unchanged.

The spectrofluorometric experiments provided the quantitative fluorescence data the fluorescent imaging system was unable to offer. Muscle isolates exposed to lower concentrations of crotoxin (1 μg/mL and 5 μg/mL) produced maximum percent changes in fluorescence of 115%, which were not significantly different from the negative control values. The higher concentration of crotoxin (10 μg/mL) did significantly depolarizes murine muscle isolates in the nine muscles studied. The maximum change in membrane potential was about 22% over negative controls during the 20 minute time period.

Lastly, the damage caused to the muscle isolates was confirmed using a creatine kinase release assay. The basal enzyme release rate for 10 muscles was determined to be 0.32 $\pm$ 0.09 U/g/hr. Eight omohyoid muscle isolates, exposed to crotoxin (10 μg/mL), showed a time-dependent, increasing release rate. Creatine kinase release rates were found to be: 5.27 $\pm$ 0.53 U/g/hr after 10 minutes; 10.93 $\pm$ 1.12 U/g/hr after 20 minutes; and 19.51 $\pm$ 3.39 U/g/hr after 30 minutes.

Conclusions. This series of experiments was successful in describing some of the early effects of crotoxin. Although a great amount of research has been conducted on crotoxin, very little is known regarding its mechanism of action. Crotoxin was found to depolarize mouse omohyoid muscle isolates within a relatively short amount

of time. Further, this study was able to provide fluorescent images of this depolarization event and light micrographs to show the details of the histopathological changes induced by crotoxin exposure. The muscle tissue necrosis resulting from the venom component was quantified using the creatine kinase release rate assay and found to be a time-dependent, increasing loss of the enzyme from the damaged muscle tissue. Taken individually, each of these techniques offer limited insights, however, when examined collectively, they offer significant proof of the myotoxicity associated with crotoxin.

Although a detailed description of crotoxin's mechanism of action remains to be made, the rapid change in membrane potential, shown in these experiments, may be an initial step in the process leading to the histopathological changes and myotoxic effects induced by crotoxin exposure. However, additional experiments are required to gather the data which would reveal such a mechanism.

The techniques used in this study were successful in producing a picture of the myotoxic effects of crotoxin. The use of the electrochromic dye, Di-4-ANEPPS, provided not only the quantitative data, as in the lasalocid and folic acid/α-chaconine projects, but was also instrumental in producing interesting fluorescent images of crotoxin's effects. Both the spectrofluorometry data and the fluorescent images serve to illustrate the versatility and the persuasive value of this analytic technique. In addition, recording the changes in membrane potential, either spectrofluorometrically or using fluorescent imaging, may provide some information as to the mechanism of action of this compound. The importance of maintaining ionic homeostasis is so

fundamentally important to the viability of cells and tissues, that monitoring its changes immediately following insult can serve as an important determinant of potential toxicity. Given the degree of muscle damage seen in the fluorescent images after 20 minutes of exposure; the detailed pathologic effects on myofibrils seen in the light micrographs after 30 minutes; and the continual release of creatine kinase over 30 minutes, it seems that the change in membrane potential, seen within the first four minutes of crotoxin exposure, is a good predictor of toxic possibilities.

This Thesis Illustrated the Value of Spectrofluorometry and Di-4-ANEPPS in Toxicology Research

An Overall Review of This Project

Concluding comments. The intent of this thesis project was, primarily, to study the potential toxicity of various compounds exposed to different cell types and tissues. However, this thesis also served to illustrate the utility of monitoring cell membrane potentials using the electrochromic dye, Di-4-ANEPPS, and spectrofluorometry. The three studies detailed in this report each involved very different compounds, involved very different cell types and tissues, and involved very different applications of this basic methodology. Additionally, important information relating to potential toxicity, levels of exposure, and mechanisms of action for these different compounds was acquired in a relatively short amount of time.

Further, this report has demonstrated not only the usefulness of this methodology, but has also shown its flexibility and adaptability. The basic

spectrofluorometric recording of cell membrane potentials was performed on different cells and tissues: embryos, erythrocytes, and isolated whole-muscle tissue. Spectrofluorometric analyses could record the membrane potentials of different preparations: stacked embryos, suspended erythrocytes, and stretched whole-muscle isolates. It was performed using not only a dual-wavelength spectrofluorometer but also a digital imaging system. Lastly, measurements were taken either continuously or at set intervals over time. The basic spectrofluorometric measurement of cell membrane potentials could be adapted for a variety of scenarios.

In closing, the use of the methodology described in this thesis will give researchers an additional tool in studying the many, complex interactions between the chemical and the biological. The problems that are inherent to toxicology research are remedied using this protocol. The influence of dosages (or concentrations), the different toxicities to different organs, tissues, or cell types, and the slow production of results are all problems that were successfully overcome using these methods. As this thesis has shown, a wide variety of compounds can be studied and their potential toxicity(s) can be evaluated in a short period of time. As the list of compounds that remains to be studied is a sizable one, having a tool which can rapidly screen the toxicity of these compounds is a great benefit. This project involved compounds which could cause devastating developmental anomalies, serious neuropathy, and severe muscle necrosis. However, the compounds that remain to be studied, no doubt, include some substances of far greater potency, of far greater gravity, and of far greater toxicity. The importance of toxicology research to the general public's health

and well-being warrants the continued development of these spectrofluorometric techniques to understand the toxicity of the myriad of chemical insults that each of us experiences, everyday.

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Thesis: A SPECTROFLUOROMETRIC STUDY USING DI-4-ANEPPS OF THE EFFECTS ON MEMBRANE POTENTIAL FROM LASALOCID, CROTOXIN, FOLIC ACID, AND α-CHACONINE EXPOSURES

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