INTERACTION OF GLYCOALKALOIDS AND PTERIDINES WITH FROG EMBRYO MEMBRANES

By

MISTY LEA MCWILLIAMS

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Oklahoma State University

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WITH FROG EMBRYO MEMBRANES

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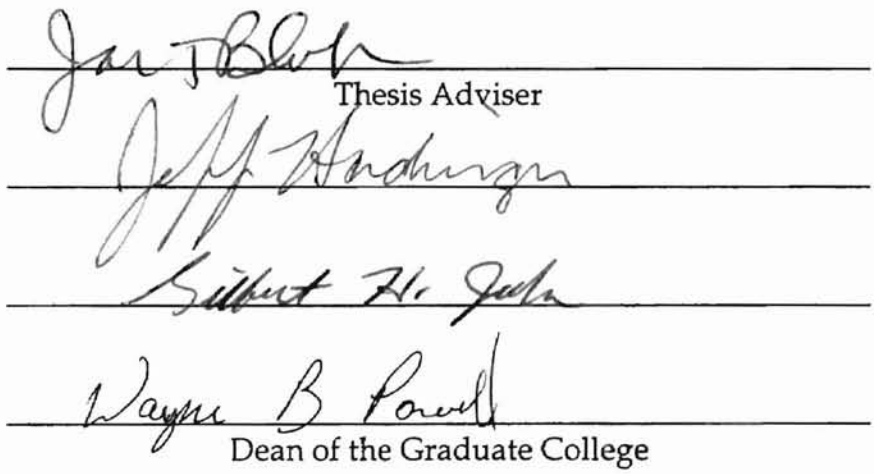

Thesis Adviser

Dean of the Graduate College

PREFACE

This study was conducted to characterize (1) the toxicity of glycosides, compounds naturally occurring in eggplant, on *Xenopus laevis* embryos; (2) the interaction of glycoalkaliods, compounds found in potatos, with pteridines, compounds in the family including folic acid, with *Xenopus laevis* embryonic membranes.

I would like to thank my major advisor, Dr. James T. Blankemeyer, for outstanding supervision, guidance, and hamburgers. I would like to extend my appreciation to my other committee members, Dr. Gilbert John and Dr. Jeff Hadwiger. I would also like to thank Dr. Mendel Friedman of the USDA, Albany, CA for his financial support over the last two years and Mrs. Cathy Butchko for her technical support.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Overview of Study

Scope and purpose. Toxicology concerns itself with the study of the impact of the chemicals, natural and 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 and living materials is of vital importance.

This study investigated the potential toxicities of two related and relatively unstudied plant secondary metabolites plus the interaction of another secondary metabolite and pteridine compounds. I compared the toxicity of solamargine and solasonine to other glycoalkaloids plant metabolites (Blankemeyer et al, 1992, 1997; Keeler et al., 1991). Previous work found that folic acid, a B-vitamin and member of the pteridine family, was able to counter the effects of α-chaconine, a potato secondary metabolite (Friedman et al., 1997). I evaluated the interaction of α-chaconine, a known toxin (Blankemeyer et al., 1992), with pteridine compounds for similar activity.

Evaluation of potential toxicity and/or interaction with known toxins was achieved with a combination of methodologies. Early stage toxicity was determined using the membrane potential of the embryos, measured by an electrochromic dye, Di-4-ANEPPS (Blankemeyer et al., 1993). It was also used to

detect if a compound has the ability to protect the cell and/or tissue from a toxin (Blankemeyer et al., 1992, 1997; Friedman et al., 1997). As previous work has shown (Blankemeyer et al., 1992, 1997; Friedman et al., 1997), the procedure is easily performed and offers results within a few minutes to a few hours. Extended exposure toxicity was evaluated using the FETAX protocol (ASTM, 1991).

Introduction to the Three Studies

Solamargine and Solasonine Glycoalkaloids Disrupt Cell Membranes and Induces Malformations in Frog Embryos

Origin of the Compounds. Solamargine and Solasonine are secondary metabolites found in many of the Solanaceae family of plants, primarily in eggplant (Aluned, 1996), and classified as glycoalkaloids. They are often called solasodine glycosides because they share the common aglycone, solasodine, while differing in the carbohydrates attached to the aglycone.

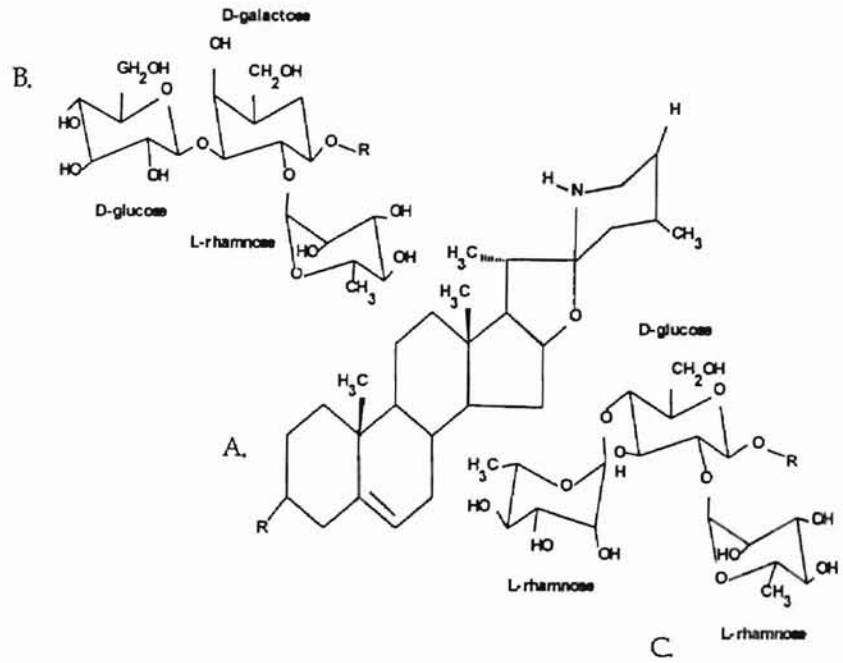

Figure 1. Structures of A) Solasodine component, B) solasonine carbohydrates, and C) solamargine carbohydrates.

Prior research of Glycosides. Potato glycoalkaloids have been extensively studied in contrast to solamargine and solasonine. The eggplant glycoalkaloids have been reported to induce embryotoxicity and teratogenicity in hamsters and effect the central nervous system of humans (Keeler et al., 1991; Vohora et al., 1984). Further

studies have found the disruption of cell membranes (Fewell et al., 1994; Roddick et al., 1990, 1992) and beneficial effects in skin cancer research (Cham et al., 1991).

Study in Brief. The two glycosides were evaluated for their toxicity in the early stage of *Xenopus laevis* development as well as after 96 hours of exposure. Solamargine and solasonine were evaluated for properties at pH 8 and 6. The relative toxicities were determined for early stage and 96 hour effects. The EC50 (membrane) dosage induced a change in fluorescence that represents 50% of the total effect. The EC50 (96 hours) dosage resulted in 50% mortality whereas the LC50 (96 hours) corresponds to the dosage that induced 50% of the total malformations occurring in the surviving subjects.

The Interaction of L-Monapterin and Potato Glycoalkaloids with Xenopus laevis.

The Glycoalkaloid, α-Chaconine. α-Chaconine, a secondary metabolite synthesized by potatoes (*Solanum tuberosum*), consists of a solanine aglycone with carbohydrates attached. The moiety is composed of two rhamnose and one glucose. α-Chaconine may be capable of inducing developmental abnormalities, including neural tube defects (Morris and Lee, 1984; Keeler et al., 1991) ; and disruption of membranes (Blankemeyer et al., 1992).

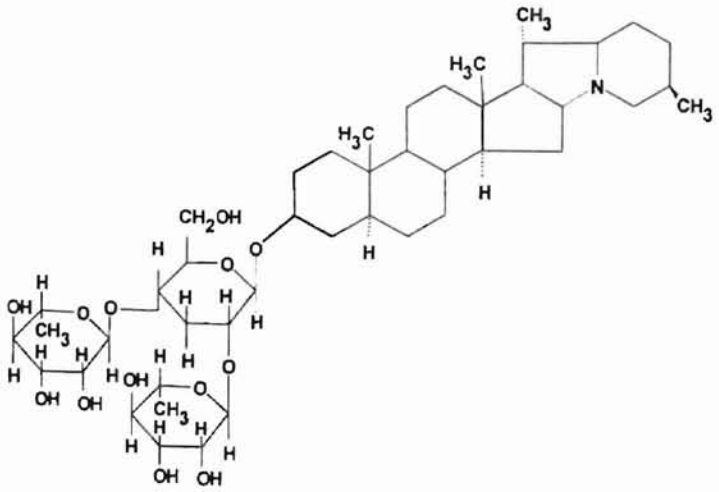

Figure 2. Chemical Structure of α-Chaconine.

