USE OF FLUORESCENCE SPECTROSCOPY TO STUDY CELLULAR EFFECTS OF TOXIC CHEMICALS ON XENOPUS EMBRYOS

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CHAPTER I

OVERVIEW

Concern over the effect of chemicals on organisms probably began sometime after the first large scale synthesis of organic chemicals (Zapp, 1980). This concern remained unfocused for many years, finally appearing in the popular consciousness after the publication of Rachel Carson's Silent Spring (Carson, 1962). The public and political concern of long-term effects of pesticides and other toxicants in water provided the political force necessary for the establishment of the EPA and NIEHS. The toxicological community responded to congressional mandates by developing a wide variety of bioassays. Some of these bioassays have fallen into disuse due to complexity and cost (see Sprague, 1969; 1970; 1971, for review). The remaining bioassays can be classified into three groups: (1) chronic bioassays (test length of 96 hours or greater); (2) acute bioassays (test length of 96 hours or less); (3) short-term bioassays (test length of 15 minutes).

Chronic bioassays have been used to establish the long-term effect of diluted toxicants by establishing a NOEC (no observable effect concentration) or a LOEC (lowest observable effect concentration). The NOEC establishes the

safe dilution of a discharge into an aquatic environment and has supplanted the less sensitive but statistically more robust EC50 and LC50 derived from acute bioassays.

Two freshwater chronic tests are currently in widespread use for NPDES permitting purposes: the 7 day Cerriodaphnia survival and reproduction and the Fathead minnow survival and growth test (Buikema, et al., 1982)

The seven day Cerriodaphnia survival and reproduction test (Sprague, 1971), is a sensitive test for aquatic pollutants. Since both survival and reproduction are measured, the probability of capturing the effect of single mode toxicant is improved. The negative criticism of the chronic Cerriodaphnia test has centered on the phylogenetic distance from daphnia to vertebrates. Although this criticism may be valid when invertebrate tests are used for estimating risk to vertebrates, the importance of small invertebrates in the aquatic food chain makes the Cerriodaphnia test quite valuable.

The Fathead minnow survival and growth test (Nebecker, 1982), uses a common minnow to test for direct effects of aquatic toxicants. The week-long length of the test provides sufficient opportunity for test waters to affect the growth and survival of the sensitive minnows. In some effluents, particularly those from very toxic sites, the effect on Cerriodaphnia and Fathead minnows will be identical. Many times the two tests will not agree, showing varying sensitivities. Unfortunately, no mode of action or cause of

death is established; it is usually difficult to evaluate or understand the differences in toxic response between the two tests. Principal criticism of the Fathead minnow test is that the minnows require skilled technicians to maintain a breeding colony and high cost ($500-800 per test).

Principal acute assays are the 96 hour Fathead minnow, Cerriodaphnia, and FETAX (Frog Embryo Teratogenesis Assay-Xenopus). The Fathead Minnow and Cerriodaphnia tests are time-abbreviated versions (see 4) of the chronic tests of the same name. In the 96 hour assays, only a survival endpoint is used. As might be expected the 96 hour tests are less sensitive because only survival is used as an endpoint and because the 50 % lethality is used rather than NOEC (No Observable Effect Concentration). FETAX uses South African Clawed frog embryos in assessing survival, growth, and malformation as endpoints (Bantle, et al., 1989). Use of the embryonic stage of the organism probably exposes the most sensitive sector of the life-cycle to the toxicant. Use of multiple endpoints provides multiple signals, enabling capture of multiple effects of toxicants.

Short-term bioassays attempt to predict the acute or chronic effects of a toxicant by exposing a sensitive organism to a toxic chemical for a short time-interval. One of these bioassays is Microtox, a registered trademark of Microbics. In the Microtox assay, a photoluminescent bacterium is used as the target organism for toxicants. Decreases in emitted light probably reflect alterations of

the membrane bound electron transport system of the bacterium. The assay takes about five minutes and operates using an instrument that requires little handling. The criticism of the Microtox assay is that the EC50 values are markedly less sensitive that other assays (e.g. 96 hour Cerriodaphnia).

About two years ago, it became apparent that a multiple-endpoint assay using vertebrates could be developed using optical measurements of fluorescence, transmission, and nephelometry. We have developed an instrument and assay that can provide rapid measurements of response to toxicants and use a computer controlled optical platform to control the experiment and to acquire and process the data. We have chosen to call the technique Cell Health Assay of Water Quality-CHAWQ.

SIGNIFICANCE

Since the 1960's, toxicity assays have been developed that provide reproducible and reliable endpoint data. These assays frequently involve the whole organism and often take several days to complete. The Fathead minnow (Buikema, 1982) and Cerriodaphnia are classic examples of these types of tests. These whole organism tests are expensive and time consuming. However, the classic tests produce results that are easy to understand and interpret. Cellular and intracellular tests, on the other hand, are faster and less expensive. Current cellular tests are usually single

endpoint (e.g. appearance of stress proteins, cytochrome P-450 activation, decrease in bacterial luminescence), but are difficult to correlate with whole animal toxicity. Clearly, toxicity is detected by the assay but the relationship of the cellular endpoint to whole animal toxicity is poorly understood. Another problem with single endpoint assays is that a molecular biology lab may be required when several different assays are required. A complimentary set of tests (with individual endpoints which does not require homogenization of the sample) performed simultaneously on a single instrument offers tremendous data quality, cost, and time advantages over single endpoint assays. CHAWQ has the ability to measure the effect of toxicants on several intracellular processes using a single, self contained instrument and whole embryos or organisms.

Another significant problem with cellular and intracellular assays has been relating the response to whole animal toxicity. CHAWQ is able to attack this problem because it can identify which intracellular mechanism(s) is being affected by the toxicant using the same species as in the whole animal test. The eventual whole animal response to the toxicant can be more accurately linked and correlated with a subcellular response in CHAWQ. CHAWQ also has the capability of detecting toxicity in resident populations of organisms thereby providing an important biomonitoring role.

CHAWQ uses fluorescent probes to measure cellular endpoints such as membrane potential, calcium levels,

intracellular pH, DNA content and structure, energy metabolism, etc. Table 1 shows the CHAWQ endpoints and the probes used to monitor those endpoints. The work proposed for this project was to assemble the CHAWQ instrument and study the utility of a number of the endpoints as shown in Table 1.

TABLE I

CHAWQ PROTOCOL

BIOMARKERS	ENDPOINTS	FLUORESCENT PROBES
Membrane Potential	Na^+ Equilibrium	Di-4-ANEPPS
Intracellular pH	± 2 Units	BCECF, SNARF
DNA Synthesis	Decreased Fluorescence	Hoechst 33258 Thiazol Orange
DNA Structure	Increased Fluorescence	Hoechst 33258 Thiazol Orange
Calcium	?	Fura-2

The organism that will be used are albino Xenopus laevis embryos. This organism was chosen for a number of reasons. First, they provide an animal that has been used in malformation and mortality assays (FETAX) at Oklahoma State University in Dr. John Bantle's lab (Bantle, et al., 1989). This body of work provides a large data base for comparison of the CHAWQ to an accepted toxicity assay.

Second, the albino animal will be used in order to negate any fluorescence of cellular pigment that might interfere with the fluorescent probe.

This dissertation is comprised of 4 manuscripts. These manuscripts are complete as written and do not require additional support material. The manuscripts include: Chapter II, Correlation With FETAX of a Cellular Bioassay-Cell Health Assay of Water Quality-CHAWQ; Chapter III, Rapid Measurement of Toxicity Using Electrochromic Dyes and Frog Embryos; Chapter IV, Effect of Potato Glycoalkaloids, α-Chaconine and α-Solanine, on Membrane Potential of Frog Embryos; and Chapter V, Measurement of DNA Integrity and Structure in Xenopus Embryos in the Presence of Hydroxyurea, Actinomycin-D, and Triethylenemelamine Using the Fluorescent DNA probe Hoechst 33258.

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CHAPTER II

CORRELATION WITH FETAX OF A CELLULAR BIOASSAY

CELL HEALTH ASSAY OF WATER QUALITY

CHAWQ

ABSTRACT

We used albino Xenopus laevis embryos to test the correlation of a short-term, cellular, multiple-endpoint assay (Cell Health Assay of Water Quality- CHAWQ) with a 96 hour assay FETAX (Frog Embryo Teratogenicity Assay: Xenopus). We describe the hardware and instrumentation to perform the assay. We also describe the endpoints and methods to measure them. Exemplary endpoint measurements are presented: 6-Aminonicotinamide produced a decrease in the membrane potential of the embryo as measured by dye fluorescence. A potato glycoalkaloid, α-chaconine, increased dye fluorescence due to intracellular pH. Measurements of DNA using fluorescent dyes showed a normal increase in DNA until gastrulation. A clastogen -triethylenemelamine- was detected with hex-bisbenzamide dye. These data suggest that the CHAWQ assay may contain endpoints that predict acute or chronic macroscopic effects on an organism.

INTRODUCTION

During the mid-1980's, our lab began investigating the impact of aquatic toxicants on active transport of sodium in frog skin (Blankemeyer 1984). From these experiments we learned that organic toxicants, heavy metals, and pH changes had a dramatic effect on the ability of frog skin to actively transport sodium (Blankemeyer and Hefler 1990). While we were investigating the frog skin as a model for active transport interaction with toxicants, we also investigated the response of membrane potential of epithelial cells using electrochromic dye (Blankemeyer and Kidder, 1986). Electrochromic dyes change fluorescent intensity or undergo a spectral shift in response to changes in membrane potential. A number of these dyes have been developed; one of the best is Di-4-ANEPPS developed by Loew (Fluher et al. 1985). CHAWQ -Cell Health Assay of Water Quality- was developed from the fusion of interest in the effects of toxicants on active transport and an interest in simultaneously observing the membrane potential of many cells. Using the electrochromic dye Di-4-ANEPPS to detect changes in membrane potential, we developed a system to observe changes in membrane potential optically rather than electrically.

The membrane potential across the cell plasma membrane results from the interaction of the ion-transporting channels in the membrane with the ions inside and outside

the cell and the active transport pumps that maintain the gradients of ions between the inside and outside of the cell. Any alteration in the channels or the pumps -or damage to the cell membrane- will result in a change in the membrane potential.

Electrochromic dyes make the detection of membrane alteration or damage an optical event.

FETAX uses South African Clawed Frog embryos to assess survival, growth, and malformation as endpoints in aquatic and developmental toxicology (Dumont et al. 1982, Bantle et al. 1989). Embryos are reared for 96 hours and then evaluated for growth, malformation, and survival. We hypothesized that toxicity, as measured by the FETAX assay, would be predicted by cellular toxicity detected by several cellular endpoints including changes in membrane potential. We chose to use mid-to-late blastula embryos and use short exposure periods. Use of the embryonic stage of the organism probably exposes the most sensitive stage of organism's life-cycle to the toxicant. We chose to use several cellular endpoints including membrane potential, intracellular calcium, mitochondrial cytochrome reduction levels, cellular pH relating to cell viability, and alterations in DNA quantity and quality.

We then designed an instrument that would permit concurrent evaluation of these endpoints over a large number of embryos and also concurrently measure optical signals from both control and experimental embryos. Use of multiple

endpoints provides multiple signals, enabling detection of numerous toxicant effects.

Here, we present details of the instrument design, the measurement protocol, and data that has been from a series of positive control experiments were performed to evaluate the efficacy of CHAWQ and compare CHAWQ results with FETAX.

EXPERIMENTAL METHODS

The purpose of CHAWQ is to rapidly assess the quality of water. The rationale of the invention is that long-term deleterious effects on an organism, living in or exposed to water, will be reflected in short-term alterations of the cells that comprise the organism. CHAWQ is designed to evaluate indicators of cellular health using cells of organisms exposed to water-borne contaminants.

HARDWARE DESCRIPTION

Figure 1 is a schematic layout of the instrumental portion of CHAWQ. The mechanical hardware in Figure 1 is a modified DeltaScan (Photon Technology Inc., Princeton, N.J.). Starting at the left side of Figure 1, a xenon arc-lamp (LAMP) delivers a light output to a conventional optical chopper (CHOPPER) which has its frequency controlled by a computer (not shown). The light is split by the optical chopper into two separate, approximately equal, beams. One light beam passes into one of the two monochromators (MONO1); the other to the second monochromator (MONO2). The

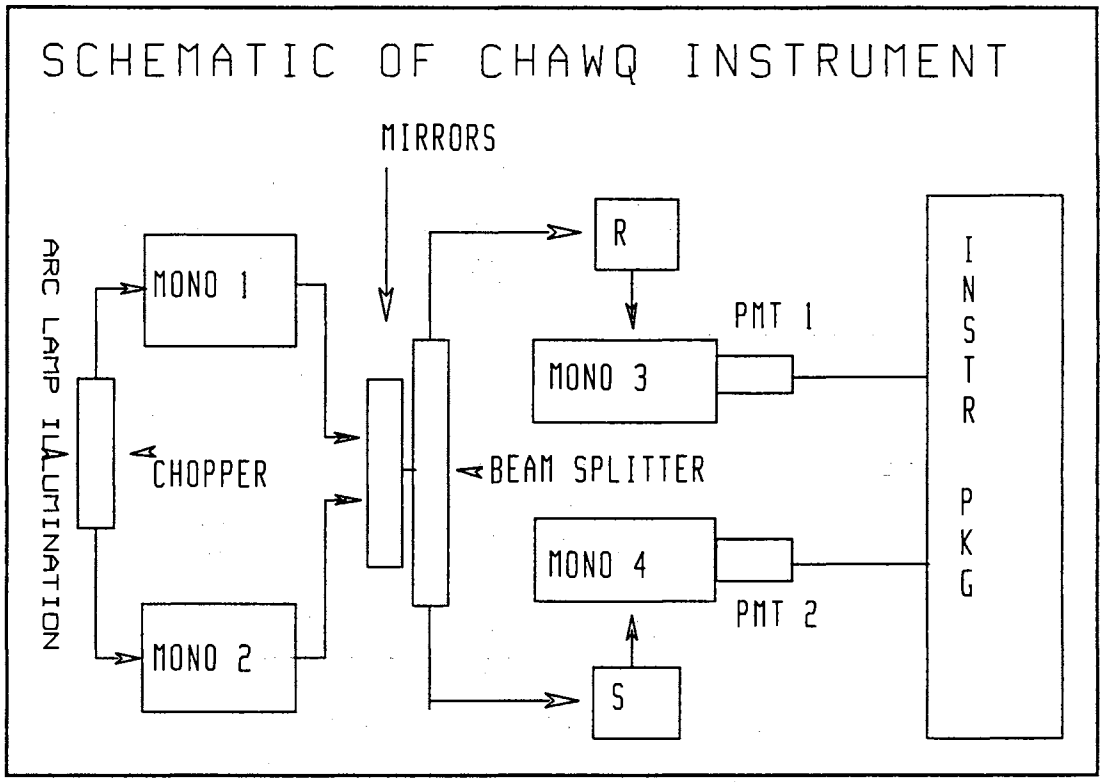

Figure 1. Schematic Diagram of Excitation Portion of CHAWQ.

monochromators have manually operated slits and electro-mechanically operated monochromator drives (not shown). The light outputs of the monochromators are combined by first-surface and parabolic mirrors into a single exit beam consisting of two temporally separate monochromator outputs.

Once the light beam has been combined into two temporally separated beams, the combined outputs are split into two beams by a beam splitter. One beam goes through a reference chamber (R); the other passes through a sample chamber (S). Both chambers are made of quartz. Inside both the sample chamber and the reference chamber are the test organisms, for example, albino Xenopus laevis embryos.

The 90 degree axis (light that scatters or is emitted at 90 degrees relative to the input beam) contains, after separation of wavelengths by a monochromator in the 90 degree light exit path, fluorescent emissions from the organisms. The fluorescent emissions are captured by a photon-counted output from a photomultiplier tube. The counting is performed by the instrumentation amplifiers and digital counting circuits.

The fluorescence and emission/excitation spectra are acquired and compared between the test sample chamber and reference sample chamber (R,S). Use of the sample chamber and the reference chamber provides an internal self-control.

BIOCHEMICAL ENDPOINTS

CHAWQ uses indicators of the intracellular status of the test cells to evaluate the toxicity of test water. Following are the indicator dyes and cellular endpoints currently used in CHAWQ.

Intracellular calcium is measured using the chelator dye, Fura-2 (Molecular Probes, Eugene, OR 94702), developed by Tsien and collaborators (Grynkiewicz et al. 1985). Fura-2 crosses the plasma membrane and is trapped inside the cells and is capable of measuring intracellular calcium activity at physiological concentrations. Since the quantity of Fura-2 loaded into cells is difficult to determine, Fura-2 and other dyes use a two-wavelength ratioing technique. In this protocol, one excitation

wavelength measures calcium activity while the second excitation wavelength measures the amount of Fura-2 loaded in the cell. The first wavelength is the measuring excitation wavelength and the second is the isosbestic excitation wavelength. We use 340 nm as the measuring excitation wavelength and 380 nm as the isosbestic excitation wavelength. The ratio of the emission (at 480 nm) at the two excitation wavelengths determines intracellular calcium independent of dye loading.

In the CHAWQ protocol, BCECF (2',7'-bis-carboxyethyl-5-(and-6) carboxyfluorescein, acetoxymethyl ester (Molecular Probes, Eugene, OR 94702) is the best indicator for detecting cell death and/or intracellular pH changes (Grandin and Charbonneau 1988). We use the excitation pair of 500 and 440 nm and the 540 nm emission to measure pH and the amount of BCECF loaded.

The oxidation-reduction status of the mitochondria is measured by using the dual-wavelength absorbance difference between a particular cytochrome wavelength and an isosbestic point. For example, to measure cytochrome c oxidation-reduction, we use the wavelengths of 550 nm to detect reduction and 530 nm as an isosbestic point. All of the cytochromes can be measured by absorbance (Kidder and Blankemeyer 1978).

DNA quantity is measured (in the presence of RNA) with bisbenzamide dye (Molecular Probes, Eugene, OR) which detects small amounts of DNA (Latt and Stretten, 1976). It

is selective for AT regions, vital, and binds in the minor groove. Unfortunately, bisbenzamide dye has no clear isosbestic point so we can not use dual wavelength ratioing to control for variations in dye loading. Currently, we use an excitation wavelength of 340 nm and an emission of 480 nm.

Another dye for DNA quantification is thiazole orange. Thiazole orange (Molecular Probes, Eugene, OR) has a high quantum efficiency and is also supravital (Terstappen and Loken 1988). We use thiazole orange with an excitation of 501 nm and an emission of 547 nm. As with the bisbenzamide dyes, thiazole orange has no isosbestic point to use excitation ratioing.

Cell membrane potential is measured with the styryl dye Di-4-ANEPPS (Molecular Probes, Eugene, OR 94702). This dye has no fluorescence in water and appears to reside in the cell membrane leaflet. It responds to an increase (a depolarization) of membrane potential with an increase in fluorescence at 490 nm excitation while the fluorescence at the 535 nm isosbestic point is relatively constant (Fluher et al., 1985). Thus the fluorescence measurement of membrane potential can be ratioed so that the fluorescence measurement of membrane potential is independent of dye loading and number of cells.

In our studies we have found that the 590 nm isosbestic point is much more reliable (see Figure 4) than the published 535 nm isosbestic point. We use an emission wavelength of 619 nm to measure fluorescence.

TEST DESCRIPTION

Frog embryos-obtained from matings of albino Xenopus laevis- were obtained from Xenopus I (Ann Arbor, Michigan). Conditioned males and females were injected with HCG (Sigma, St. Louis, MO) approximately twelve hours before amplexus. After amplexus, embryos were sorted into viable and non-viable groups. The embryos were then treated with 2 % cysteine to remove jelly and placed in Petri dishes in groups of 25. The embryos were at mid-to-late blastula to neurula stage at the time of experimental measurement (although each experimental group was compared to a control group of the same age). Embryos were reared at a constant temperature of 24 degrees C.

After four hours growth in FETAX solution (Dumont et al. 1982), the embryos were placed in a cuvette and inserted in the sample chamber. The monochromators were moved to the appropriate wavelength (e.g. 490 nm for Di-4-ANEPPS) and measurements of the fluorescent emission begun. The data was collected to magnetic storage and retained for analysis. Data were fitted with linear regression (to determine the best mean for the sample period).

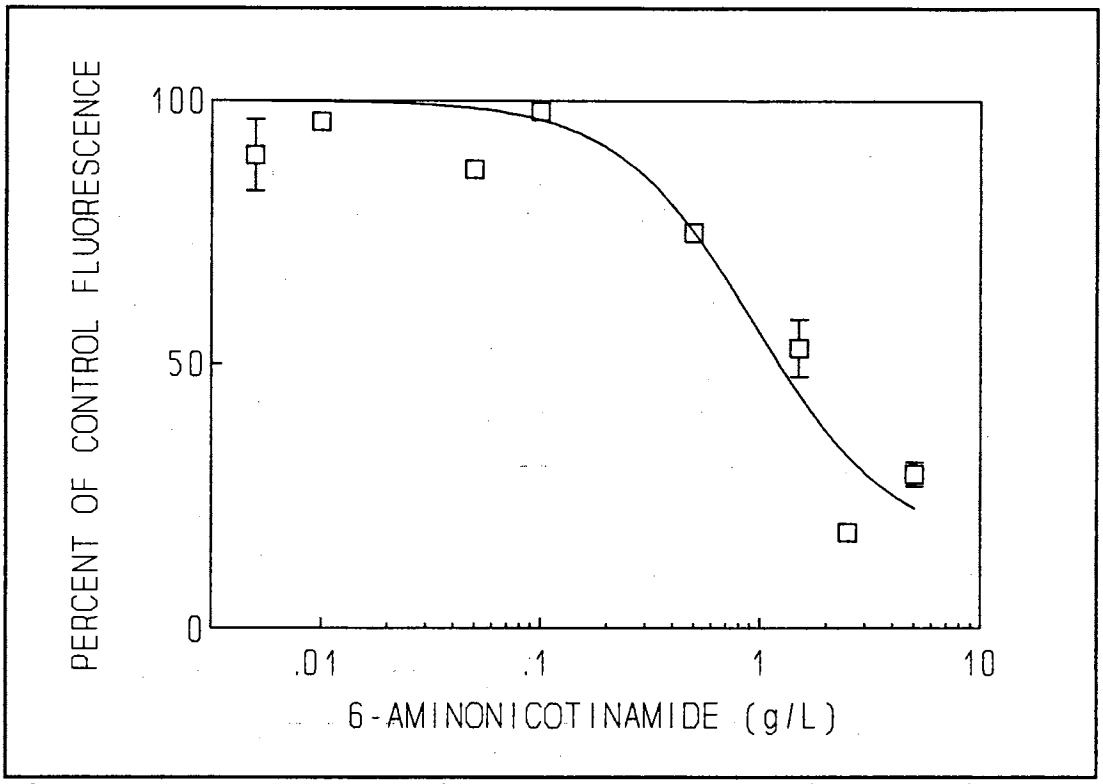

Figure 2. Concentration-Response Experiment with 6-Aminonicotinamide and Xenopus embryos

EC50's were determined by non-linear regression using a sigmoidal model (GraphPad, Los Angeles, CA.).

We used test compounds to evaluate CHAWQ that have been well established with the FETAX assay. 6-amino nicotinamide was obtained from Sigma (St. Louis, MO). The teratogen 6-Aminonicotinamide (6-AN), has been extensively tested with animals. Using FETAX, Dawson et al. (1989) found that the LD50 and EC50 differed by two orders of magnitude thus producing a numerically high Teratogenicity Index (TI). The LD50 found by Dawson et al. was ca. 3000 mg/L whereas the EC50 (for malformation) was found to be 5.7 mg/L. The ratio of these two was 602 (TI). We were interested to discover

how the EC50 for CHAWQ measurements would compare with the 96 hour endpoints reported by Dawson et al.

One of the difficult tasks in using a fluorescent probe to measure membrane potential is the calibration of the fluorescence as a function of membrane potential. We used gramicidin, an ionophore that passively transports cations across the cell membrane (Clement and Gould, 1981) to change the embryonic cell membrane potential from its resting potential to a value near zero. Gramicidin (Sigma, St. Louis) depolarizes the electrical potential across cell membranes and was used to evaluate the optical signal measured by Di-4-ANEPPS after depolarization of the membrane potential. α-Chaconine (Roth, Basel) is one of a family of glycoalkaloids occurring in potatoes. It has been tested extensively with FETAX.

Hex-bisbenzamide dye (Molecular Probes, Eugene, OR) was used to assess the amount of DNA strand breakage and the amount of DNA exposed by the clastogen.

RESULTS

In the experiment diagrammed in Figure 2, the membrane potential was measured optically using Di-4-ANEPPS and the following protocol. Embryos were exposed to 6-AN for 30 minutes using various concentrations of 6-AN and a constant concentration of Di-4-ANEPPS (Figure 2). The dye was excited by a 490 nm blue beam and emitted a red fluorescence measured at 619 nm. Figure 3 shows that the ratio of

experimental to control fluorescence decreased upon exposure to 6-AN. This indicates that the membrane potential (as measured by the electrochromic dye Di-4-ANEPPS) hyperpolarized upon 30 minutes of exposure to 6-AN. Fitting of this data via non-linear regression to a sigmoidal model produced an EC50 of 1.05 ± 0.2 g/L. The CHAWQ EC50 is near the observed LD50 for FETAX exposure to 6-AN (3 g/L) but much higher than the malformation EC50. Note that the tail of the plot (low levels of 6-AN) doesn't reach the control level of 100 %. It is possible, although difficult to discern from the data in Figure 2, that there may be a second concentration-response curve hidden in the noise of the low concentrations of 6-AN. Planned instrument improvements will explore the low concentration region of 6-AN. The relatively close EC50 of CHAWQ and the LC50 of FETAX suggests a cause and effect relationship between membrane potential and death of the embryo although the two events may be entirely independent.

When we incubated frog embryos with 96 μM gramicidin for thirty minutes, we found that the fluorescence increased relative to the control. This data is presented in Figure 3. It shows the Di-4-ANEPPS fluorescence (emitted at 619 nm) as a spectrum of the excitation wavelength of light. This shows that the decrease in fluorescence observed during incubation with 6-AN is a hyperpolarization of the embryo's cellular membrane potential. Note that the 490 nm region- the peak of the excitation wavelength- increased with

gramicidin whereas the 590 nm fluorescence was independent of wavelength. The 590 nm point is a useful point that is used to normalize dye loading between samples.

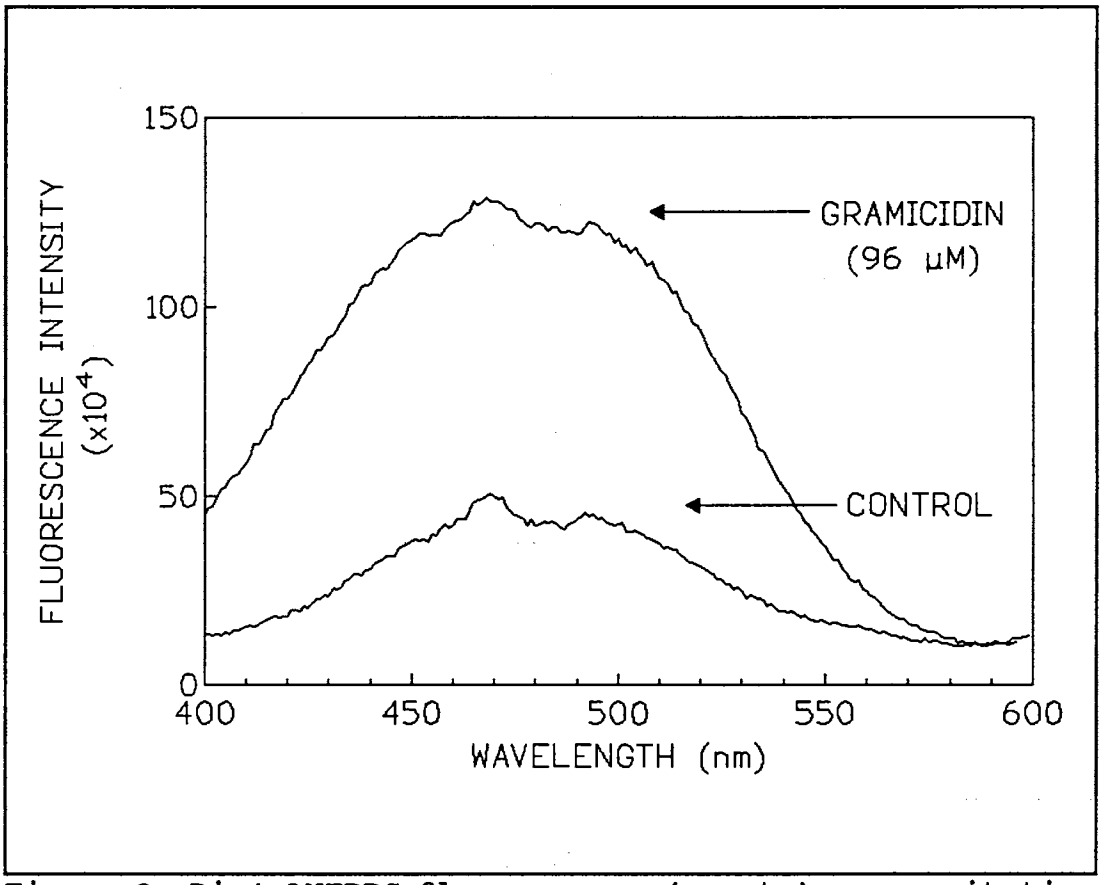

Figure 3. Di-4-ANEPPS fluorescence (counts) vs. excitation wavelength(nm).

Figure 4 represents the fluorescence of BCECF. This dye measures not only intracellular Ph, but also membrane viability as well (Grandin and Charbonneau 1988). The embryos were exposed to α-chaconine for 3 hours. α-Chaconine has been shown (Blankemeyer et al. 1992) to cause a hypopolarization (depolarization) in embryos incubated in this compound for 30 minutes; the EC50 for depolarization was 2.03 ± .005 mg/L. Figure 4 indicates that after 3 hours

has elapsed, another event takes place. The increase in the 500 nm region of Figure 4 shows that the intracellular pH has numerically increased from about 7 to near 8. Increasing concentrations of α-chaconine produce increasing fluorescence suggesting a concentration-response relationship between α-chaconine and fluorescence. Since the fluorescence is a function of intracellular pH, the α-chaconine probably causes an increase of cellular pH over the three hour incubation period.