The Pterin, L-Monapterin. L-Monapterin, a pteridine ring with a trihydroxypropyl chain, is synthesized by microorganisms such as *Echerichia coli* and *Serratia marcescens* (Klein, 1993). The release of L-monapterin from *E. coli* has been associated with the switch from logarithmic growth phase to the stationary phase. This peak of production is

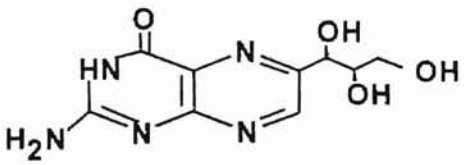

Figure 3. Chemical Structure of L-monapterin.

linked to its function as an intercellular messenger and regulator of growth by activation and inactivation of inhibitory factors such as repressors and chalones (Wachter et al., 1983).

The Pterin, Folic Acid. Folic acid is a B vitamin found in leafy greens, vegetables, legumes, nuts, whole grains, and brewer's yeast. Folic acid is known to break down homocysteine, a serum amino acid (AHA, 1997). Individuals having low blood levels of folic acid show an increase risk of fatal heart attack and stroke (Morrison et al., 1996). As yet, however, there has been no treatment study linking folic acid supplements to reduced atherosclerosis according to the American Heart Association (1997). Folic acid deficiencies have also been linked to birth defects involving neural tube closures and carcinogenesis (Finnell et al., 1998).

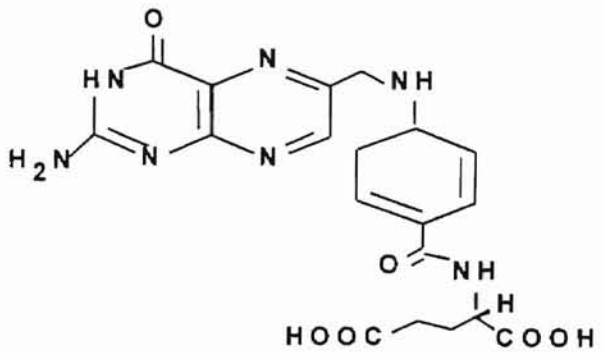

Figure 4. Chemical Structure of Folic Acid.

Prior Research with α-Chaconine and Pterins. Previously, our laboratory showed (Friedman et al., 1997) that the toxic effects of α-chaconine on the membrane potential, mortality and malformations of *Xenopus laevis* was reduced by folic acid. The potato glycoalkaloid/folate combination was found to hyperpolarize the strong deplorarization of the embryonic membrane caused by glycoalkaloid treatment alone.

The α-Chaconine/L-Monapterin study, in brief. L-Monapterin was analyzed for its ability to protect against the activity of α-chaconine. Both spectrofluorometric and survival data (mortality and malformation) is reported. Pre- and post- α-chaconine treatments in relation to L-monapterin treatments are provided to determine any variation in activities (spectrofluorometricly based).

Relationship Between Methotrexate and α-Chaconine:

Effects on Membranes of Early Stage Xenopus laevis Embryos.

The Glycoalkaloid, α-Chaconine. Work performed by Dr. James Blankemeyer and associates has successfully illustrated the effects of α-chaconine on the membrane potential, mortality, and malformation rate of *Xenopus laevis* (Blankemeyer et al., 1992; Rayburn et al., 1995a). It was also determined that disruption of sodium active transport or some interaction with sodium channels occurred in frog skin exposed to α-chaconine (Blankemeyer et al., 1995).

The Folate Analog, Methotrexate. Methotrexate, 4-amino-4-deoxy-10-methylfolic acid, is known to inhibit the growth of bacteria, fungi, and a number of parasitic protozoa (Blakely, 1969). *Rana pipiens* (Blakely, 1969) and *Xenopus laevis* (Bantle et al., 1990) embryos do not develop past the blastula stage when exposed to high levels while lower levels of methotrexate produce abnormalities it the regions of the embryo exhibiting high mitotic activity (Blakely, 1969).

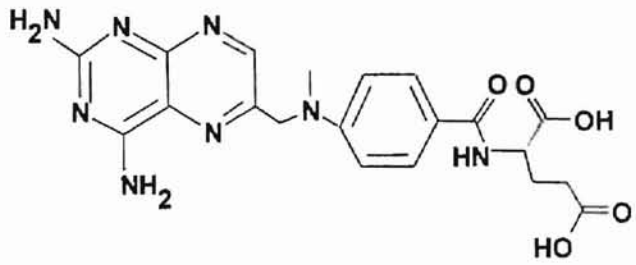

Figure 5. Chemical Structure of Methotrexate.

Methotrexate also inhibits the activity of dihydrofolate reductase resulting in a block in folate and dihydrofolate conversion to tetrahydrofolate and its derivatives (Rosowsky, 1987). The resulting effect is similar to a folate deficiency but the crucial effect is the repression of thymidylate synthesis so DNA

synthesize can not be maintained (Blakely, 1969). In rapidly proliferating cells, the arrest of cell division can have lethal effects.(Blakely, 1969) With its activities, methotrexate is administered as an chemotheraputic agent as well as an antimicrobial agent. The cytotoxicity of the drug limits its use in this manner (Rosowsky, 1988).

Prior Research with Methotrexate. Bantle et al. (1990) reported that methotrexate has the potential to be teratogenic since it produced up to 50% reduction in overall size and induced miscoiling of the gut at concentrations >20 mg/L of *Xenopus* embryos after 96 hours. Methotrexate's TI (LC50/EC50) value was 11.5; scores below 1.5 are considered to be non-teratogenic (Bantle et al., 1990).

The α-Chaconine/Methotrexate study, in brief. Methotrexate was analyzed for its ability to prevent depolarization of the *Xenopus laevis* embryonic membrane by α-chaconine. Previous work by Bantle et al. (1990) showed that prolonged exposure to methotrexate generated malformations in the embryos. Therefore, this study only addressed the effects within the first 24 hours. This study also evaluated the effects of α-chaconine in relation to pre-treatment, post-treatment, and continuous exposure to methotrexate.

THE METHODOLOGY

Spectrofluorometry

Basic concepts. Fluorophores release photons of light upon exposure to light of a certain wavelength. This fundamental light adsorption and emission property serves as the basis for this spectrofluorometry. When a fluorophore absorbs the excitation energy, an electron is elevated from the ground state to a higher electronically excited state. The energy absorbed by the fluorophore is not only used in the elevation of an electron to a higher energy level, but is also transformed into vibrational modes. Since, the excited state fluorophore is in contact with surrounding solvent molecules, it gives up energy causing the excited molecule to step down the ladder of vibrational levels, releasing energy as fluorescent emissions. Therefore, the use of fluorescent dyes in biological studies relies on a fundamental energy transition of the dye molecules (Johnson, 1996).

The fluorophore used in this study is an electrochromic dye, Di-4-ANEPPS, 1-(3-sulfonatopropyl)-4-[P-[2-(di-n-butylamino)-6-naphthyl]vinyl] pyridinium betaine, which fluoresces in response to a change in membrane potential. Di-4-ANEPPS becomes embedded into the membrane where it responds to the membrane potential (Haugland, 1996).

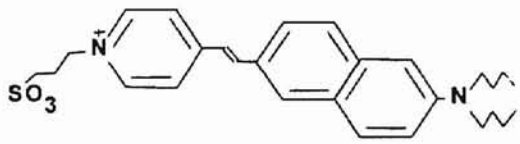

Figure 6. Chemical Structure of Di-4-ANEPPS.

The influx or efflux of ions across a membrane can significantly affect a cell's normal physiology, structure, and viability by altering the electrochemical gradient. The monitoring of changes in membrane potential by Di-4-ANEPPS predicts the toxicity of a compound resulting from a change in the movement of ions. A change in ion flow suggest some change in membrane permeability (Darnell et al., 1990). In general, Di-4-ANEPPS increases its fluorescent emission as the cell depolarizes (Blankemeyer et al., 1995) or hypopolarizes (Darnell et al, 1990). In conjunction with the spectrofluorometric data, survival testing was performed as described in the *Standard Guide for Conducting the Frog Embryo Teratogenesis Assay - Xenopus* (FETAX) (ASTM, 1991). FETAX allows for the evaluation of mortality as well as developmental malformations due to exposure to test compounds from blastula to tadpole.