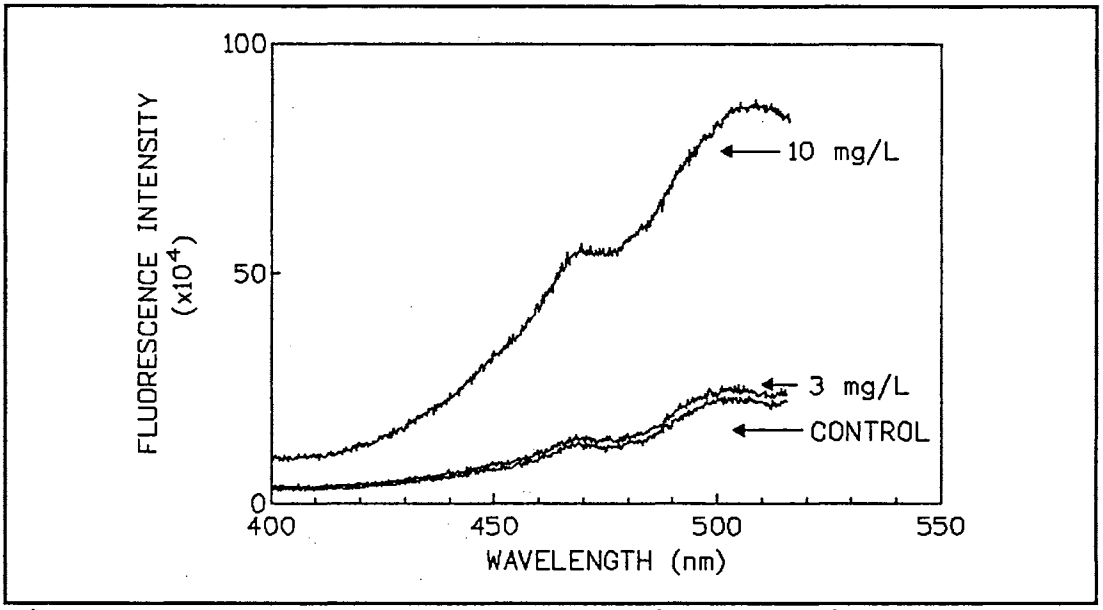

Figure 4. Cellular pH experiment using BCECF-AM. Fluorescence of BCECF (counts) vs. Excitation wavelength (nm) in the presence of α-Chaconine.

One of the major design elements of CHAWQ is the ability to measure embryonic DNA without digesting or altering the embryo. Figure 5 represents an experiment that uses thiazole orange, a DNA-binding fluorescent dye (Latt and Stetten 1976), to measure the DNA content of the embryo during normal growth and development. As expected, there is

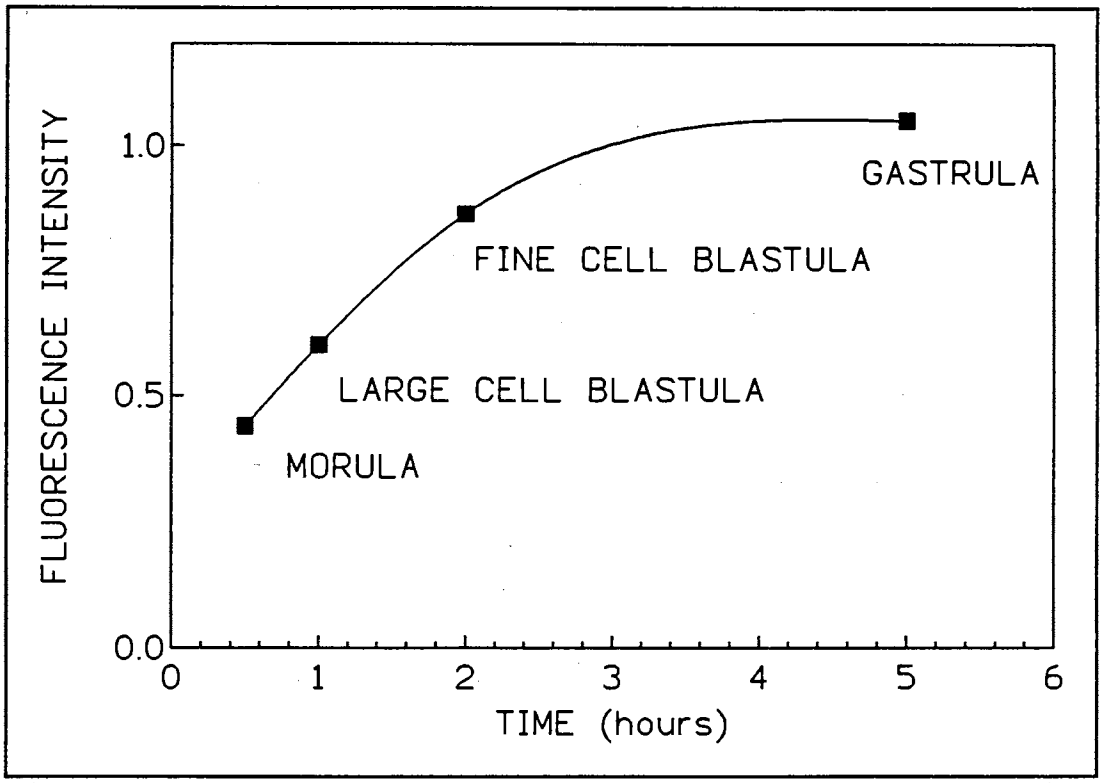

Figure 5. Measurement of Thiazole Orange fluorescence with embryonic development.

a steady increase in DNA binding, and therefore fluorescence, until gastrulation. Anything that alters DNA synthesis rates will alter this curve and be measured as a difference between the amount of DNA in the experimental and control groups.

Figure 6 represents a series of experiments with the clastogen triethylenemelamine (TEM) (Evenson et al, 1989). The results are presented in a concentration-response format. The embryos were treated for four hours with the clastogen. Control embryos were incubated in FETAX solution. Thirty minutes before the assay, hex-bisbenzamide dye is added to both the control embryos and the experimental embryos. The fluorescence resulting from excitation of the

bisbenzamide dye at 340 nm was measured at 480 nm using a photon counter as previously described. The concentration-response curve in Figure 6 demonstrates that hex-bisbenzamide dye can detect a clastogen is living embryos.

Two endpoints, cytochrome oxidation-reduction and calcium activity were not affected by the test chemicals. We found that mitochondrial cytochromes are essentially inactive in frog embryos. Apparently the frog embryos rely on non-oxidative pathways to provide energy for growth. When using Fura-2 to measure intracellular calcium, we found no difference in intracellular calcium between control and experimental embryos thirty minutes after incubation with test chemicals.

CONCLUSION

The methods and results demonstrate that a multiple-endpoint assay can be used to evaluate the toxicity of chemicals on organisms. Although the data herein are from frog embryos, a variety of other organisms (dahpnia, sea urchins, fish embryos) are possible candidates for this technique. The test materials correlated well with FETAX results demonstrate the versatility of the assay.

The membrane potential endpoint correlated well with FETAX showing in one case a hyperpolarization of the membrane potential (6-AN) and a depolarization in another (gramicidin). Blankemeyer et al. (1992) demonstrated that potato glycoalkaloids depolarize membrane potential and that

the CHAWQ EC50's for the potato glycoalkaloids were near those of FETAX. Note that the change induced in membrane potential by 6-AN (Figure 2) was opposite in sign to the change induced by potato glycoalkaloids. Thus the sign of the potential change does not directly indicate toxicity, only the presence of a difference between the membrane potential of the experimental and control embryos. The detection of membrane potential as a biochemical endpoint is reliable and valid when compared to FETAX.

Intracellular pH was an important endpoint, although the incubation period in toxicant required to measure pH differences is much longer than the incubation time required to obtain changes in membrane potential. In this study, when we incubated α-chaconine with embryos for thirty minutes using the CHAWQ tests outlined in the Methods section, we found an effect only on embryonic membrane potential. However, after three hours, we found effects in intracellular pH (numerically increased).

When we used a known clastogen, TEM, we found that a significant increase in fluorescence of the hex-bisbenzamide dye. Thus the DNA quantity and quality endpoint is detecting DNA effects on quantity of DNA and quality of DNA. Interestingly TEM was the only test compound that did not produce a change in membrane potential in the embryos.

The lack of effect of the test chemicals on mitochondrial oxidation-reduction and on intracellular calcium demonstrates the usefulness of a concurrent multiple

endpoint assay. By using multiple endpoints, CHAWQ is more likely to detect the cellular events associated with toxicity than single endpoint assays.

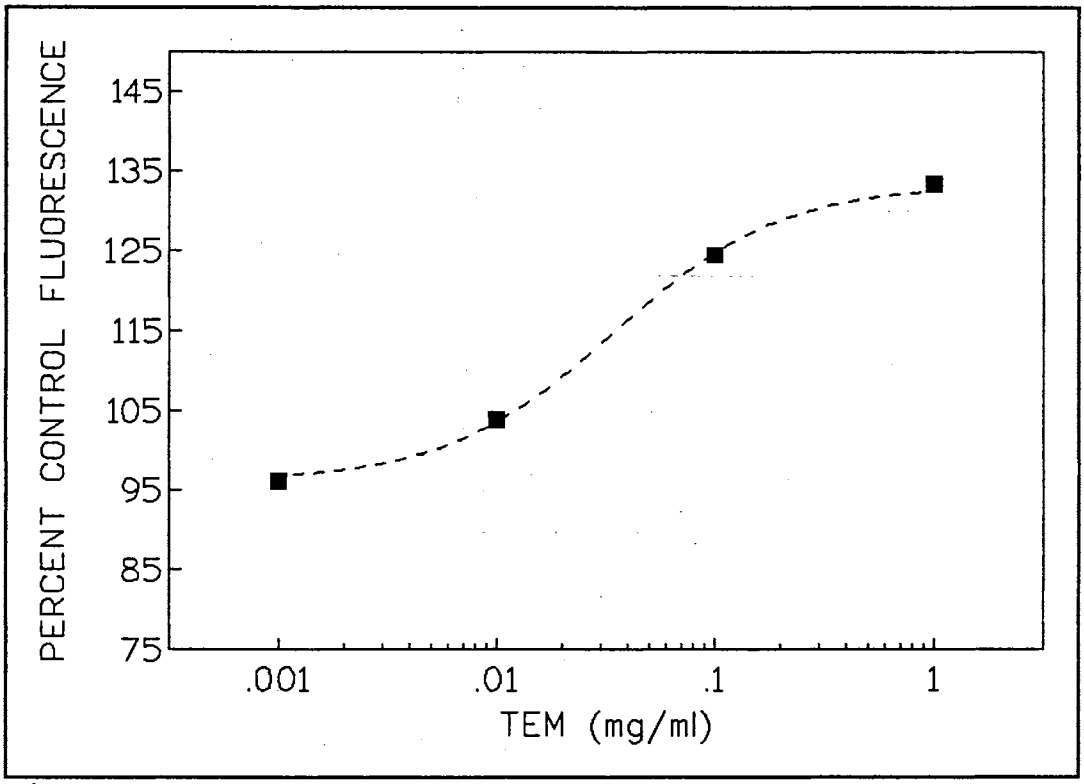

Figure 6. Effect of clastogen on DNA as measured by bisbenzamide dye. Triethylenemelamine concentration vs percent control fluorescence

We have presented information that outlines an instrument and assay that shows promise in establishing the relationship of short-term assays with conventional bioassays. Because CHAWQ uses multiple endpoints to detect toxicity on a cellular or subcellular level, CHAWQ is able to better predict the outcome of a longer term bioassay because it is more probable that CHAWQ will capture the particular cellular event that is the cause of the measured death, malformation, or lack of fitness that the longer term

assay documents. Of course it is also possible that CHAWQ may detect cellular events that do not result in measurable effects on the survival, development, or fitness of the organism.

The cellular endpoints that we have described are not the final set. We see a continual evolution of endpoints to best measure and assess the cellular events that underlay and possibly cause death and developmental errors. Particularly important in the CHAWQ design is the ability to concurrently assay both the experimental and control embryo sets. The simultaneous exposure and measurement allows matched pair statistics to be used to analyze the data. The use of matched pair "t" statistics should permit us to detect very small differences between control and experimental embryos. We believe that these small differences, followed over long periods, may illustrate the mechanism and promote understanding of the concentration-response relationship that exists at the low-concentration end of the concentration-response curve.

CHAWQ is not limited to use on frog embryos. We have successfully tested CHAWQ on fish embryos, daphnia, and blue-green algae. The fully operational CHAWQ will be able to measure the time course of both the experimental and control embryos over a short or a long time-span. The fully implemented CHAWQ will have continuous observation capabilities so that we will be able to determine the precise time-course of embryonic responses to toxicants.

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CHAPTER III

RAPID MEASUREMENT OF TOXICITY USING ELECTROCHROMIC DYES AND FROG EMBRYOS

INTRODUCTION

The membrane potential of a cell is affected by the ionic concentrations inside and outside of the cell and by the carriers and ion pumps located in the cell membrane. If any are disrupted, the membrane potential across the cell will change. One way to measure the membrane potential of many cells is to use a fluorescent dye, usually termed an electrochromic dye, to measure the membrane potential of cells. Di-4-Anepps is a styryl dye that is apparently interspersed in the membrane leaflet. Di-4-Anepps changes fluorescence directly in response to changes in membrane potential (Fluher et al 1985).

Because alterations in a membrane bound ATPase or alterations in ion channels could explain some toxicity at the cellular level (Blankemeyer and Hefler 1990; Blankemeyer and Bowerman 1992), we examined the effect of 6-aminonicotinamide (6-AN) on the membrane potential of embryos of the South African Clawed Frog, Xenopus laevis. We chose 6-AN because the EC50 for malformation differs greatly from the LC50 (Dawson et al 1989). We hope to

discern whether the response of the embryonic membrane potential, measured by fluorescent dye, predicts the EC50 or LC50 as determined by FETAX.

FETAX-Frog Embryo Teratogenicity Assay-Xenopus was developed as an environmental bioindicator for the presence of toxicant in water (Dumont et al 1983). FETAX has been useful in assessing the developmental, environmental, and genetic changes wrought by toxic substances (Bantle et al 1990). FETAX uses 96-hr endpoints of overall length, survival, and malformation to assess the effect of chemical compounds. A teratogenic index is calculated by dividing the LC50 (survival) concentration by the EC50 (malformation) concentration. The teratogenic index

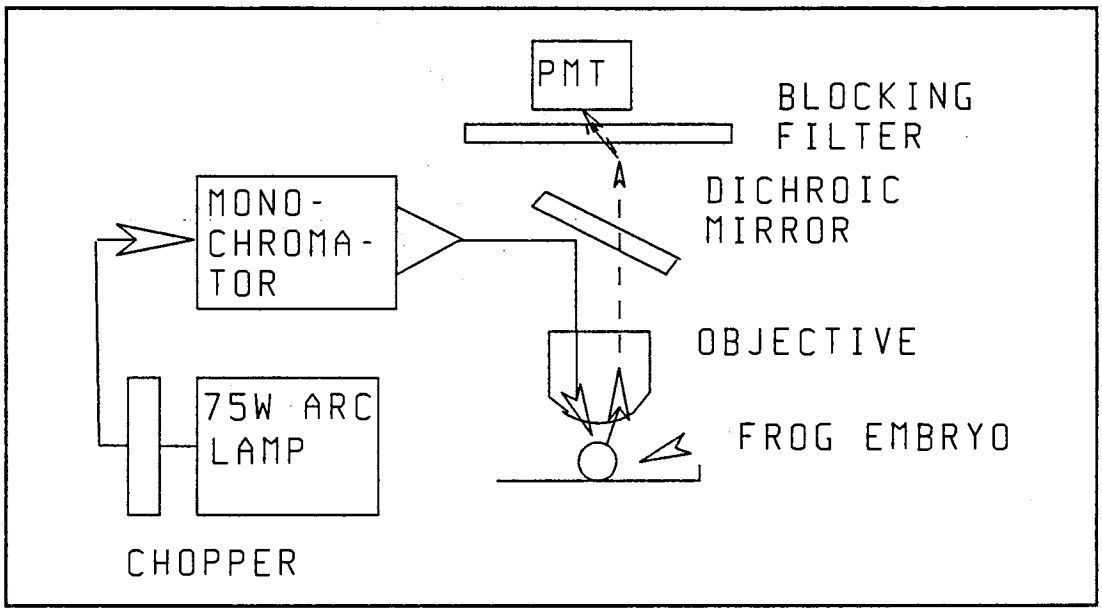

Figure 1. Syntopic diagram of embryo fluorescence instrument including monochromators, microscope, embryo, and photomultiplier tube. Line represents light path.

(TI) gives a measure of the teratogenicity of a chemical relative to its lethal concentration.

MATERIALS AND METHODS

Albino Xenopus laevis frogs were purchased from Xenopus-I (Ann Arbor, Michigan). Breeding pairs were conditioned for 1 mo to 6 wk prior to usage. Twelve hours prior to amplexus both male and female frogs were injected with Human Chorionic Gonadotropin (Sigma, St. Louis). Following successful amplexus, eggs were collected, dejellied with 2% (w/v) cysteine, adjusted to pH 8.1, and separated into viable and non-viable groups using a dissecting microscope. Twenty mid-to-late blastula stage embryos were selected; viable embryos were collected in covered plastic petri dishes. Embryos were maintained in FETAX solution which contained 10.8 mM NaCl, 1.2 mM $NaHCO_3$, 0.58 mM $MgSO_4$, 0.44 mM $CaSO_4$, 0.4mM KCl, and 0.14 mM $CaCl_2$ and was at pH 8. Valinomycin, gramicidin, and 6-aminonicotinamide were purchased from Sigma (St. Louis, Missouri) and dissolved into FETAX solution without a carrier solvent. Di-4-Anepps was purchased from Molecular Probes (Eugene, Oregon).

Each concentration of 6-AN was tested with at least three groups of embryos. Each set of experimental embryos was compared against a control of 20 embryos co-cultured with the experimental embryos. 6-AN was added to the petri dish containing the embryos at the same time as the styryl dye Di-4-Anepps. The final concentration of Di-4-Anepps was nominally 10^{-6} M and was identically concentrated in control

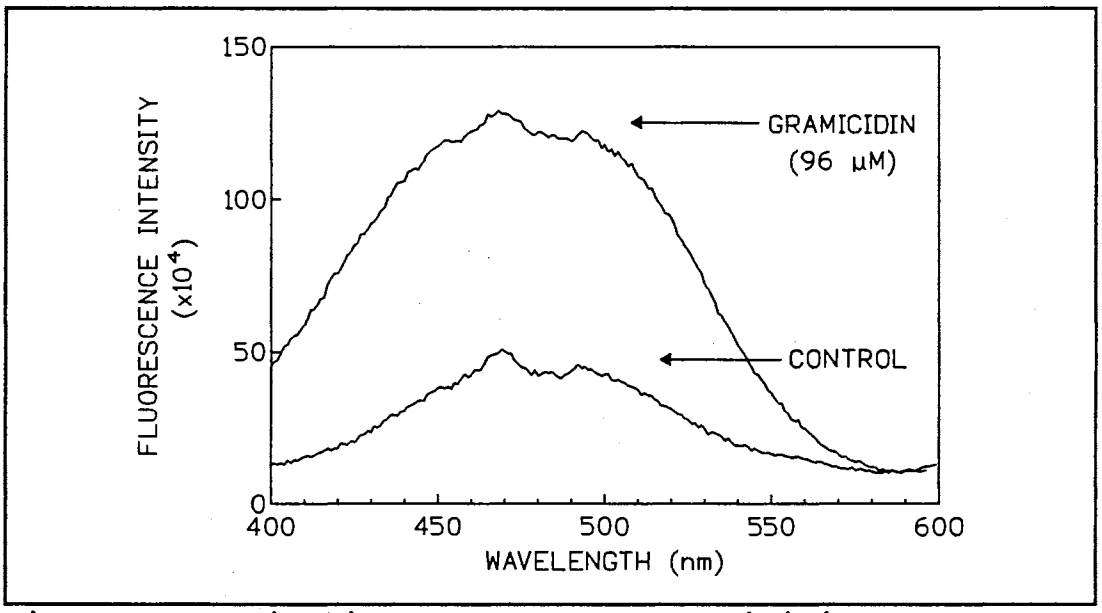

Figure 2. Excitation spectrum of Gramicidin and control embryos

and experimental petri dishes. After 30 min of exposure to Di-4-Anepps, and to 6-AN for the experimental, the embryos were placed on a microscope well-slide and the fluorescence data collected. The embryos were excited with 480 nm light from a PTI DeltaScan (Photon Technology Inc., Princeton, New Jersey) monochromator-based excitation source. The excitation light passed through a dichroic mirror and microscope objective (see Figure 1). The reflected excitation light and the fluorescence from the embryos returned through the objective. The excitation light was removed by a high-pass optical filter (580 nm). The fluorescence was measured by a photomultiplier tube operating in photon-counting mode for increased sensitivity. The experimental fluorescence counts were divided by the control fluorescence counts to normalize the data for the age and condition of the embryos.

Statistical analysis of the data was performed with GraphPad (San Diego, California). EC50's were determined using the 50 % point of the maximum effect of the chemical. Concentration-response curves were fitted by non-linear regression techniques using iteration to find the best fit employing a sigmoidal model.

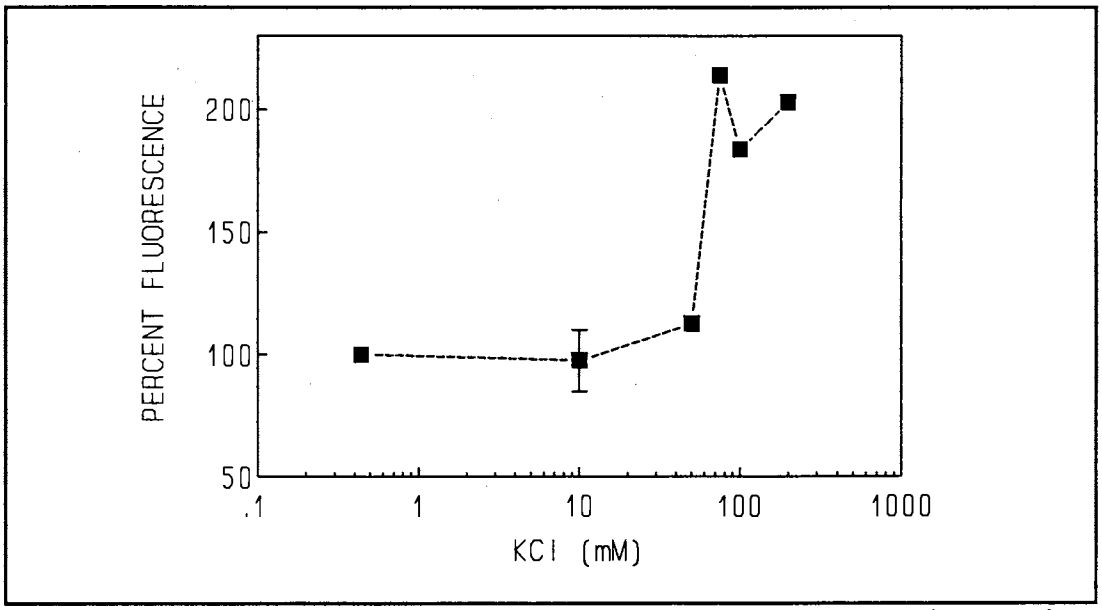

Figure 3. Concentration response plot for valinomycin-treated embryos. Concentration of valinomycin was 20 μM.

RESULTS AND DISCUSSION

Although the relationship of Di-4-Anepps fluorescence to membrane potential has been developed using liposomes, we extended the voltage calibration to frog embryos. Figure 2 illustrates the results of an experiment to calibrate the response of the embryos to gramicidin, an ionophore. Gramicidin forms large cation-selective channels that make the cell membrane permeable to any cation in the bath solution. The membrane potential will be near zero volts

when gramicidin is applied. In Figure 2, the fluorescence of a gramicidin-treated embryo, loaded with Di-4-Anepps, is plotted against excitation wavelength. Also plotted in Figure 2 is the fluorescence of a control embryo, loaded only with Di-4-Anepps. Figure 2 shows that the fluorescence of Di-4-Anepps, loaded into the embryos, increases when gramicidin is added. Since the normal membrane potential of the embryo cells is negative, the increase in fluorescence represents a depolarization of the embryonic cells. When gramicidin was mixed with Di-4-Anepps(without embryos), the fluorescence did not increase above the background fluorescence of the Di-4-Anepps.

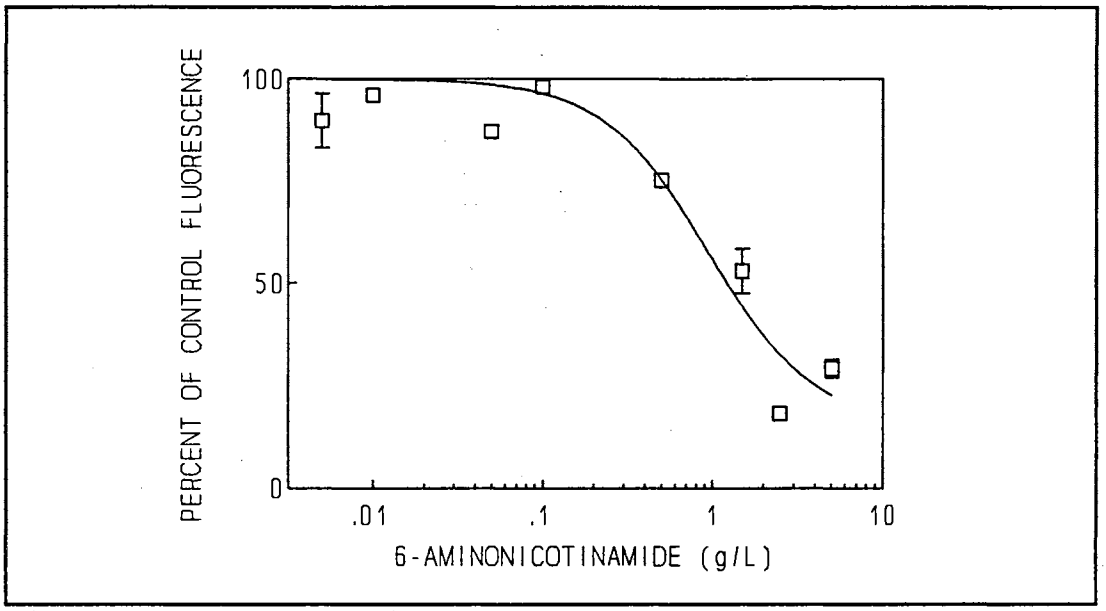

Figure 4. Concentration response of fluorescence versus 6-AN concentration

Valinomycin is a potassium-selective ionophore that makes the membrane permeable to potassium. When 20 μM valinomycin was mixed with 1 μM Di-4-Anepps and excited by 480 nm illumination, the fluorescence did not increase above

the background fluorescence of the Di-4-Anepps. The cell membrane potential is a function of the ion concentrations inside and outside the cell membrane and any electrogenic ion transport. The membrane potential of a cell treated with ionophore permeable to only one ion has a membrane potential predicted by the Nernst equation. When various concentrations of potassium are in the bath solution with valinomycin, the cell membrane potential can be calculated by using the Nernst equation for potassium:

$$V_{i-o} = \frac{R*T}{F} * \ln \frac{K_o}{K_i}$$

where:

V_{i-o} is the membrane potential referenced to the outside of the embryo (Volts).

R is the gas constant (8.314 Joules $°K^{-1}$ mol^{-1}).

T is the temperature in degrees Kelvin (°K).

F is Faraday's constant (96,486 Coulombs mol^{-1}).

K_o is potassium activity outside the embryo (molar).

K_i is the intracellular K activity (molar).

In the experiment reported in Figure 3, various concentrations of potassium with 20 μM valinomycin were used to calibrate the fluorescent response of the frog embryos. Note that the fluorescence decreased as the bath potassium decreased showing that the decrease in fluorescence represents a hyperpolarization of the membrane potential. The data in Figures 2 and 3 are a calibration curve for the embryos and present a framework to estimate the effect of toxicants on the embryonic membrane potentials.

Figure 4 represents the results of several trials with the teratogen 6-aminonicotinamide. Note that as the concentration of 6-aminonicotinamide increased, the fluorescence from the dye Di-4-Anepps decreased. We interpret this result, based on the calibration in Figures 2 and 3, to mean that 6-AN causes a hyperpolarization of the embryo's membrane potential. Higher concentrations of 6-AN produced greater hyperpolarization.

The data in Figures 2 and 3 demonstrate that the response of the embryos to standard ionophores is predictable and consistent with literature reports on Di-4-Anepps. Although there is still a possibility of direct dye interaction with the ionophores wherein the interaction is mediated by the embryo, our control experiments with Di-4-Anepps and both gramicidin and valinomycin suggest that the dye is detecting the membrane potential of embryonic cells. The data in Figure 4 show that the teratogen 6-aminonicotinamide effected a response in membrane potential

with an EC50 at 0.9 g/L. Since the reported 96 hr LC50 for 6-AN is 3 g/L in FETAX whereas the EC50 for malformation is 5.5 mg/L (Dawson et al 1989), we conclude that the membrane potential EC50 is predictive of the lethal effect of 6-AN rather than the teratogenic effect of 6-AN. Thus EC50 from the membrane potential assay using blastula-stage embryos and requiring a thirty minute assay is near the LC50 of 6-AN on 96 hr frog embryos. We can speculate that membrane potential is a bioindicator signaling the effect of toxicant on frog embryos.

The membrane potential assay is part of a bioassay named CHAWQ- Cell Health Assay of Water Quality. CHAWQ uses cellular bioindicators with quantitative endpoints to rapidly assess water quality through optical transduction of bioindicators. The assay can be used in water quality testing, assessment in bioremediation and toxicity reduction, and in determination of mode of action of toxicants. Validation of the membrane potential bioassay in progress.

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CHAPTER IV

EFFECT OF POTATO GLYCOALKALOIDS, α-CHACONINE AND α-SOLANINE, ON MEMBRANE POTENTIAL OF FROG EMBRYOS

ABSTRACT

To demonstrate whether potato glycoalkaloids can alter the integrity of membranes of frog embryo, albino frog embryos were incubated with α-chaconine and α-solanine. Di-4-Anepps, an electrochromic fluorescent dye, was added to measure embryonic membrane potential. α-Chaconine increased the Di-4-ANEPPS fluorescence up to 1600 % of control, α-solanine increased the fluorescence up to 400 %, and solanidine had no effect. Increases in fluorescence, when plotted in a concentration-response format, produced EC50 values near published values for FETAX (Frog Embryo Teratogenicity Assay-Xenopus). Possible mechanisms and the significance of the fluorescence to food safety are discussed.

INTRODUCTION

Solanaceous plants, including such agronomically important crops as potatoes, synthesize secondary plant metabolites including glycoalkaloids. In commercial potato

cultivars, the primary glycoalkaloids are α-chaconine and α-solanine. These compounds can have toxic effects in animals and humans (Jelinek et al., 1976; Munn et. al., 1975). Thus potatoes may represent a potential source of undesirable compounds, especially if improperly stored or processed (Morris and Lee, 1984).