Overview of Procedure. Each trial consisted of at least three replicas of seventy-five albino *Xenopus* embryos. The exposure of embryos involved incubating in the test solution plus 2mg/mL Di-4-ANEPPS for thirty minutes. Next, the embryos were loaded into a microcuvette as previously described in Blankemeyer et al. (1993). The embryos were scanned with a dual-wave beam spectrofluorometer, using Delta Scan (Photon Technologies II, NC) software to record the data. Numerical calculations were performed on y-intercepts obtained from the linear fit of the ratio of scans made at optimal wavelengths (EX

470, 580; EM 620) for the dye. All data were normalized by division of average control fluorescence and expressed as percent of control.

Frog Embryo Teratogenesis Assay - *Xenopus*

Basic Concepts. The FETAX assay allows for the evaluation of development over a 96 hour period beginning in the blastula stage. *Xenopus laevis* breeding pairs were conditioned for 1 to 2 months prior to usage. Four hours prior to mating, both frogs were injected with human gonadotropin (HCG). Following successful amplexus, the eggs were collected and dejellied with 2% (w/v) cysteine adjusted to pH 8.1. The embryos were sorted according to viability with those in mid to late blastula stage being isolated for utilization. Embyros were maintained in FETAX solution (10.8 mM CaSO4, 0.4 mM NaCl, 1.2 mM $NaHCO_3$, 0.53 mM $MgSO_4$, 0.44 mM $CaSO_4$, 0.4 mM KCl, and 0.14 mM $CaCl_2$) at pH 8. The FETAX solution is adjusted to the appropriate pH for testing as to not change the final pH. Twenty-five embryos were exposed to 10 mL of solution of the compound(s) of interest. The solutions were changed daily to maintain concentrations and potency (some compounds are light and/or air sensitive) of the compounds. The embryos were incubated at a constant temperature to promote even development. The embryos were scored daily for survival (ASTM, 1991).

At the end of the 96 hours, the embryos were fixed with 3% formalin so they may be scored for malformations, as determined by the ASTM (1991) guide. Overall growth and organ malformations were recorded for each individual specimen.

CHAPTER 2

DEVELOPMENTAL TOXICITY OF SOLAMARGINE AND SOLASONINE GLYCOALKALOIDS IN FROG EMBRYOS

INTRODUCTION

Members of the Solanaceae family of plants synthesize secondary metabolites including glycoalkaloids and polyphenols, presumably to protect themselves against phytopathogens. These plants include potatoes, tomatoes, and eggplants (Friedman, 1997; Friedman and McDonald, 1997; Friedman et al., 1997). Solasonine and solamargine are two major glycoalkaloids found in eggplants (*Solanum melongena*) and exist in at least one hundred other Solanum species (Aluned, 1996; Aubert et al., 1989; Bajak et al., 1979; de Almeida and Rocca, 1995; Ehmke et al., 1995; El-Badaoui et al., 1996; El-Khristy et al., 1986; Jaggi and Kapoor, 1994; Neszrnelyi et al., 1988; Ridout et al., 1989). Structurally, these two glycoalkaloids have the same steroidal part of the molecule (aglycone), solasodine, but differ in the nature of the carbohydrate side chain. The trisaccharide chain attached to the 3-hydroxy group of solasonine has a branched α-L-rhamnopyranosyl-β-D-glucopyranosyl-β-galactopyranose (solatriose) structure. The corresponding trisaccharide of solamargine consists of a branched *bis*-α-L-rhamnopyranosyl-β-glucopyranose (chacotriose) structure. The two carbohydrate side chains are identical in structure to the corresponding side chains in the two major potato glycoalkaloids, α-chaconine and α-solanine. The

eggplant glycoalkaloids differ from those found in potatoes only in the structure of the steroidal part of the molecules.

There is widespread interest in inexpensive plant sources for the aglycone solasodine, formed on hydrolytic removal of the carbohydrate side chains from solamargine and solasonine, since it can be readily converted to 16-dehydropregnenolone, a key intermediate in the synthesis of steroidal medicines (Jaggi and Kapoor, 1994). Solasodine itself has antifertility properties in primates (Dixit et al., 1989) and induces a reversible liver enlargement in mice (Friedman et al., 1996).

Although biological and toxicological properties of potato glycoalkaloids have been studied extensively (Caldwell et al., 1991; Friedman and Henika, 1992; Friedman and McDonald, 1997), this does not appear to be the case for the two eggplant glycoalkaloids, often called solasodine glycosides. Reported studies include (a) induction of embryotoxicity/teratogenicity in hamsters (Keeler et al., 1991), effects on the central nervous system (Vohora et al., 1984), disruption of cell membranes (Fewell et al., 1994; Roddick et al., 1990, 1992), and beneficial effects in skin cancer (Cham et al., 1991).

Previously, we have shown that the potato and tomato glycoalkaloids alter the membrane potential of Xenopus laevis frog embryos and the active transport of sodium by frog skin (Blankemeyer et al., 1992, 1995, 1997). These results suggested that one possible mechanism of action of glycoalkaloids is direct or indirect effects on active transport across cell membranes.

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 of these are disrupted, the membrane potential across the cell will change. One way to measure the membrane potential is with the aid of a fluorescent dye, usually termed an electrochromic dye. One such probe, Di-4-ANEPPS, is a dialkylaminostyryl pyridinium sulfonate with both positive and negative charges. Its effectiveness as a probe is presumably due to its ability to intersperse in the membrane leaflet. It changes fluorescence directly in response to changes in membrane potential. Because alteration in membrane pumps and ion channels could explain glycoalkaloid toxicity at the cellular level, we also examined the effects of two eggplant glycoalkaloids on the membrane potentials, survival, and organ malformations of the South African Clawed Frog, *Xenopus laevis*. Our results show that the two glycoalkaloids differ in potency, presumably due to differences in the structure of the respective carbohydrate side chains.

MATERIALS AND METHODS

Test material

Solamargine was isolated from berries of *Solanum khasianum* Clarke (Weissenberg, 1979; Yaniv et al., 1981). Solasonine was obtained from Biosynth AG, Basel, Switzerland. α-Chaconine was isolated from potato sprouts (Friedman et al., 1993). Each compound produced a single peak on HPLC chromatograms (Friedman and Levin, 1992; Magrini et al., 1989; Vogel et al., 1990). Di-4-ANEPPS dye (99% pure) was obtained from Molecular Probes, Eugene, OR. The Albino Xenopus laevis frogs came from Xenopus-I, Ann Arbor, MI.

Methods

Frog 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 pH 8 FETAX solution which contained 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 $CaCl_2$ (Friedman et al., 1991).

The glycoalkaloids were dissolved in FETAX solution and Di-4-ANEPPS was dissolved in ethanol, as described previously (Blankemeyer et al., 1992;

Friedman et al., 1991). The embryos were evaluated either in the fluorescence assay for effects on cell membranes or in the frog embryo teratogenicity assay (FETAX) for survival and teratogenicity (ASTM, 1991; Bantle et al., 1991). The experimental setup for measuring fluorescence emanating from embryos loaded with Di-4-ANEPPS dye is described elsewhere (Blankemeyer et al., 1993, 1995, 1997). This protocol uses cellular dyes to probe the mode and mechanism of toxicity. Each concentration of glycoalkaloid was tested with at least three replicates of 75 embryos each per concentration. Each replicate was compared against the average of three control replicates, cultured with the experimental embryos. A positive control, α-chaconine, was used in at least one Petri dish of embryos in each trial to establish repeatable measurements from trial to trial. The FETAX protocol requires placing the embryos in plastic Petri dishes and treating duplicate replicates at each concentration, including negative and positive controls. The embryos were scored for survival and malformations. PROBIT analysis was used to generate the EC5O and LC5O values (Tallarida and Murray, 1990). Curves were fitted by non-linear regression using GraphPad (Los Angeles, CA). Iteration was used to find the best fit, employing a sigmoidal model.

RESULTS

Solasonine

Figure 7 shows the results from several experiments for solasonine at pH 6 and 8. Groups of embryos were tested to obtain fluorescence ratio of experimental to control values after exposure to several concentrations of the glycoalkaloid for 30 min. Each data point represents the average of two trials of three replicates each at each concentration. The data were plotted as percent of control fluorescence on the ordinate (with error bars denoting standard error) and concentration on the abscissa. Only solasonine concentrations of greater than 10 mg/L at pH 8 produced fluorescence significantly greater than the control fluorescence; differences were judged significant at P = 0.05. The maximum change in fluorescence was 310% of the control embryos at pH 8. The corresponding value at pH 6 was 130%. The increase in fluorescence represents a decrease in membrane potential

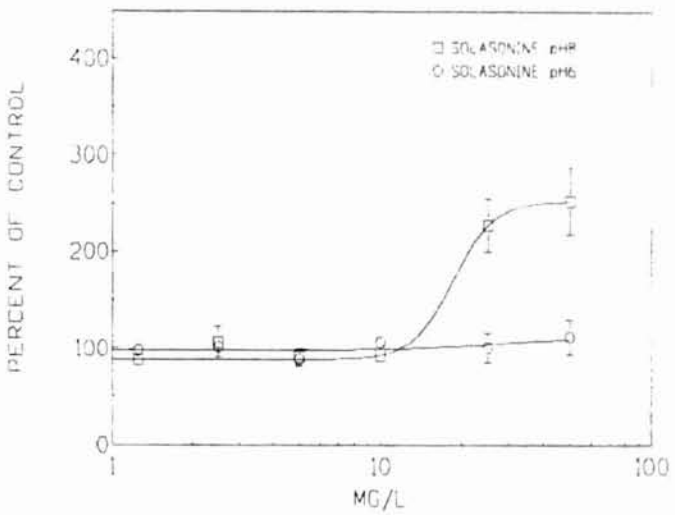

Figure 7. Effects of solasonine buffered at pH 6 and pH 8 on the membrane potential of albino *Xenopus* embryos. Data were from three trials of three replica of seventy-five embryos.

(hypopolarization) of cells in the embryo. The calculated EC5O (membrane) value for solasonine at pH 8 is 18.6 mg/L. The corresponding value at pH 6 is 26.6 mg/L.

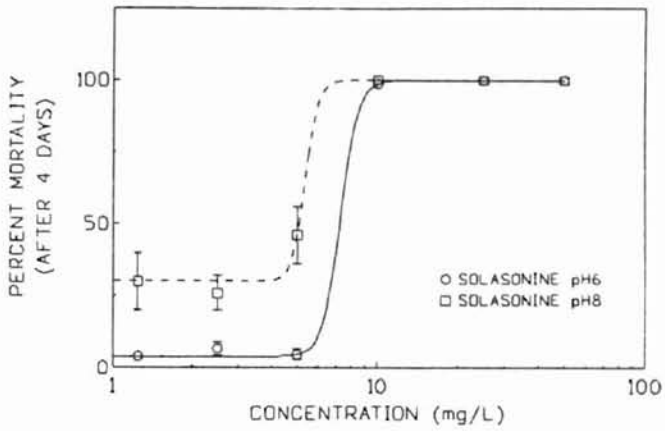

Figure 8. Effects of solasonine buffered at pH 6 and pH 8 on the survival of albino *Xenopus* embryos under the protocol of the FETAX assay. Data were three trials of three replicas of seventy-five embryos.

We also incubated *Xenopus laevis* embryos and determined the percent survival using the FETAX protocol. Figure 8 illustrates the results of this experiment at pH 6 and 8.

When the embryos were exposed to solasonine at pH 6, there was no mortality at a concentration of 5 mg/L. In contrast, at pH 8 mortality was almost 50% at this concentration. The calculated LC5O for pH 6 is 7.26 mg/L and for pH 8 is 5.35 mg/L. The latter value is identical within experimental error

Concentration (mg/liter)	Mortality (%)	Malformation (%)	(type)
Controls	4	5.2	gut
0.1	5	7.9	gut
0.5	8	5.4	gut
1.0	0	7.5	gut
1.5	8	10.8	gut
2.0	8	13.5	gut
2.5	10	16.7	gut
3	53	36.8	moderate*
4	98	100	severe*

* See text for definition.

Table 1. Solamargine concentration response for mortality and malformation in FETAX.

Compound	EC50 (mg/L)	LC50 (mg/L)	TI
Solamargine	2.43 (2.03-2.91)	2.88 (2.64-3.15)	1.19
Solasonine	5.06 (4.42-5.92)	5.86 (5.30-5.92)	1.11

Table 2. Comparison of developmental toxicity of solamargine and solasonine based on LC50 (lethality) and EC50 (malformation) value in FETAX.

with the value of 5.86 reported previously for solasonine (Friedman et al., 1992). The results show that solasonine is more toxic to frog embryos at pH 8 than at pH 6.

Solamargine

Figures 9 describes effects of solamargine on *Xenopus laevis* membrane potentials. Solamargine produced a 660% (of control) change in dye fluorescence at pH 6 with an EC5O (membrane) value of 8.2 mg/L. Lack of material precluded determining the corresponding values at pH 8.

Previously, we reported on the extent of organ malformations induced by solasonine in the FETAX assay (Friedman et al., 1992). The results were compared with those obtained in this study for solamargine (Tables 1 and 2). Two criteria were used to define the nature of malformations described in Table 1 (ASTM, 1991; Bantle et al., 1991). The first is the number of organ Systems affected. For a "moderate" designation shown in Table 1, three major organs must be malformed e.g. tail-kinking, gut coiling, and brain abnormality. Most organs must be affected for "severe" designation. The second criterion is based on a subjective numerical rating of the malformations ranging from 1 to 10. On this scale, the value assigned to a "moderate" malformation ranges between 3-5. Numbers above 5 describe a "severe" malformation.

DISCUSSION

The solasonine experiments demonstrated that the effect of pH on fluorescence in the membrane potential assay was similar to the corresponding effect in the embryo survival assay. Solasonine and solamargine proved to be more potent at pH 8 than pH 6 in both assays. Although the EC5O (membrane) and LC5O values differ, the embryos were exposed to solasonine for only 30 min. in the membrane potential assay compared to 96 hr in the embryo survival assay.

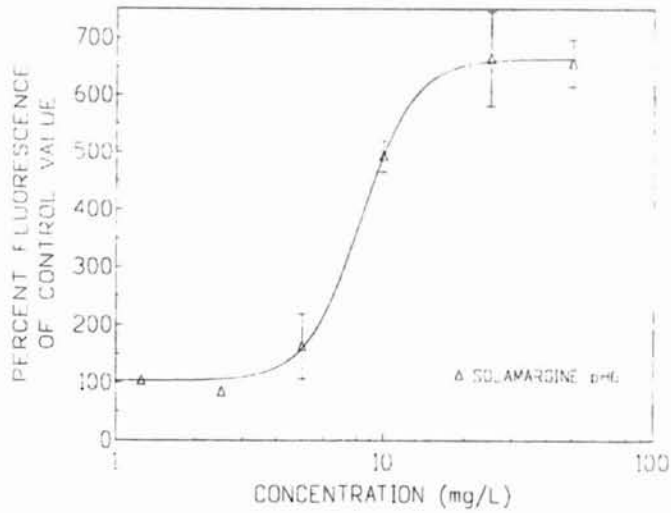

Figure 9. Effects of solamargine on the membrane potential of albino *Xenopus* embryos at pH 6. Data were two trials of three replicas of seventy-five embryos.

Since the pK value of the NH group of the terminal ring of the glycoalkaloids is near 6 (Ripperger and Schreiber, 1981), this group is partially protonated at pH 6 but not at pH 8. This implies that the greater observed potency of solasonine at pH 8 compared to 6 is probably due to the fact that the unshared electron pair of the unprotonated amino group (:NH) probably participates in binding of the glycoalkaloids to cell membrane receptor sites and that the protonated form probably does not. To add to this, one must remember that the pH change is global and it could be influencing more than just the

protonation of the glycoalkaloids. More studies are needed in support of this hypothesis. Solamargine at pH 6 generated more fluorescence intensity than did solasonine at either pH 6 or 8. It was also more lethal in the survival assay than solasonine. Tables 1 and 2 show that the teratogenic potency of solamargine is about twice that of solasonine. The results from both assays therefore suggest that solamargine is more embryotoxic than solasonine.

Since the extent of fluorescence increase in the membrane assay seems to parallel toxicity in the frog embryo survival assay, the membrane potential assay is apparently probing one of the primary effects of solasonine and solamargine on the survival and malformations of the embryos. Di-4-ANEPPS measures the membrane potential of cells in the developing embryo. 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 solasonine and solamargine change the characteristics of ion channels in the developing frog embryo. The membrane assay makes it possible to develop a better understanding of the molecular events governing the physiology and toxicology of glycoalkaloids.