As part of a program designed to lower the potential toxicity of glycoalkaloids in potatoes, the toxicity of several potato alkaloids was tested with the Frog Embryo Teratogenesis Assay- Xenopus (FETAX). Using FETAX, α-chaconine was about three times more toxic than α-solanine (Friedman et al., 1991).

One proposed mechanism of action for the toxic action of the glycoalkaloids is disruption of membranes. Roddick and Rienberg (1987) and Roddick et al., (1988), reported that potato and tomato glycoalkaloids disrupted liposome membranes. They also reported that mixtures of α-chaconine and α-solanine acted synergistically in lysing rabbit erythrocytes, red beet cells, and Penicillum notatum protoplasts. They speculated that the nature of the carbohydrate side chain affected the cell disruption process and that cell disruption appears not to be directly related to binding of the glycoalkaloids to membrane steroids.

The membrane potential of a cell is affected by the ionic concentrations inside and outside of the cell and by the permeabilities of the carriers and ion pumps located in or near the cell membrane. If any are disrupted, the

membrane potential across the cell will change. One way to measure the membrane potential of many cells is to use a fluorescent dye, usually termed an electrochromic dye, to measure the membrane potential of cells. Di-4-ANEPPS is a dialkylaminostyrl pyridinium sulfonate dye with positive and negative charges (Figure 1) that is apparently interspersed in the membrane leaflet and that changes fluorescence directly in response to changes in membrane potential (Fluher et al., 1985).

Because alteration in an ion pump or ion channels could explain alkaloid toxicity at the cellular level (Blankemeyer and Hefler, 1990), we examined the effect of glycoalkaloids on the membrane potential of embryos of the South African Clawed Frog, Xenopus laevis. We hope to establish whether the frog embryo can serve as a model and an index for mammalian toxicity studies.

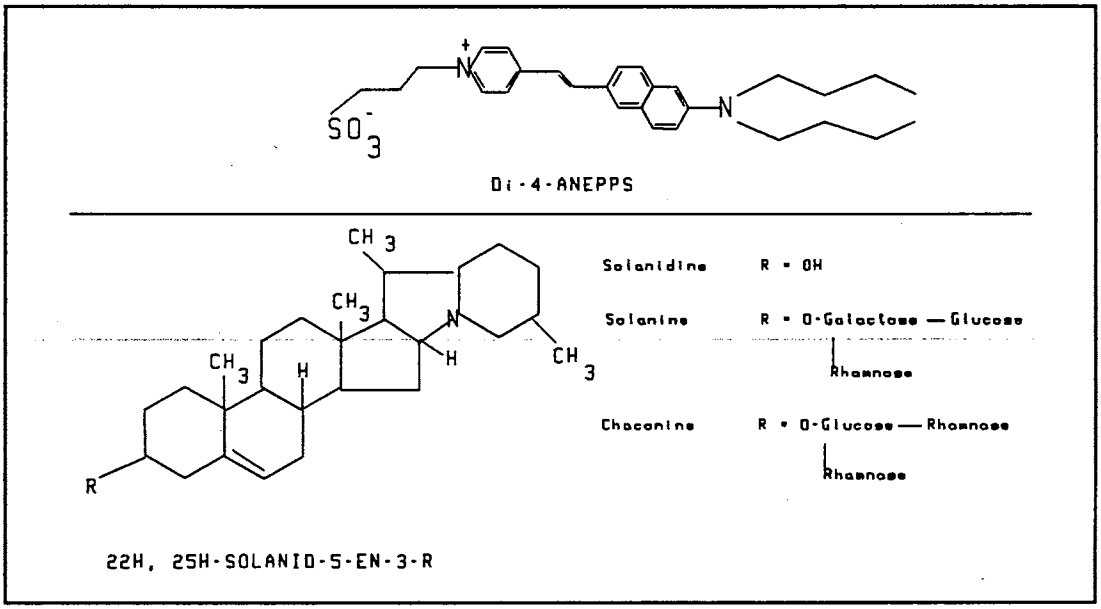

Figure 1. Structures of glycoalkaloids and the membrane potential dye Di-4-ANEPPS.

The two glycoalkaloids evaluated in this study, α-chaconine and α-solanine, are found in approximately equal concentrations in the potato plant, Solanum tuberosum, and in several other Solanum and Veratrum species. The sugar side chain of α-chaconine has a branched bis-α-L-rhanmopyranosyl-ß-D-glucopyranose trisaccharide side chain. The sugar side chain of α-solanine is a branched α-L-rhanmopyranosyl-ß-D-glycopyranosyl-ß-galactopyranose (Roddick et al., 1988). For comparison we also evaluated their common aglycone, solanidine, which lacks the carbohydrate side chain (Figure 1). Our results will show that the trisaccharide side chains have a strong influence on the Di-4-ANEPPS fluorescence and thus the membrane potential of frog embryos.

MATERIALS AND METHODS

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. Four hours prior to mating, the frogs were injected with Human Chorionic Gonadotropin (Sigma, St. Louis, MO). Following successful amplexus, eggs were collected, dejellied with 2% (w/v) cysteine adjusted to pH 8.1, and separated into viable and non-viable groups. Mid-to-late blastula to neurula stage embryos were selected under a dissecting microscope. Groups of 20 viable embryos were collected in covered plastic dishes. Embryos were maintained in FETAX solution which

contained 10.8 mM NaCl, 1.2 mM $NaHCO_3$, 0.58 mM $MgSO_4$, 0.44 mM $CaSO_4$, 0.4mM KCl, and 0.14 mM $CaCl_2$ and was at pH 8. α-Chaconine (95% purity) and α-solanine (95% purity) were purchased from Sigma (St. Louis) and dissolved into FETAX solution as described previously (Friedman et al., 1991). Solanidine (99 % purity) was purchased from Roth (Basel, Switzerland) and was dissolved in FETAX solution by first dissolving solanidine in 10 ml of DMSO then dissolving the mixture in FETAX. The purity of the alkaloids was confirmed by high-performance liquid chromatography and thin-layer chromatography. Di-4-ANEPPS (99% purity) was purchased from Molecular Probes (Eugene, OR).

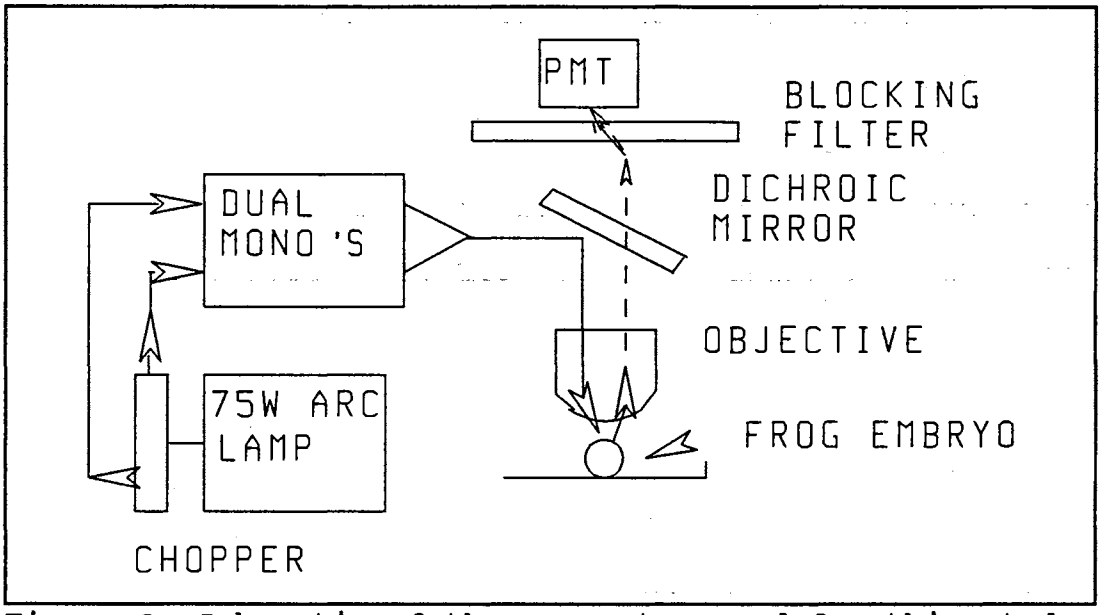

Figure 2. Schematic of the apparatus used for this study.

Each concentration of glycoalkaloid was tested with at least three groups of embryos. Each set of experimental embryos was compared against a control triplet of 20 embryos, co-cultured with the experimental embryos. The

test chemical, α-chaconine or α-solanine or solanidine, was added to the petri dish containing the embryos at the same time as the styryl dye Di-4-ANEPPS. The final concentration of Di-4-ANEPPS was nominally 10^{-6} M and was identically concentrated in control and experimental petri dishes. After thirty minutes, the embryos were placed on an immersion slide and the fluorescence data collected.

Figure 2 describes the setup for measuring of fluorescence emanating from embryos loaded with Di-4-ANEPPS. A 75 watt xenon arc lamp (USHIO) provided the light source. The collimated beam from the xenon lamp passed from a collimating and focusing mirror to a monochromator set to 490 nm. The 490 nm excitation passed through a dichroic mirror and was reflected through a microscope objective onto the embryo. Reflected light and fluorescent emission from the embryo returned through the objective. The reflected 490 nm light was filtered out by the dichroic mirror and a blocking filter (590 nm high pass). The intensity of fluorescent emission was measured by a photomultiplier tube operating at 900 volts in photon-counting mode. The photon count was divided by 10 and collected via a module attached to a PC (Photon Technology Inc, Princeton, N.J.). The emission data (in counts per second) was collected to disk and fitted by linear regression over the sample period (20 seconds). The calculated value for the midpoint in the time-based record was used as the emission intensity.

Statistical analysis of the data was performed with GraphPad (Los Angeles). EC50's were determined using the 50 % point of the maximum effect of the glycoalkaloid on fluorescence. Curves were fitted by non-linear regression techniques using iteration to find the best fit employing a sigmoidal model.

RESULTS

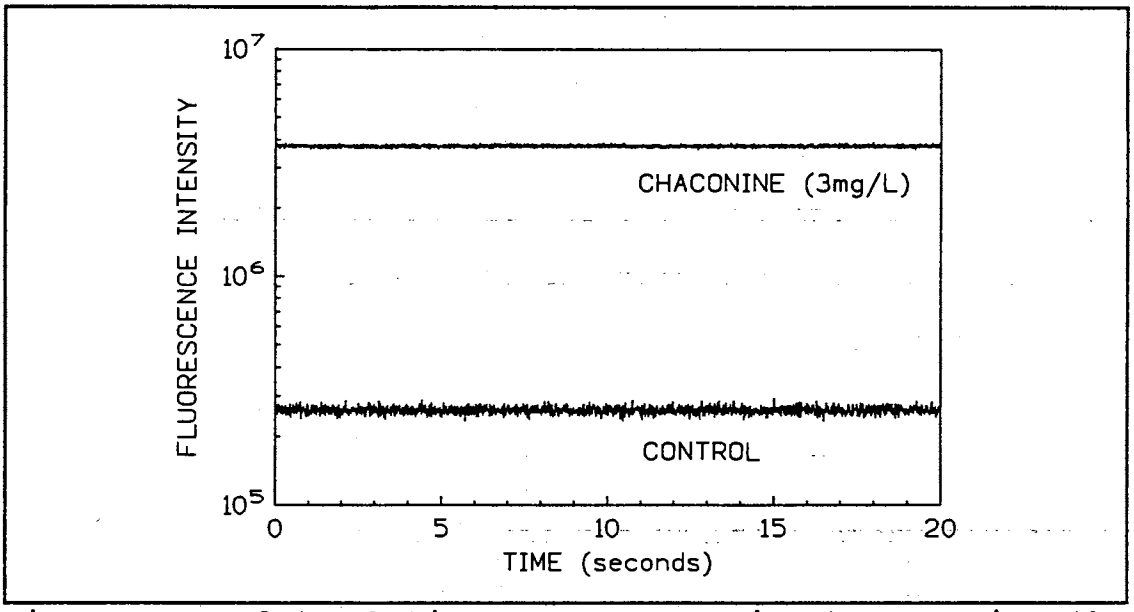

Figure 3. Plot of time course experiment comparing the effect of 3 mg/L α-chaconine (upper trace) to the control of no α-chaconine (lower trace) on the membrane potential of *Xenopus* embryos.

The structures of the compounds used in this study are illustrated in Figure 1. A typical trace of the fluorescent emission is displayed in Figure 3. As the Figure shows, the Di-4-ANEPPS fluorescence (for the experimental and control) was nearly constant over the twenty minute period. The ordinate is counts per second of the photons detected by the PMT and photon counter. These traces were retained on disk.

Subsequent linear regression analysis produced a value of 2.59 $x10^5$ counts per second for the control trace and 3.74 $x10^6$ counts per second for the 3 mg/L α-chaconine trace. The regression was obtained by fitting a linear equation to the data and then using the calculated counts at the midpoint as the data. For each concentration, the fluorescence was corrected for control by dividing the experimental counts by the control embryo counts. In Figure 3, the fluorescence ratio was 14.4.

Figure 4 shows the results of an experiment wherein groups of twenty Xenopus embryos were used to obtain fluorescence ratios of experimental to control at various concentrations of α-chaconine. The embryos were exposed to α-chaconine for 30 minutes. The data are plotted as percent of control fluorescence on the ordinate (with error bars denoting standard error) and log α-chaconine concentration on the abscissa. The EC50 for fluorescence was 2.03 ± 0.005 mg/L. The maximum change in fluorescence was a 1600 % increase over the control embryos. Each data point was significantly different from control fluorescence except the 1.3 mg/L point. Differences were judged statistically different at the $p > 0.05$ level. The increase in fluorescence determined for α-chaconine represents a decrease in membrane potential (hypo-polarization) of some, most, or all of the cells in the embryo.

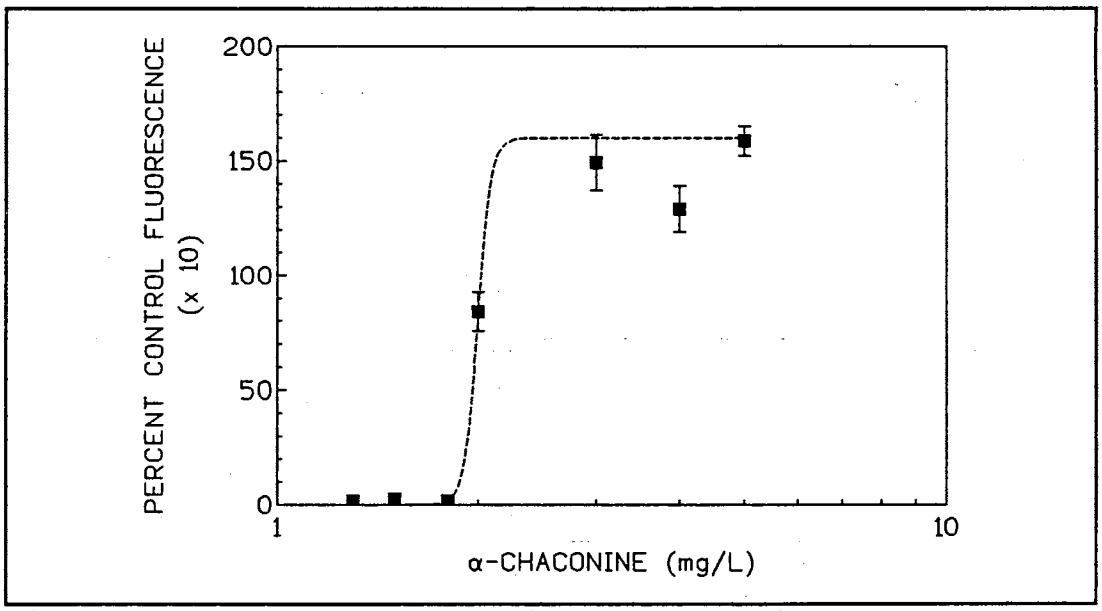

Figure 4. Plot of α-chaconine data showing the effect of α-chaconine on Di-4-ANEPPS fluorescence in a concentration-response format.