It is instructive to compare the present findings with solasonine and solamargine to those reported earlier for the potato glycoalkaloids α-solanine and α-chaconine. The present results show that solamargine is more potent in disrupting cell membranes than is solasonine by a factor between 2 and 3. Since the two glycoalkaloids share the same steroidal aglycone (solasodine), this

difference presumably arises from the different structures of the carbohydrate side chains. With respect to the potato glycoalkaloids, our previous results indicated that α-chaconine was 3 to 4 times more potent than α-solanine. Since these two glycoalkaloids also share the same aglycone (solanidine), while the carbohydrate side chain of α-solanine corresponds to that of solasodine and that of α-chaconine to that of solamargine, the current findings reinforce our hypothesis about the key role of the carbohydrate moieties in influencing cell membrane disruptions and embryotoxic/teratogenic effects of glycoalkaloids (Rayburn et al., 1994). Evidently, the trisaccharide of solamargine in which two rhamnose units are connected to a glucose moiety binds more efficiently to cell receptor sites than the corresponding trisaccharide of solasonine in which one rhamnose and one glucose units are bridged by a galactose.

A main objective of these studies is to develop a relative potency scale of structurally different glycoalkaloids and metabolites to serve as a guide for the removal of the most toxic compounds from plant foods through plant breeding and plant molecular biology techniques (Moehs et al., 1997). This is a challenging problem because relative potencies of individual glycoalkaloids may not reflect their toxicities in foods containing more than one glycoalkaloid, in view of the apparent synergism observed with both potato and eggplant glycoalkaloids (Rayburn et al., 1995a; Roddick et al., 1994). A need exists to further define such synergism following oral consumption in order to obtain a more realistic assessment of possible risk associated with consumption of

glycoalkaloid-containing foods. Possible protective effects of nutrients against adverse effects of eggplant glycoalkaloids (Friedman et al., 1997; Rayburn et al., 1995b), and the applicability of the cell membrane assays to studies of mechanisms of action and inactivation of bacterial toxins, which like glycoalkaloids also disrupt the integrity of cell membranes (Louise and Obrig, 1995), also merit study.

CHAPTER 3

THE INTERACTION OF L-MONAPTERIN AND POTATO GLYCOALKALOIDS WITH *XENOPUS LAEVIS*

Introduction

Pteridines, including the B vitamin folic acid, are compounds that consist of a pterin ring structure plus a varying glutamic acid residue. Pteridines play roles in nature ranging from pigments to cofactors for numerous redox and one-carbon transfer reactions (Ayling, 1993). The first pteridines were isolated and studied in butterflies (Pfleiderer, 1993).

Folates, a class of pteridines, are essential in interconversion of amino acids and nucleotide synthesis (Gissen, 1993). Recent literature (Blount et al., 1997; Finnell et al., 1998; Morrison et al., 1996) and FDA dietary recommendations (Gissen, 1993) suggest that folate deficiency can result in anemia, central nervous birth defects such as *spina bifida* and other neural tube defects, elevated homocysteine levels resulting in premature vascular disease, and in some organisms, death (Gissen, 1993).

α-Chaconine, a secondary metabolite of solanaceous plants, has the ability to disrupt the membrane potential of developing *Xenopus laevis* embryos (Blankemeyer et al., 1992,1995). Previous studies have described protective effects of glucose-6-phosphate, nicotine adenine dinucleotide phosphate (NADP) (Rayburn et al., 1995), and folic acid against α-chaconine induced malformations (Friedman et al., 1997). Our data has shown that membrane potential produced

concentration dependent results similar to that of a full-scale FETAX assay (Friedman et al., 1991) for the folic acid and α-chaconine response.

The findings that folic acid can prevent malformations in *Xenopus laevis* has prompted further investigation into other pteridine compounds. Folic acid acts at concentrations above recommended nutritional levels. When investigating other work with pteridines, L-monapterin showed a strong chemotactic induction of the actin response in *Dictyostelium* development at 10 nM, whereas at concentrations below 100 nM, folate failed to induce a response (Tillinghast and Newell, 1987). This led to the investigation with L-monapterin to compare it with folic acid using potato glycoalkaloids and *Xenopus laevis* embryos.

L-Monapterin, consisting of a pteridine ring plus a trihydroxypropyl chain (see Figure 3), exists in plants, animal tissues, and some microorganisms. In bovine retinal pigment epithelium, monapterin was found to be the second most prevalent pterin (Gerding et al., 1993). L-Monapterin is synthesized by microorganism such as *Escherichia coli* and *Serratia marcescens* (Klein, 1993). In *E. coli*, this release of L-monapterin has been associated with the switch from logarithmic growth phase to stationary phase. This peak of production is linked to its function as an intercellular messenger and regulator of growth by activation or inactivation of inhibitory factors such as repressors or chalones (Wachter et al., 1983). In humans, L-Monapterin has been associated with detection of conditions such as urinary tract infection. The alternate isomeric

form, D-monapterin, has been associated with the urine of cancer patients (Ogiwara et al., 1992).

Materials and Methods.

Test Materials.

α-Chaconine was isolated from potato sprouts (Friedman et al., 1993). Folic acid was purchased from Sigma, St. Louis, MO and L-monapterin was obtained from Fluka, Ronkonkoma, NY. Di-4-ANEPPS dye (99% pure) came from Molecular Probes, Eugene, OR. The *Xenopus laevis* frogs were purchased from Xenopus-I, Ann Arbor, MI.

Methods

Frog breeding pairs were conditioned for one month to six weeks prior to breeding. Four hours prior to mating, the frogs were injected with human chronic gonadotropin, 0.25cc per 100mg weight (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. Embryos were maintained at pH 8 in FETAX solution which contained 10.8 mM NaCl, 1.2 mM $NaHCO_3$, 0.58 mM $MgSO_4$, 0.4 mM KCl, and 0.14 mM $CaCl_2$ (ASTM, 1991).

The glycoalkaloid and pterins were dissolved in FETAX solution and adjusted to pH 7. Di-4-ANEPPS was dissolved in ethanol, as described previously (Blankemeyer et al., 1992; Friedman et al., 1991). They embryos were evaluated either for fluorescence or survival and teratogenicity (ASTM, 1991).

The experimental setup for measuring fluorescence from embryos loaded with DI-4-ANEPPS is described in Blankemeyer et al. (1993, 1995, 1997). The protocol uses cellular dyes to probe the mechanism of toxicity; specifically, the change in membrane potential. For each concentration, at least three replicates of 75 embryos each were tested.

Figure 10 illustrates the alteration of fluorescence protocol to obtain time course (multiple treatment) measurements of toxicity. The embryos were incubated with Di-4-ANEPPS at a final concentration of 2mg/mL for 30 min. Each exposure of the embryos to the solutions, compound plus Di-4-ANEPPS (2mg/mL), started with a 4 minute dish incubation. Afterwards, the embryos were transferred to a microcuvette (Blankemeyer et al., 1993) containing the test solution to allow for a continuous exposure throughout data collection. The microcuvette was placed into the spectrofluormeter chamber for readings at

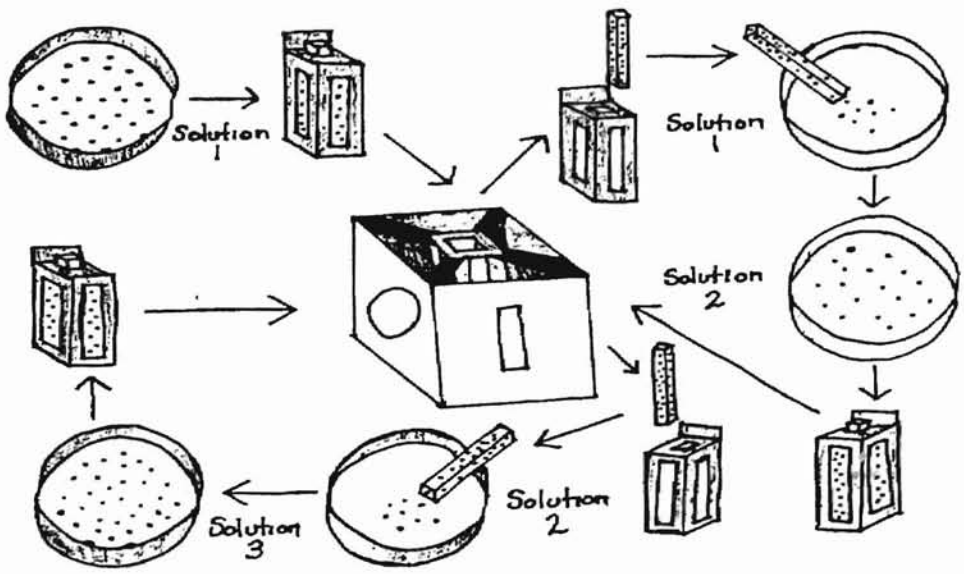

Figure 10. Illustration of time course treatment procedure.

defined time intervals. A continuous reading was not obtained since the compounds are photoreactive. Various combinations of pre-treatment and post-treatment were utilized to test the interaction of the glycoalkaloids and pteridines.

To test for survival and malformations, the FETAX protocol was utilized (ASTM, 1991). This involves placing 25 embryos into the appropriate concentration which was obtained by dilution of stock solution (50 mg/L for glycoalkaloids and 10 mg/L for monapterin) into FETAX solution. The solution was changed every 24 hours for 96 hours and then survival and malformations were scored.

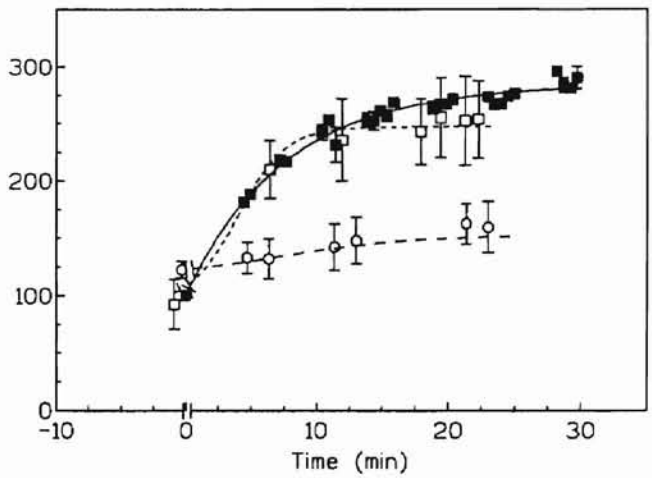

Figure 11. Time Course Fluorescence Assay. Negative values represent pre-treatment. Positive values represent post-treatment. The closed squares are 10 mg/L α-chaconine treatment alone. The open squares are pre-treatment of 10 mg/L α-chaconine then post-treatment of 10 mg/L α-chaconine and 0.5 mg/L L-monapterin. The open circles are pre-treatment of 0.5 mg/L L-monapterin then post-treatment of 10 mg/L α-chaconine and 0.5 mg/L L-monapterin. Data were three trials of 1 replica of 75 embryos. For pre-treatment of L-monapterin, one trial was unresponsive so data were ommitted from graph. Each point represents thirty seconds of data.

Data was fit into linear regressions using GraphPad (Los Angeles, CA). L-Monapterin is known to naturally fluorescence (Klein, 1993) although a test of fluorescence versus L-monapterin produced a negligible slope. That is, the fluorescence from L-monapterin was not a factor in our measurements.

Results

Figure 11 is the time course of embryo fluorescence with various monapterin treatments. Two modes of evaluation were set up to determine if pre monapterin or α-chaconine exposure caused a variation in the final fluorescence upon treatment with α-chaconine. At 0.5 mg/L L-monapterin and 10 mg/L α-chaconine, neither a pre-exposure of α-chaconine or L-monapterin produced a significant decrease (α=0.05) in fluorescence compared to α-chaconine alone.

Figure 12 indicates the survival upon exposure to L-monapterin and α-chaconine

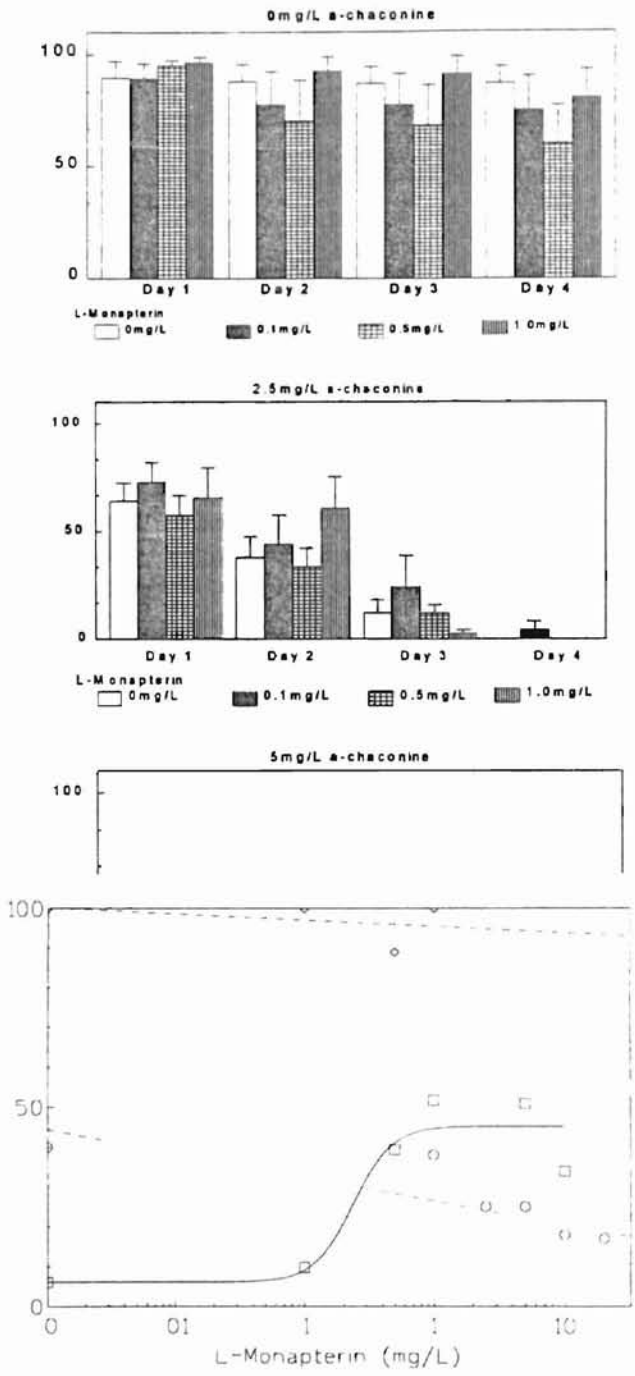

Figure 13. Percent Malformation of surviving population from FETAX assay after 96 hours. The open squares-solid line represents L-monapterin alone. The open diamonds-dash line represents 2.5 mg/L α-chaconine at the corresponding L-monapterin concentrations. The open circles-dash line is 2.5 mg/L α-chaconine and corresponding folic acid concentrations (Friedman et al., 1997).

over 96 hours. For the first 24 hours, L-monapterin seems to have a positive effect on the survival at concentrations ≤1.0 mg/L with the maximum effect being at 1.0 mg/L. Through 96 hours, 2.5 mg/L α-chaconine and 0.1 mg/L L-monapterin shows a 10% increase in survival over that of 2.5 mg/L α-chaconine alone. At concentration >0.1 mg/L, the survival is at or below that of 2.5 mg/L α-chaconine alone. This shift in maximum concentration indicates that higher concentrations of L-monapterin may have a secondary effect on developing embryos. This can also be seen among the malformation data in Figure 13. When the embryos were exposed to L-monapterin alone, at concentrations greater than 0.5 mg/L, the malformation rate is considerably higher than that of the controls with the majority of these malformations occurring in the gut. All other malformations accounted for only 16.2% of the total percentage. Folic acid and 2.5 mg/L α-chaconine resulted in a lower malformation rate than monapterin alone (figure 13).

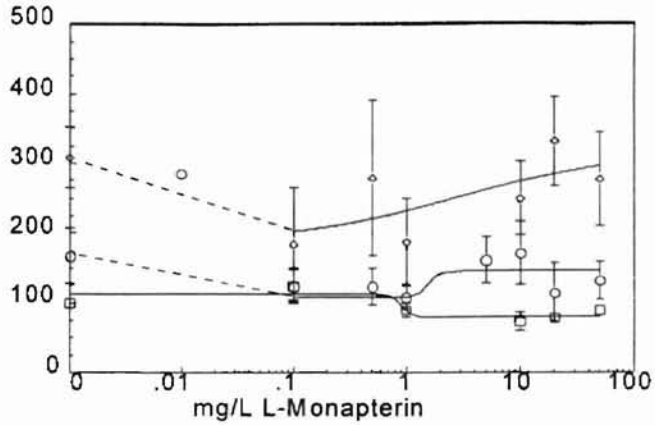

Figure 14. Fluorescence Assay. Data reported as percent of control fluorescence. Bars represent standard error. Open square is L-monapterin only, open circle is 5 mg/L α-chaconine, and open diamond is 10 mg/L α-chaconine.