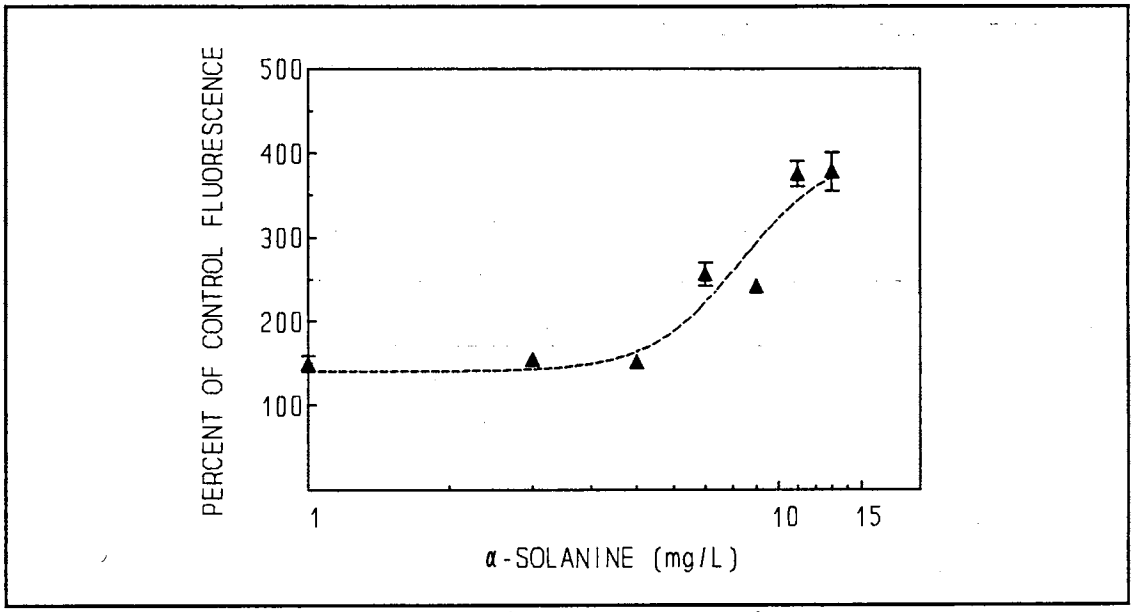

Figure 5. Plot of α-solanine data showing the effect of α-solanine on Di-4-ANEPPS fluorescence in a concentration-response format.

In a separate set of experiments, groups of 20 embryos were also incubated with various concentrations of α-solanine and a fixed concentration of Di-4-ANEPPS for 30

minutes. Figure 5 illustrates the result of those incubations in a concentration-response format. Each data point for each concentration of solanine was collected at least in triplicate as described above for α-chaconine. The error bars on the figure represent the standard error. All data points on the α-solanine figure were significantly different from control ($p > 0.05$). The Di-4-ANEPPS fluorescence increased in the α-solanine treated embryos as did the fluorescence in the α-chaconine experiments. However the maximum increase for solanine was 400 % of the control fluorescence, much less than the α-chaconine. The EC50 for α-solanine was 8.3 ± 0.03 mg/L.

Solanidine, the aglycone of α-chaconine and α-solanine, was also tested at the solubility limit (10 mg/L) in FETAX solution. Solanidine had no effect on fluorescence of frog embryos using Di-4-ANEPPS when compared to control embryos in FETAX solution with DMSO.

CONCLUSIONS

FETAX-Frog Embryo Teratogenicity Assay-Xenopus was developed an environmental bioindicator for the presence of toxicant in water (Dumont et al., 1982). FETAX has been useful in assessing the developmental, environmental, and genetic changes wrought by toxic substances (Bantle et al., 1990). FETAX uses 96 hour endpoints of overall length, survival, and malformation to assess the effect of chemical compounds. A teratogenic index (TI) is calculated by

dividing the LC50 (survival) concentration by the EC50 (malformation) concentration. TI is a measure of the teratogenicity of a chemical relative to its lethal concentration.

These data show that the membrane potential, as measured by the styryl dye Di-4-ANEPPS, changes rapidly and markedly upon administration of α-chaconine, showing an increase of up to 1600 %. The EC50 of the 30 minute α-chaconine exposure (2.03 mg/L) was close to the average EC50 of the full-scale 96 hour FETAX assay (2.85 mg/L, Friedman et al., 1991). It is probable that the cellular event that we have observed in these young embryos is causal to the effects observed in the 96 hour FETAX embryos.

The α-solanine data showed a similar increase in fluorescence when compared to control embryos, although the amount of the maximum increase was 400 %, one-fourth of the α-chaconine effect. The EC50 for α-solanine in the thirty minute exposure was 8.3 mg/L also was close to FETAX EC50 (9.65 mg/L) (Friedman et al., 1991). Again, the EC50 for the α-solanine assay was close to the EC50 for the full-scale FETAX assay.

Since the EC50/LC50 derived from the FETAX data was near the EC50 of the membrane potential change, we suggest that the effect of the glycoalkaloids on membrane potential (measured by fluorescence) correlates with the eventual malformation and/or death of the embryo. That is, the effect of the potato glycoalkaloids determined in the 96 hour FETAX

assay is predicted by the effect of the glycoalkaloids on the membrane potential of recently fertilized embryos. We speculate that the membrane potential assay is measuring a fundamental mechanism of action of the glycoalkaloids and that the mechanism of action affecting membrane potential is the same mechanism of action for the teratogenicity/lethality determined in the 96 hour FETAX assay.

A striking result of this study is the fourfold-greater fluorescent intensity induced by α-chaconine compared to α-solanine. These two glycoalkaloids have the same aglycone but differ only in the nature of the carbohydrate attached to the 3-OH position of the aglycone (Figure 1). The three carbohydrate residues associated with α-chaconine are one glucose and two rhamnoses; the three carbohydrate residues associated with α-solanine are galactose, glucose, and rhamnose. In a previous study, Friedman et al. (1991a) proposed that the carbohydrate residues influence the relative toxicities of the glycoalkaloids by participating in binding to sugar molecules associated with receptor sites of cell membranes. Supporting this suggestion is our negative result for solanidine which has no carbohydrate moiety. However the negative data for solanidine could be due to the marked difference in solubility between the aglycone (almost insoluble in water) and the glycoalkaloids (moderately soluble in water). The present results with the two glycosides and with solanidine are in agreement with the

previous suggestion (effect of carbohydrate residues) since otherwise α-chaconine and α-solanine should not differ in toxicity since the steroid moiety is identical in each.

This study does not resolve the nature of the atomic and electronic interactions between the membrane, fluorescent dye, and the glycoalkaloid. Di-4-ANEPPS measures the membrane potential of the cells of the developing embryo. Whether the change is directly on a surface receptor on the cells or through a secondary or tertiary messenger is not clear from our results. However since the membrane potential change is associated with changes in ionic permeability of the membrane or with ionic activities on either side of the membrane, we conclude that (a) α-chaconine and α-solanine change the characteristics of ion channels, either passive, active, or both, in the developing frog embryo; (b) the fluorescence assay is potentially useful to evaluate the relative embryotoxicities of potato alkaloids and possibly other dietary ingredients; (c) the assay makes it possible to develop a better understanding of the molecular events and mechanisms governing the physiology and toxicology of the alkaloids. Such an understanding is needed to guide our efforts to develop safe foods.

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CHAPTER V

MEASUREMENT OF DNA INTEGRITY AND STRUCTURE IN XENOPUS EMBRYOS IN THE PRESENCE OF HYDROXYUREA, ACTINOMYCIN-D, AND TRIETHYLENEMELAMINE USING THE FLUORESCENT DNA PROBE HOECHST 33258.

INTRODUCTION

Recently, we have reported the design and implementation of a new, rapid assay to test the toxicity of compounds on cellular functions (Blankemeyer et al., 1993; Blankemeyer et al., 1992; Stringer and Blankemeyer, 1993). This assay, Cell Health Assay of Water Quality-CHAWQ, utilizes whole Xenopus laevis embryos and cellular biomarkers that can be measured by fluorescent probes that fluoresce in response to specific cellular events such as membrane potential, intracellular pH, intracellular calcium levels, and DNA content and structure (Blankemeyer et al., 1993). Utilizing numerous cellular biomarkers, the effects of a wide variety of toxic compounds with dissimilar cellular mechanisms can be detected.

This study is a continuation of the Cell Health Assay of Water Quality (CHAWQ) that has been described previously

(Blankemeyer, et al., 1993). Our primary purpose was to use Hoechst 33258 to measure the effects of compounds that are known to affect DNA. We have shown that it is possible to successfully measure membrane potential and intracellular pH changes in response to deleterious compounds (Blankemeyer et al., 1993; Blankemeyer et al., 1992); we now expand the CHAWQ assay by measuring an additional endpoint. Additionally, interactions of the compounds with Xenopus DNA will be discussed in terms of the known mechanisms of action and the fluorescence behavior of Hoechst 33258 in the presence of either double stranded DNA or chromatin.

The focus of this study is to measure the effects of three classes of compounds that either act to inhibit DNA synthesis, DNA structure, or DNA conformation. We have chosen to use Triethylenemelamine (TEM), a clastogen, Hydroxyurea, a DNA synthesis inhibitor, and Actinomycin-D, a compound that both binds DNA and inhibits mRNA transcription.

TEM is a compound that is used as an intermediate and monomer for oil-additive compounds, ion-exchange resins, surfactants, and cosmetic manufacture (Verschaeve and Kirsch-Volders, 1990). This compound has been shown by numerous investigators to cause base mutations in DNA, as well as gross malformations in mice and rats such as cleft palates, dwarfism, exencephalus, and polydactyly (Yamamura et al., 1992; Nagao and Fuikawa, 1990). Verschaeve and Kirsch-Volders (1990) report that TEM also arrests mitotic

and meiotic cell division during metaphase and anaphase in plants, insects, and mammals, although no mechanism was proposed.

Hydroxyurea is a compound that is routinely used in chemotherapy in the treatment of cancer (Eastman et al., 1991). This compound has been shown to inhibit DNA synthesis during the S-phase of the cell cycle (Broadie and Bate, 1991; Holme et al. 1991). This drug has also been shown to cause gross malformations and death in 96-hr Xenopus embryos (Courchesne and Bantle, 1985) using the FETAX malformation and mortality assay (Dumont et al., 1982). Slabaugh et al., (1991) has shown that hydroxyurea reduces the amount of substrate for the enzyme ribonucleotide reductase. Inhibition of DNA synthesis via substrate depletion would most likely cause the chromatin structure to remain in an uncondensed state and to not reassociate with histone proteins.

Actinomycin-D has been shown to both inhibit mRNA synthesis (Boggaram et al, 1991, Hickey et al, 1990) as well as to bind DNA directly (Baily and Graves, 1993; Jones et al, 1993; Basu et al., 1990). Courchesne and Bantle (1985) and Courchesne et al., (1986) have shown that Actinomycin-D also causes malformation and death in Xenopus embryos.

We used the fluorescent probe Hoechst 33258, a dye that binds DNA in the minor groove, specifically binding AT-rich regions (Latt and Stetten, 1976). Labarca and Paigen (1979)

have shown that Hoechst 33258 can be used to quantify DNA; the fluorescence increase is linear with respect to increasing DNA concentrations. These investigators also determined that Hoechst 33258 fluorescence is decreased in the presence of chromatin, presumably because a number of dye binding sites are blocked by histone proteins. Hoechst 33258 has also been used to measure the DNA content of epithelial cells during neoplastic transformation in vitro after treatment with chemical carcinogens (Knowles and Franks, 1977).

METHODS

Albino Xenopus laevis breeding pairs were purchased from Xenopus I (Ann Arbor MI). Double stranded calf thymus DNA, calf thymus histones, deoxyribonuclease I (DNA_{asc}), Actinomycin-D, and Hydroxyurea were purchased from Sigma (St. Louis, MO). TEM was a generous gift from Dr. Karen McBee. All toxicants were dissolved in FETAX solution (10.8 mM NaCl, 1.2 mM $NaHCO_3$, 0.58 mM $MgSO_4$, 0.44 mM $CaSO_4$, 0.4 mM KCl, and 0.14 mM $CaCl_2$ adjusted to pH 8.0). Hoechst 33258 was purchased from Molecular Probes (Eugene, OR) and dissolved in FETAX solution at 2 mg/ml.

Embryo assay protocol. Frogs were mated and embryos were collected as previously described (Dawson and Bantle, 1987; Blankemeyer et al., 1993). Embryos were sorted into viable and non-viable groups and put into petri dishes in groups of 25 per dish. Fine cell blastula stage embryos

were incubated in FETAX solution or toxicant diluted with FETAX for 4 hours in the case of actinomycin-D and TEM, or for 18 hours for tests with hydroxyurea. Hoechst 33258 was added 30 minutes before measurement at a final concentration of 20 μg/ml. Fluorescent levels of the control embryos were compared to those of embryos incubated in toxicant; control embryos were incubated for the same period of time as those in the presence of toxicant.

Measurement of DNA content in developing embryos was performed by first determining the developmental stage with a dissection scope as outlined in Bantle, et al. (1991) and Nieuwkoop and Faber, (1975). Groups of 20 embryos at approximately stage 4 were put into each of 13 dishes; embryos were incubated at 25 degrees C. At 30 minute or 1 hour intervals, embryos were removed and Hoechst 33258 was added at a final concentration of 20 μg/ml. Embryos were incubated in Hoechst for thirty minutes. Before fluorescence measurements were taken, the embryos were staged using a dissection microscope; stages were determined according to Nieuwkoop and Faber, (1975).

A Photon Technologies Inc, (PTI), (Princeton, NJ) dual wavelength spectrofluorometer was utilized to make all fluorescent measurements (Blankemeyer et al., 1993; Stringer and Blankemeyer, 1993). Embryos were placed in a 3x3 mm quartz cuvette in a specially designed cuvette holder and dye fluorescence was measured at 90° incident to the excitation. Fluorescent emission wavelengths were focused

on a second monochrometer through a fiber optic cable. The second monochrometer was set to the fluorescent emission wavelength to block any excitation light. The intensity of the fluorescent emission was measured by a photomultiplier tube. Excitation and emission wavelengths for Hoechst 33258 were 358 nm and 480 nm, respectively.

Excitation spectra for Hoecht 33258 was obtained by scanning the sample from 300-450 nm with the emission wavelength set to 480 nm. Steady-state and time-resolved fluorescence measurements were made by holding the excitation wavelength constant at 358 nm and emission wavelength at 480 nm.

Isolated DNA protocol. Experiments with calf thymus DNA and histones were carried out at DNA concentrations of 10 μg/ml and histone concentrations of 50 μg/ml according to Latt and Stetten, (1976). Calf thymus DNA and calf thymus histones were dissolved in FETAX solution; experiments were carried out at room temperature. DNA and histones were allowed to incubate for 15 minutes before the addition of Hoechst 33258; dye concentration was 20 μg/ml incubated 15 minutes.

Time-resolved fluorescence measurements of calf thymus DNA were carried out at DNA and Hoechst 33258 concentrations as stated above. DNA was incubated with either FETAX solution, 2.0 mg/ml hydroxyurea or 100 μg/ml actinomycin-D for 30 minutes. The sample was placed in the spectroflurometer and stirred with a Helma Cuv-O-Stir (New

York, New York). Data acquisition commenced and Hoechst 33258 was added; fluorescence data was taken for 50 seconds. Rate constants for fluorescence increase were determined from an exponential fit of the data using Kinfit (Jefferson, GA). The following single exponential model was used:

$$Y=\exp*^{(k*t)}+b$$

Data analysis. Concentration-response data were analyzed with GraphPad (Los Angeles) and EC_{50} values were determined using the 50 % point of the maximum effect of the test compounds on fluorescence. Concentration-response curves were fitted by non-linear regression using iteration to find the best fit. Excitation spectra were smoothed once by the data acquisition package Delta Scan (Princton, NJ.) using the Savitzski-Golay algorithm.

RESULTS and DISCUSSION

The data shown in Figure 1 indicates that the fluorescence of Hoechst 33258 increases as the embryo develops. As the Xenopus embryo develops from stage 4 through stage 13, the fluorescence of the dye increases. We

interpret this change as being indicative of an increase in the amount of DNA in the embryo.

The data presented in Figure 1 correspond well with previous studies. Dawid (1965), isolated DNA from developing Xenopus embryos and quantified the material by ultraviolet spectroscopy, CsCl gradient banding, melting curves, and measurement of ^{32}P incorporation during DNA synthesis; DNA content was measured for embryos in stage 8 through 45. Dawid indicates that detecting DNA synthesis earlier than stage 8.5 to 9 is difficult at best and is not easily demonstrated during the first few divisions beyond fertilization. The DNA content versus embryonic stage data published by Dawid indicates that DNA content increases linearly with increasing embryonic stages up to approximately stage 19. The data we present in this study agrees with Dawid's quantification data in that fluorescence increases are not measured until the embryo reaches mid-cleavage or stage 8; beyond stage 8, larger fluorescence increases are measured. Although the present study does not attempt to quantify DNA content in Xenopus embryos, the data suggests that the fluorescence changes observed are due to increasing amounts of DNA as the embryo develops.

Figures 2 and 3 are exemplary results from control experiments designed to test the fluorescence response of the dye under varying conditions. In Figure 2, DNAse I was added to a sample of double stranded calf thymus DNA. Trace 1 is the fluorescence of Hoecht 33258 in the presence of DNA

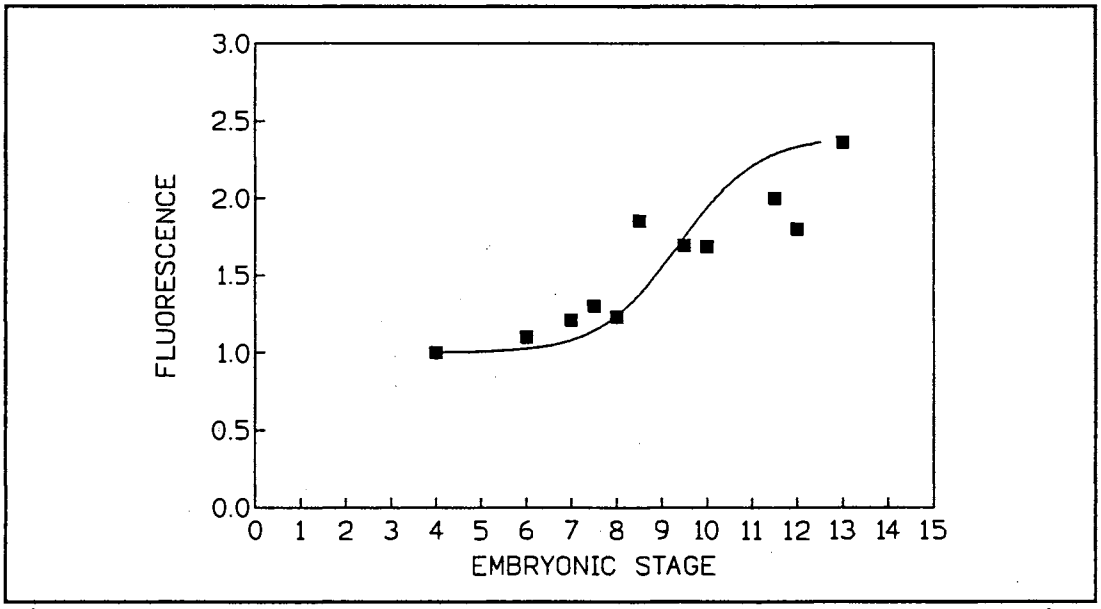

Figure 1. Measurement of Hoechst 33258 fluorescence with embryonic development. Embryos staged according to Nieuwkoop and Faber, 1975.

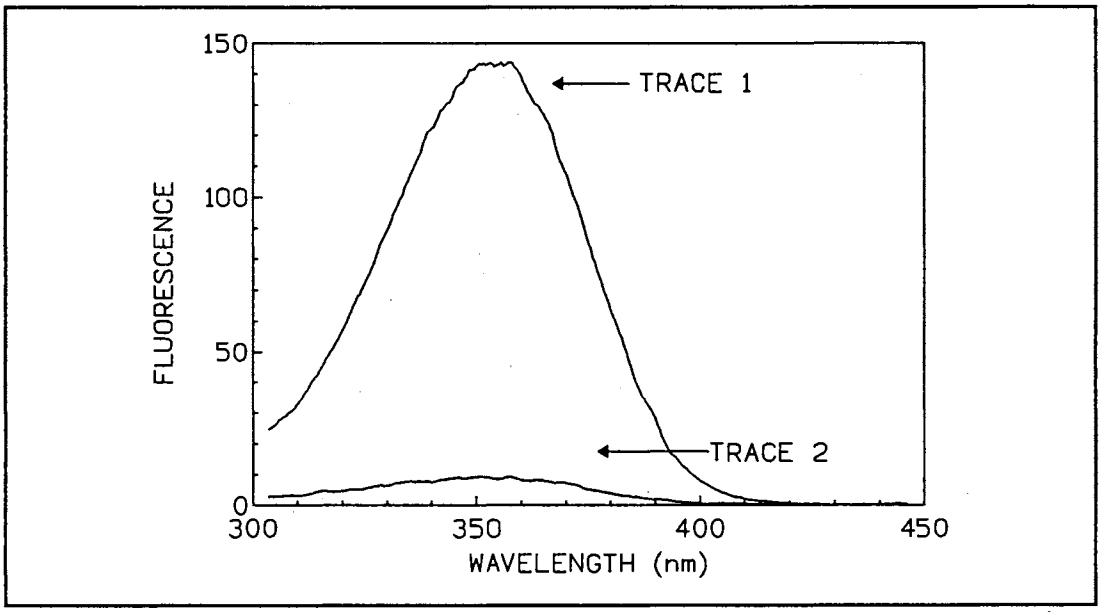

Figure 2. Measurement of Hoechst 33258 fluorescence with calf thymus DNA. Trace 1: 10 μg/ml DNA, 20 μg/ml Hoechst 33258.
Trace 2: 20 μM Deoxyibonuclease I added to sample.

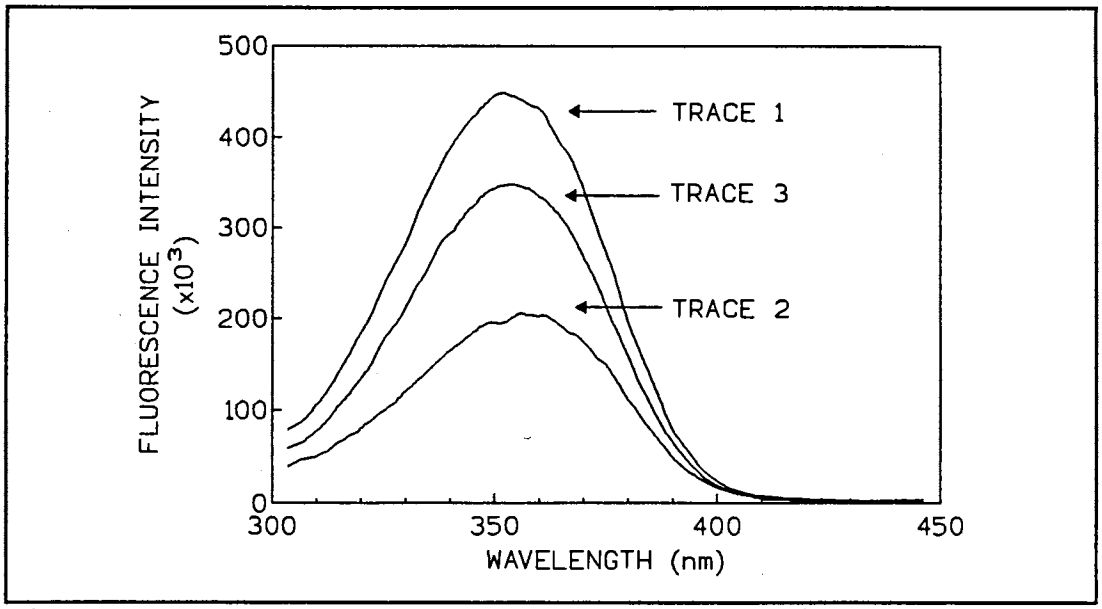

Figure 3. Hoechst 33258 fluorescence with calf thymus DNA.
Trace 1: 10 μg/ml DNA, 20 μg/ml dye.
Trace 2: Calf thymus chromatin
Trace 3: 2 M NaCl added to chromatin sample, incubated 30 minutes.

only. Upon addition of DNAse I, a decrease in the fluorescence of Hoecht 33258 was measured (Figure 2, Trace 2). This indicates that when DNA is cleaved by an enzyme, the effect can be detected by Hoechst 33258, resulting in a decrease in fluorescence. The fluorescence of the dye is greatly diminished in the presence of reconstituted chromatin (Figure 3, Trace 2), indicating that a number of the dye's binding sites on the DNA are not accessible. Addition of 2 M NaCl (Figure 3, Trace 3) to calf thymus chromatin causes the DNA to dissociate from the histone protein (Labarca and Paigen, 1980), thereby exposing additional dye binding sites on the DNA; the fluorescence increases to approximately half that of double stranded DNA alone (Figure 3, Trace 1). An increase in fluorescence of

Hoechst 33258 would be expected if chromatin is exposed to an agent that disrupts its structure and exposes additional dye binding sites without cleaving the DNA.

Figure 4 is representative time-resolved data for calf thymus DNA in FETAX solution. Hoechst 33258 was added 4 seconds after the start of data acquisition. Rate constants for fluorescence increases were calculated to be 550 msec. (± 0.15) for DNA incubated in FETAX alone, 600 msec. (± 0.21) for DNA incubated in hydroxyurea, and 350 msec (± 0.3) for DNA incubated in actinomycin-D. The rate-constant is indicative of the rate at which the dye reaches its maximum fluorescence level upon DNA binding. If fewer DNA binding sites are exposed to Hoechst 33258, the maximum fluorescence would be reached on a much faster time scale.

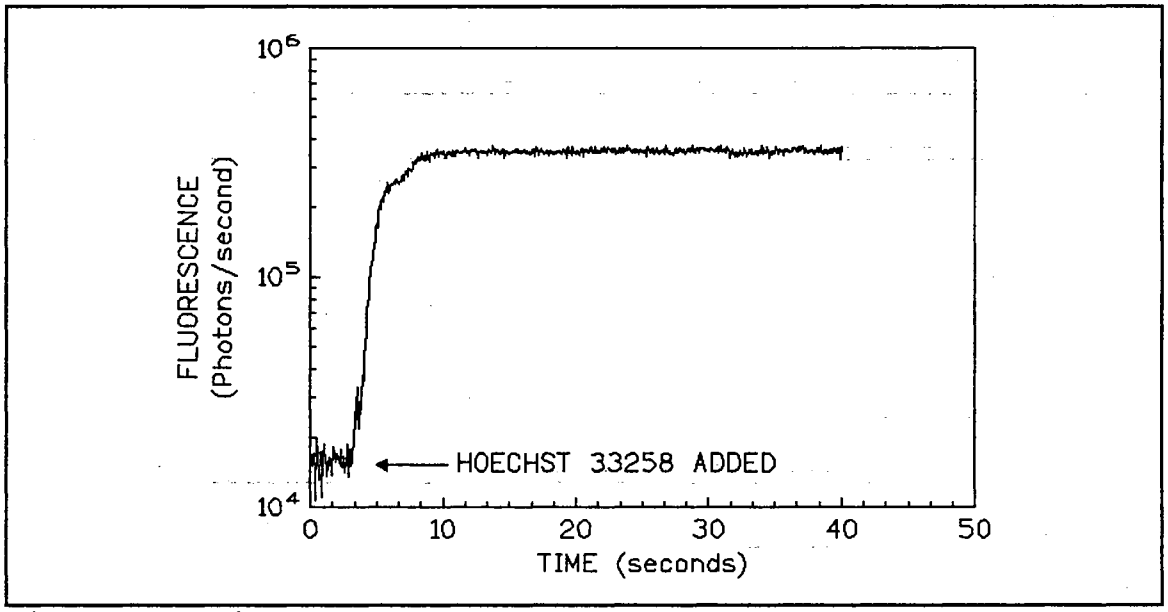

Figure 4. Time-resolved fluorescence measurements of Hoechst 33258 in the presence of calf thymus DNA. Data represents an exponential fluorescence increase due to Hoechst 33258 binding to the DNA.

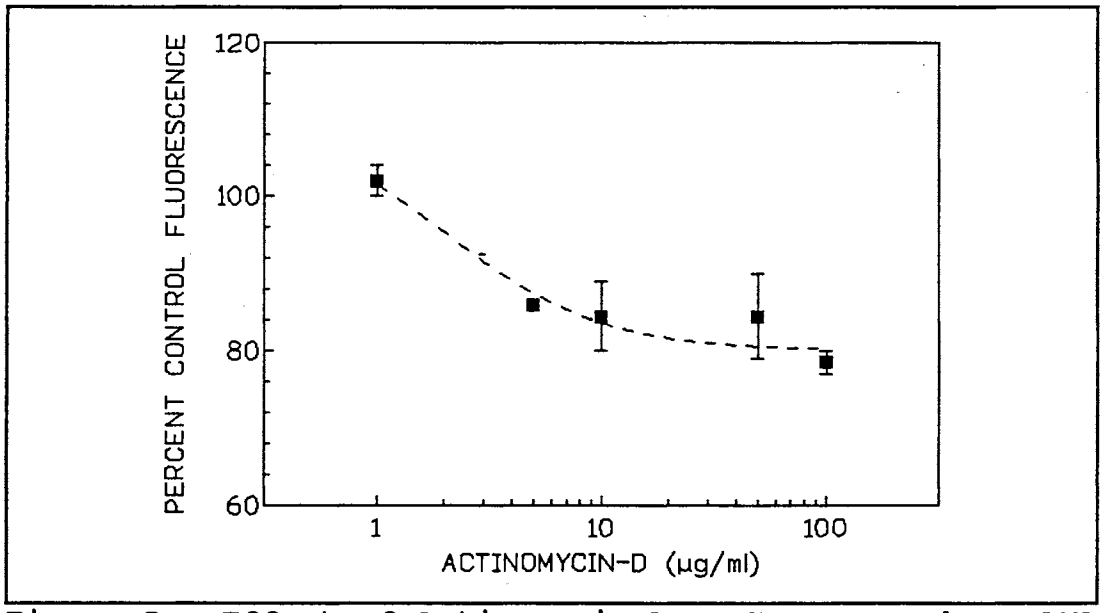

Figure 5. Effect of Actinomycin-D on Xenopus embryo DNA as measured by the fluorescence of Hoechst 33258. Concentration vs. percent of control fluorescence.