Figure 14 shows the results of the single treatment fluorescence assays with α-chaconine and L-monapterin. All data collected within a concentration were averaged prior to standardization with averaged untreated data. The data

were plotted as percent of control fluorescence. A decrease in fluorescence is seen with 0.1 mg/L and 1 mg/L L-monapterin over 10 mg/L α-chaconine alone with EC_{50} of 2.87 mg/L. For 5 mg/L α-chaconine, 1 mg/L and 20 mg/L L-monapterin returned the fluorescence to that of the control with an EC_{50} of 1.53 mg/L. It is interesting to note that L-monapterin alone does produce a hyperpolarization for concentrations above 1 mg/L.

Conclusion

The fluorescence assays shows that L-monapterin has no significant effect on the depolarization of the membrane caused by α-chaconine. Neither the time course or the single treatment assays prevented the α-chaconine induced depolarization. Therefore, if the cells bind or uptake L-monapterin, it does not effect the binding of α-chaconine to the membrane (Keukens et al., 1995).

The survival data seems to indicated that L-monapterin also has a negative action. After 24 hours, the higher the concentration of L-monapterin the lower the relative survival and higher the malformation rate. This is not a surprising finding, in that many of the known folate analogs cause a block in folate metabolism. For example, methotrexate is a known inhibitor of dihydrofolate reductase which reduces folate into its precursor molecules. If L-monapterin is taking on this type of role then as the concentration increases, more inhibition of folate metabolism would occur. An extended block of folate metabolism can induce mutations in protein and nucleic acid synthesis which would ultimately lead to cell death.

Therefore, we can conclude that L-monapterin provides no significant effects against α-chaconine induced membrane disruption. Prolonged exposure leads to an equal and/or increased cell death and malformations as compared to α-chaconine alone.

CHAPTER 4

RELATIONSHIP BETWEEN METHOTREXATE AND α-CHACONINE: EFFECTS ON MEMBRANE POTENTIAL OF *XENOPUS LAEVIS* EMBRYOS

Introduction

The findings that folic acid can prevent malformations in *Xenopus laevis* has prompted further investigation into other pteridine compounds (Friedman et al., 1997). Methotrexate, 4-amino-4-deoxy-10-methylfolic acid (see figure 5), serves as an antibiotic and cytoxic drug in the treatment of cancer, autoimmune diseases, and psoriasis (Kamen, 1997). *Rana pipiens* (Blakley, 1969) and *Xenopus laevis* (Bantle et al., 1990) embryos do not proceed with development past the blastula stage when exposed to high levels with lower levels producing abnormalities in the regions of the embryo exhibiting high mitotic activity.

In man, methotrexate has earned a place in the treatment of an increasing number of diseases. It has an emerging role in the treatment of inflammatory bowel disease (Tang and Neuberger, 1996) and systemic lupus-erythematosus (Kipen et al., 1997). Methotrexate is an effective agent in the treatment of juvenile rheumatoid arthritis and is becoming widely accepted as the second-line agent of choice. In adult patients, MTX has been associated with significant morbidity, increased risk of infection, pulmonary toxicity, and development of lymphoma. Yet, in children, these toxicities are uncommon and treatment is well tolerated (Gottlieb et al., 1997). In some patients, MTX toxicity is associated with

preexisting folate deficiency. Folinic acid and folic acid supplementatin should be included with any MTX treatment (Shiroky, 1997).

Methotrexate interacts with multiple systems, including; inhibition of dihydrofolate reductase (Stone and Morrison, 1986), promotion of adenosine release, and inhibition of transmethylation reactions (Cronstein, 1997).

Materials and Methods.

Test Materials.

α-Chaconine was isolated from potato sprouts (Friedman et al., 1992). Folic acid and Methotrexate were purchased from Sigma, St. Louis, MO. Di-4-ANEPPS dye (99% pure) came from Molecular Probes, Eugene, OR. The *Xenopus laevis* frogs came from Xenopus-I, Ann Arbor, MI.

Methods

Frog breeding pairs were conditioned for one month to six weeks prior to breeding. Four hours prior to mating, the frogs were injected with human chronic gonadotropin, 0.25cc per 100mg weight (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. Embryos were maintained at pH 8 in FETAX solution which contained 10.8 mM NaCl, 1.2 mM $NaHCO_3$, 0.58 mM $MgSO_4$, 0.4 mM KCl, and 0.14 mM $CaCl_2$ (ASTM, 1991).

The glycoalkaloid and pterins were dissolved in FETAX solution and the pH adjusted to appropriate levels. Di-4-ANEPPS was dissolved in ethanol, as

described previously (Blankemeyer et al., 1992; Friedman et al., 1991). They embryos were evaluated for fluorescence.

The experimental setup for measuring fluorescence from embryos loaded with Di-4-ANEPPS is described in Blankemeyer et al. (1993, 1995, 1997). The protocol uses cellular dyes to probe the mechanism of toxicity; specifically, the change in membrane potential. For each concentration, at least three replicates of 75 embryos each were tested. The protocol was altered to obtain time course readings of the effects.

The fluorescence protocol was altered to obtain time course (multiple treatment) measurements of toxicity (see figure 10). The embryos were incubated with Di-4-ANEPPS at a final concentration of 2mg/mL for 30 min. Each exposure of the embryos to the solutions, compound plus Di-4-ANEPPS (2mg/mL), started with a 4 minute dish incubation. Afterwards, the embryos were transferred to a microcuvette (Blankemeyer et al., 1993) containing the test solution to allow for a continuous exposure throughout data collection. The microcuvette was placed into the spectrofluormeter chamber for readings at defined time intervals. A continuous reading was not obtained since the compounds are photoreactive. Various combinations of pre-treatment and post-treatment were utilized to test the interaction of the glycoalkaloids and pteridines.

Curves were fitted by non-linear regression and linear regression using GraphPad (Los Angeles, CA). Iteration was used to find the best fit, employing a sigmoidal model. EC50 and LC50 values were generated from the iterations.

Results

Figure 15 is the time course of embryo fluorescence with various methotrexate treatments. Two modes of evaluation were set up to determine if prior methotrexate or α-chaconine exposure caused a variation in the final fluorescence upon treatment with α-chaconine. At 50 mg/L methotrexate and 10 mg/L α-chaconine, a pre-exposure to methotrexate kept the fluorescence level of the α-chaconine and methotrexate treatment at a lower level than a pre-exposure to α-chaconine. This indicates that an interaction is occurring between the pterin and glycoalkaloid, although whether this is direct or indirect is still not known.

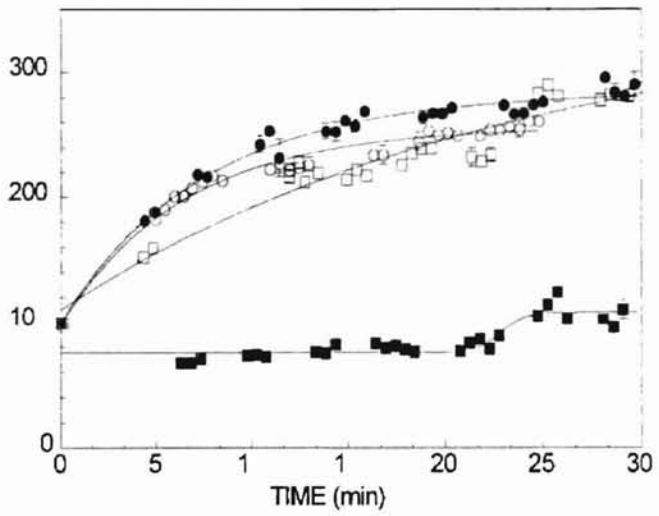

Figure 15. Time Course Fluorescence Assay. Plotted as percent control fluorescence. Closed circle is α-chaconine treatment alone. Open square is pre-methotrexate then post-α-chaconine. Open circle is pre-α-chaconine and post α-chaconine and methotrexate. Closed square is pre-methotrexate then post-α-chaconine and methotrexate. Data were two trials of 1 replica of 75 embryos.

Figure 16 shows the results of single treatment fluorescence assays with α-chaconine and methotrexate. All data collected within a concentration was averaged prior to standardization with average untreated data. The data was plotted as percent of control fluorescence. This figure shows that increasing concentrations of methotrexate decreased the fluorescence induced by α-chaconine. By itself, methotrexate had no effect on the fluorescence.