Figure 5 is a plot of actinomycin-D concentration vs. fluorescence of Hoechst 33258. As actinomycin-D concentration increased, the fluorescence decreased; the calculated EC50 was 6.34 µg/ml. The fluorescence decrease observed in the presence of actinomycin-D can be interpreted as a decrease in the number of Hoechst 33258 binding sites. The decrease in the number of binding sites in the presence of actinomycin-D suggests two possible mechanisms. One, a decreased amount of DNA synthesis, via a decrease in mRNA synthesis, (Boggaram et. al., 1991) and two, actinomycin-D and Hoechst 33258 are competing for the same binding sites. Since the rate constant for dye binding in the presence of actinomycin-D is approximately twice as fast as the rate constant in the absence of the compound, the data suggests that there are fewer DNA binding sites available for Hoechst

33258, due to actinomycin-D binding to the DNA as described by Jones et.al., 1993. That actinomycin-D cleaves the DNA, thus causing decreased dye fluorescence, can not be ruled out entirely. However, Basu et al., (1990), indicate that actinomycin-D shifts the melting curve for calf thymus DNA; as a result, an increase in the T_m is observed. They concluded that the drug is stabilizing the DNA helix. If the drug cleaves the DNA, a decrease in T_m would be expected.

We conclude that the changes in fluorescence of the dye are most likely due to actinomycin-D binding to the DNA. However, the EC50 for fluorescence decrease (6.34 μg/ml) is in the same range as the EC50 for malformation calculated for this compound using the FETAX assay (2.17 μg/ml) (Courchesne and Bantle, 1985). Although the CHAWQ assay alone cannot differentiate between DNA-toxicant binding effects and reduction in DNA synthesis, the change in fluorescence indicates that it can measure an interaction of a chemical with embryonic DNA.

Incubation of Xenopus embryos in hydroxyurea for 18 hours resulted in a biphasic fluorescence response of Hoechst 33258 as shown in Figure 6. The biphasic fluorescence data suggests that what is being measured is both a decrease in dye binding sites and an increase in dye binding sites. At low concentrations, a decrease in fluorescence is measured; at higher concentrations, an increase in fluorescence is measured. Two EC50 values were

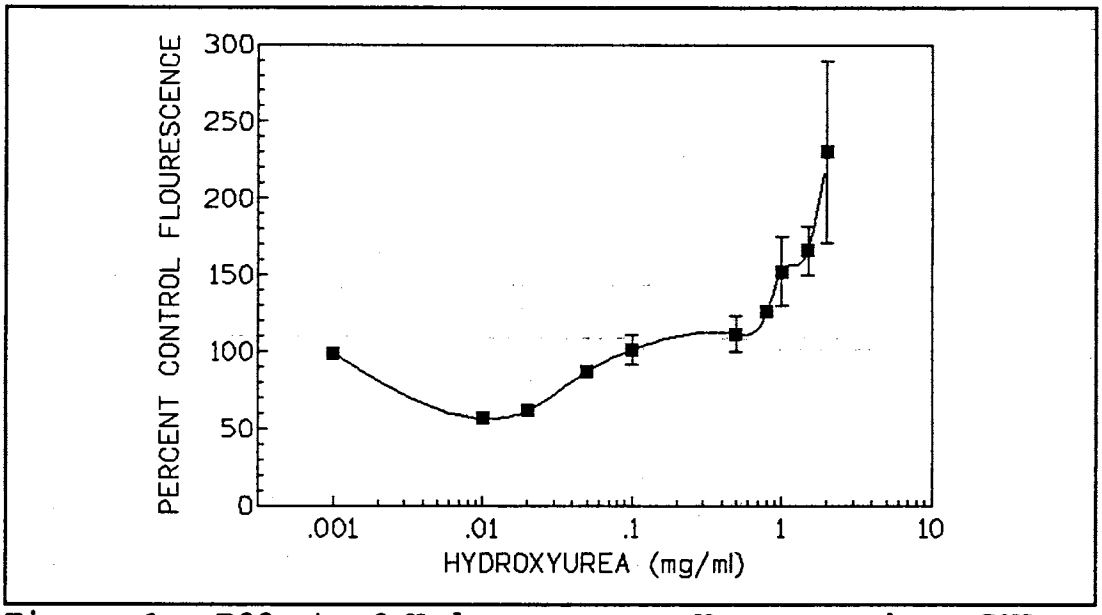

Figure 6. Effect of Hydroxyurea on Xenopus embryo DNA as measured by the fluorescence of Hoechst 33258. Concentration vs. percent control fluorescence.

calculated; the first EC50 of 0.04 mg/ml corresponds well to the EC50 for malformation for hydroxyurea using the FETAX assay (0.35 mg/ml), whereas the second EC_{50} of 1.4 mg/ml corresponds to the calculated LC_{50} using FETAX (1.82 mg/ml) (Courshesne and Bantle, 1985). We propose that low concentrations of hydroxyurea are inhibiting DNA synthesis (smaller fluorescence) and higher concentrations are compromising the structural integrity of the chromatin. That the decrease in fluorescence of Hoechst 33258 (in the presence of low concentrations of hydroxyurea) is caused by hydroxyurea binding to the DNA can be somewhat ruled out. Time-resolved fluorescence data indicate that the rate constant for Hoechst 33258 binding to calf thymus DNA is 550 msec for the control and 600 msec for 2.0 mg/ml hydroxyurea. This indicates that the fluorescence change measured is

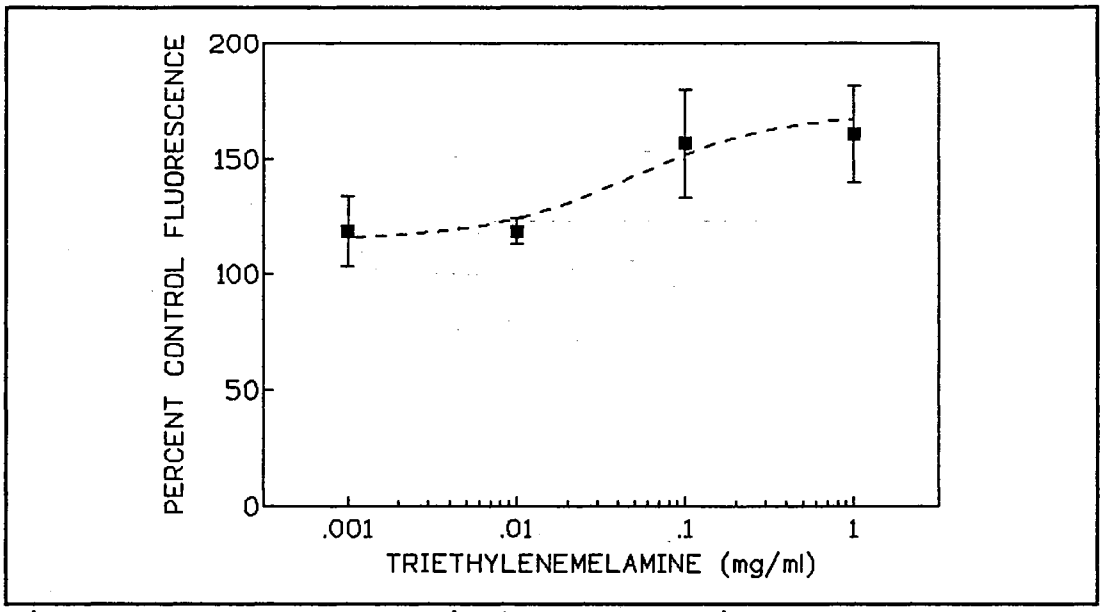

Figure 7. Effect of Triethylenemelamine (TEM) on Xenopus embryo DNA as measured by the fluorescence of Hoechst 33258. Concentration vs. percent control fluorescence.

probably not due to hydroxyurea binding to the DNA in such a way as to compete for Hoechst 33258 binding sites.

Figure 7 shows the results of an experiment wherein Xenopus embryos were incubated for 4 hours in the presence of various concentrations of TEM. The calculated EC50 for fluorescence increase was 0.1 mg/ml.

Calf thymus DNA was incubated with Hoecht 33258 for 15 minutes as shown in Figure 8, Trace 1. Trace 2 is the excitation spectra of Hoechst 33258 incubated with calf thymus chromatin. TEM was added to the chromatin sample at a concentration of 0.1 mg/ml and incubated for 15 minutes. Trace 3 is the excitation spectra of the TEM treated chromatin. The fluorescence intensity increases to about half that of the double-stranded DNA in Trace 1. The increased fluorescence of Hoechst 33258 in the presence of

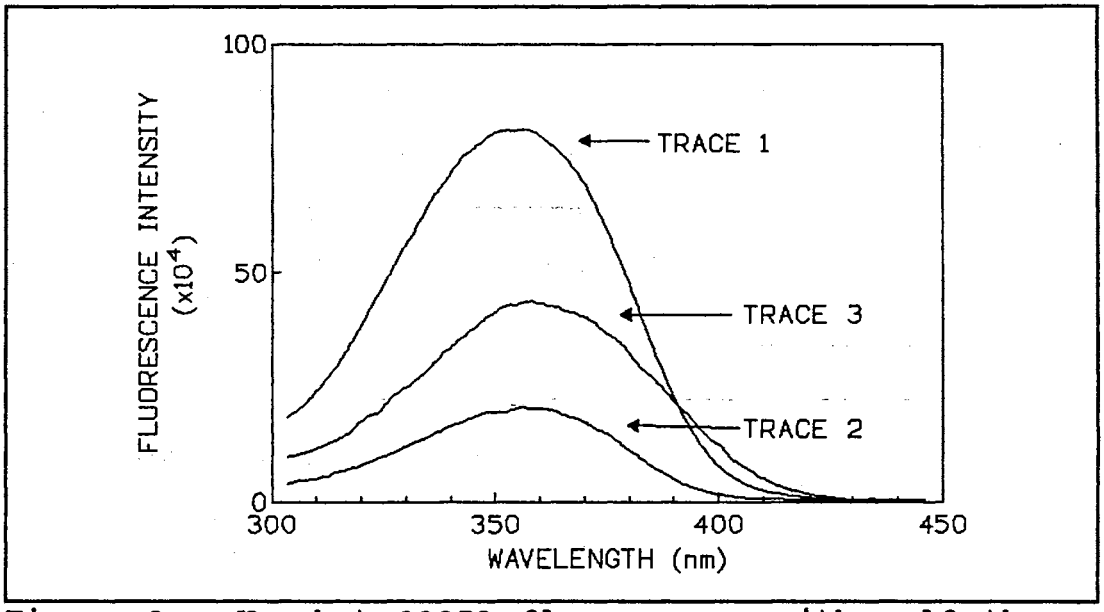

Figure 8. Hoechst 33258 fluorescence with calf thymus DNA.
Trace 1: 10 μg/ml DNA, 20 μg/ml dye.
Trace 2: Calf thymus chromatin
Trace 3: 0.1 mg/ml TEM added to chromatin sample. Incubated 30 minutes.

TEM in both embryo and in calf thymus chromatin experiments indicate that TEM is acting to alter chromatin structure. If TEM were cleaving the DNA or inhibiting synthesis, a decrease in fluorescence would be observed.

We have shown that it is possible to detect changes in DNA in the presence of compounds known to cause numerous types of malformations via a variety of mechanisms, acting either directly or indirectly on DNA. This study provides a framework with which to test other compounds and to assess their ability to act as genotoxins. The analysis of the proposed mechanisms for the three compounds used in this study are derived from what is already known about these

compounds and by observing the change in direction of the fluorescence of Hoechst 33258.

The DNA endpoint of CHAWQ is designed to test the effect of compounds on DNA; the endpoint is not designed to determine exact mechanisms of genotoxic compounds. Fluorescence changes observed using Hoechst 33258 can be of two types, either an increase or a decrease. The experiments in this study using isolated DNA and chromatin were designed to determine under what set of conditions an increase or decrease in fluorescence would be measured. We can conclude from this study that increases in fluorescence in the presence of genotoxins are most likely due to compromised chromatin integrity (exposing additional probe binding sites), although fluorescence enhancement of Hoechst 33258 through interaction with a toxicant can not be ruled out entirely. Fluorescence decreases can be caused by 1). decreased DNA synthesis, 2) cleavage or degradation of the DNA helix, or 3) binding of compounds to the DNA in such a manner as to compete for Hoechst 33258 binding sites, or 4) Hoechst 33258 binding sites could be altered by the toxin in such a way as to quench the fluorescence of the probe without necessarily competing for probe binding sites. However, whether an increase or decrease in fluorescence is measured in the presence of suspected genotoxic compounds makes little difference with respect to the ability of the CHAWQ assay to detect a compounds ability to interact with DNA. Any fluorescence change of Hoechst 33258 indicates

some type of an effect on, or interaction with, embryonic DNA that could result in malformation or death of the animal at a later time in the life cycle.

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CHAPTER VI

CONCLUSIONS

This thesis describes the development of the Cell Health Assay of Water Quality-CHAWQ. The preceding chapters have described the rationale, instrumentation, experimental protocol, and representative data for several CHAWQ endpoints.

From the beginning, CHAWQ was designed to be used as a rapid test for water quality as well as an assay to screen for chemicals that might be toxic to an organism. The Xenopus embryos used in the study were chosen as the test organism chiefly because they were readily available and toxicity data was available for comparison to CHAWQ; although, any number of organisms can be used such as Daphnia, fish embryos, or even mammalian cell lines.

We compared our data to the FETAX assay data only because we were using Xenopus embryos and there was a wealth of FETAX data available. However, unlike the FETAX assay, CHAWQ is not a embryological development assay.

The comparisons made between CHAWQ EC50's and FETAX EC50's in this study are loose at best. This study only attempted to design the assay and examine the type of data obtained from it; the way is now paved for more rigorous

validation and comparison to not only FETAX, but also to other acute and chronic bioassays.

CHAWQ is designed to measure several cellular endpoints. As a result, the assay will provide evidence for a broad range of cellular effects of a toxicant. The mechanism can be loosely defined as an effect on membrane potential, DNA, calcium levels, intracellular pH, etc. However, the assay alone can not define the exact mechanism of these changes without using other experimental techniques. In spite of this limitation, the assay does what it was conceived to do, namely, perform a rapid evaluation of toxicity of a chemical.

The rapidity of the test can not be overstated. Using the experimental technique designed in this study, one can perform the toxicity evaluation of a chemical from start to finish in less than two days; if short incubation periods are used (30 minutes to one hour), the screening procedure can be completed in one day.

The future for the CHAWQ assay looks very bright. However, as with any new assay, CHAWQ has allowed us to ask many more scientific questions than the assay is capable of answering. The data opens many intriguing avenues of experimentation to elucidate toxicity mechanisms suggested by the CHAWQ data. For instance, why is the membrane potential altered by certain compounds and not others? Ion transport is certainly altered, but using the CHAWQ assay

alone can not discover what ion transport mechanisms are being affected.

With further validation and more rigorous data comparison to other toxicity tests, CHAWQ could become an indispensable piece of equipment in a toxicology laboratory and/or an environmental testing facility.

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