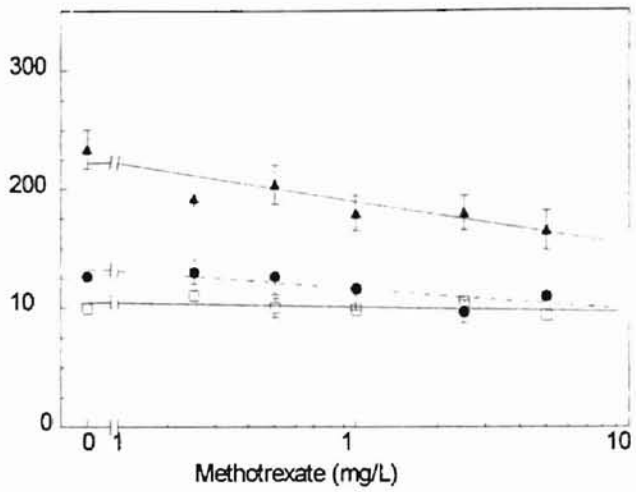

Figure 16. Percent of control fluorescence of methotrexate at 0 mg/L α-chaconine (open square), 5 mg/L α-chaconine (colored circle), and 10 mg/L α-chaconine (colored triangle). Data were two trials of three replicas of seventy-five embryos.

Conclusion

The fluorescence assays indicated that methotrexate prevented the depolarization of the membrane potential caused by α-chaconine. Folic acid (Friedman et al., 1997) and MTX produced similar results at equivalent concentration. The time course assay provided another perspective into the action of methotrexate. The pre-methotrexate treatment produced no significant change in fluorescence over the control in the presence of α-chaconine and methotrexate. When the MTX treatment is withheld till after the α-chaconine initial exposure, the fluorescence increased to that of the α-chaconine treatment

alone. This is also the result of a pre-treatment of MTX then exposure to α-chaconine alone returns the fluorescence to that of the α-chaconine treatment alone. Since MTX must be present for α-chaconine's effect to be neutralized, some competitive binding may be occurring on the membrane. Another possibility is that the methotrexate inhibition of dihydrofolate reductase has increased the internal concentration of folates, producing a result similar to that of folic acid treatments (Friedman et al., 1997).

Therefore, we can conclude that MTX prevented the α-chaconine depolarization of embryonic membrane during the first 24 hours of development at a MTX dose similar to that of folic acid. After that point, the higher concentrations of methotrexate caused a secondary effect that leads to an equal and/or increased cell death and malformations as compared to α-chaconine alone (Bantle et al., 1990).

CHAPTER 5

CONCLUSION

The results obtained allow for the characterization of the toxicity of solamargine and solasonine (eggplant glycosides) plus the interaction of pteridines and α-chaconine, a well studied glycoalkaloid.

In the solamargine/solasonine study, I found that solamargine was more cytotoxic and teratogenic than solasonine. When this was compared with existing data on potato glycoalkaloids, a correlation became apparent. The presence of the carbohydrates and their composition leads to variation in toxicity. In solamargine and α-chaconine, the carbohydrate makeup was 2 rhamnose:1 glucose. Both compounds have a significantly higher level of toxicity than their counterparts, solasonine and α-solanine, respectively; which only have 1 rhamnose, 1 glucose, and 1 galactose molecule.

In human heptoma cells, the 2′ rhamnose moiety plays a role in triggering cell death by apoptosis (Chang et al., 1998). With molecular modeling, the 2′ rhamnose was predicted to lie adjacent to the steroid which drastically changes the dihedral angle of the glycosidic bond (Chang et al., 1998) allowing interaction with the membrane. Free rhamnose inhibits the cytotoxicity of glycosides in murine sarcoma cells (Cham and Daunter, 1990).

If the interaction of the carbohydrates with the membrane has a direct correlation with the disruption by the glycoalkaloids, then blocking the binding of these sugar should decrease or even prevent the resulting damage. Previous

work with α-chaconine found that folic acid ameliorated the toxicity of the glycoalkaloids (Friedman et al., 1997).

When the interaction of folate and α-chaconine was expanded to folate analogs and derivatives, a mode of action starts to emerge. The proposed model (Keukens et al., 1995) for α-chaconine action (figure 17) was dependent on the interaction of the sterol portion of the molecule acting directly with cholesterol. The hydrophobic interaction results in the sugar moiety being on the exposed face. The sugars then interact with one another to cause the disruption (Keukens et al., 1995).

Folic acid was found to protect against α-chaconine induced cytotoxicity and teratogenesis (Friedman et al., 1997). There is evidence that folic acid is actively transported across membranes by folate receptors (Kamen et al., 1991). The binding of folic acid to its receptor may alter the membrane such that α-chaconine can no longer interact with the cholesterol. In some organism, the folate receptors may also bind folate analogs

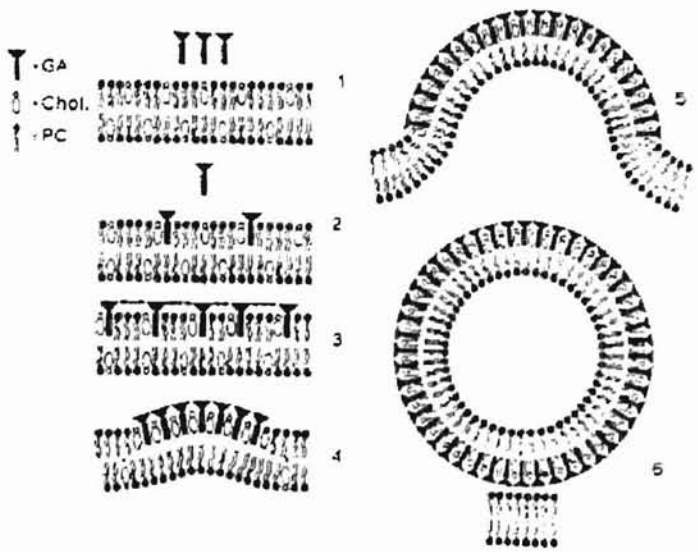

*Figure from Keukens et al., 1995.
Figure 17. The proposed model of glycoalkaloid induced membrane disruption.

and/or derivatives (Tillinghast and Newell, 1989). This resulted in the hypothesis that if these receptors were shared in *Xenopus*, the transport of any molecule via these receptors that generated a similar conformational change could provide equivalent protection from glycoalkaloid induced membrane disruption.

L-Monapterin and methotrexate were used to test this hypothesis. L-Monapterin resulted in no significant reduction of α-chaconine toxicity and induced malformations. Methotrexate, a known teratogen, acts in a two phase process. It resulted in a minimal change in membrane potential and mortality within the first 24 hours of *Xenopus* embryonic development. Extended exposure (96 hours) produced significant mortality and malformations (Bantle et al., 1990). When coexposed with α-chaconine, a hyperpolarization of the α-chaconine induced depolarization occurred within the first 24 hours. Upon further investigation, it was found that methotrexate must be present in both the pre- and post- treatment for the hyperpolarization to occur. When the MTX was removed from the post-treatment, a depolarization that mimicked the α-chaconine only treatment resulted. This implied that the MTX concentration was depleted. This depletion could be due to metabolism of the compound by the developing embryo.

Therefore, it was the conclusion of this study that both solamargine and solasonine exhibit cytotoxicity due to the presence of the carbohydrates, specifically rhamnose, with the increased solamargine toxicity due to its 2'

rhamnose. Secondly, the decrease in α-chaconine induced depolarization by methotrexate and folic acid (Friedman et al., 1997) may result from an alteration in membrane conformation inhibiting the binding of the glycoalkaloids (Keukens et al., 1995). If the action is receptor mediated, methotrexate and folic acid induce a similar change with L-monapterin generating a different effect. L-monapterin may interact without a receptor or the size of the molecule may not induce a substantial conformational change that inhibits the glycoalkaloid interaction.

Future investigation into the action of protection provided by the pterins should include (1) determination of folate receptors, (2) a folate receptor-substrate binding assays with the three pterins and (3) a binding assays between folate receptor and glycoalkaloid, and (4) a survival study between methotrexate or L-monapterin, folic acid, and glycoalkaloid to determine if folate rescue occurs. The first three studies will determine if the binding of the pterin to the receptor blocks the toxicity due to conformational changes within the membrane or competition for receptor binding site. In fourth study, folate rescue will indicate that the secondary effects, cytotoxicity and teratogenesis, were linked to inhibition of folate metabolism.

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VITA

Misty Lea McWilliams

Candidate for the Degree of

Master of Science

THESIS: INTERACTION OF GLYCOALKALOIDS AND PTERIDINES WITH FROG EMBRYO MEMBRANES.

MAJOR FIELD: Microbiology, Cell and Molecular Biology

BIOGRAPHICAL:

PERSONAL DATA: Daughter of Currie and Judy McWilliams of De Kalb, TX.

EDUCATION: Attended Angelo State University and South Plains College before graduating from Oklahoma State University with a B.S. in Cell and Molecular Biology, May 1996. Completed the Requirements for the Master Degree at Oklahoma State University in May, 1998.