AN EVALUATION OF THE DEVELOPMENTAL TOXICITY OF POTATO GLYCOALKALOIDS USING THE FROG EMBRYO TERATOGENESIS ASSAY-XENOPUS

BY

JAMES ROBISON RAYBURN

Bachelor of Science
Louisiana College
Pineville, Louisiana
1987

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1990

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
Doctor Of Philosophy
May, 1994

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Thesis Approved:

Thesis Adviser

Dean of the Graduate College

PREFACE

The Frog Embryo Teratogenesis Assay: Xenopus (FETAX) is an in vitro bioassay designed to determine the potential teratogenic hazard a compound or complex mixture poses to developing organism. FETAX may also be used to assist in determining the mechanism of action chemicals. FETAX lends itself to looking at structure activity relationships and how they effect developmental toxicity. Also FETAX has been used to determine mechanism of actions of chemicals by testing chemicals alone and together in mixture studies. By using toxic unit analysis it can be determined if chemicals work synergistically, concentration additively, response additively, or antagonistically. Also FETAX has been used to determine if chemicals are activated or deactivated by cytochrome P450 enzymes. This project was conducted to show the usefulness of FETAX in these three areas investigating the same chemicals. Chaconine and solanine are two potato glycoalkaloids which are know to be toxic to humans. These glycoalkaloids were chosen to investigate the potential usefulness of FETAX in helping to identify mechanism of actions of chemicals. By so doing, it is hoped that this research will increase FETAX usefulness to biomedical and environmental toxicology. This thesis is divided into five chapters. An introduction to the work is presented in Chapter I, with a literature review. Chapter II is a complete manuscript which has been submitted to *Food and Chemical Toxicology*. Chapter III and IV are each complete manuscripts that are being prepared for submission.

Several people were instrumental in the completion of this research project: Dr. Jack Bantle who served as my major adviser; Drs. Jim Blankemeyer, Marsha

Black, and Charles Qualls Jr. served as advisory committee members. Their help was much appreciated. Dr. Mendel Friedman who initially diverted me to work with the glycoalkaloids and provided funds for this research. Several others provided assistance including: Mendi Hull, Jason Sides, Amy Lewis.

I would also like to thank my parents Dr. A. L. Rayburn and Mrs. Flossie Rayburn for their support during my schooling.

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CHAPTER I

INTRODUCTION

Importance of Solanaceae

The *Solanaceae* family of plants, including the potato, contains many plants important to humans' food consumption. The potato ranks third to wheat and rice in annual production, with nearly 300 million tons produced on a world-wide basis (Sharma and Salunkhe 1989). Although potatoes provide an excellent source of carbohydrate energy (Jadhav et al. 1981), they also contain glycoalkaloids, which are secondary plant metabolites. The glycoalkaloids probably function in defense against insects and other pests (Norris, 1986). The most common glycoalkaloids in potatoes are α-chaconine (α-C) and α-solanine (α-S) (Sharma and Salunkhe 1989), which are toxic to man. Glycoalkaloids (GA) in low concentrations are considered necessary for the proper flavor of potatoes. However, elevated levels of GAs result in a distinctly bitter taste (Slanina 1990). The GA content also accounts for much of the potato poisoning in humans. The toxicity of the glycoalkaloids to humans and animals has been well documented (Jadhav et al. 1981, Morris and Lee 1984, Renwick 1982).

Goals and Objectives

Because GAs are toxic to humans and animals, it is important to understand their toxic mechanisms of action. If a developmental toxicity model were developed, it would assist in the prediction of which GAs would be the most toxic or teratogenic.

A model of this sort should include possible interactions that might occur between the different GAs. Also, the model should indicate the possibility of biotransformation of the alkaloids either into more toxic or into less-toxic forms. Modeling of chemicals has been used by many laboratories (Dawson et al. 1991, Eriksson et al. 1990, Fiqueroa et al. 1991, Kavlock 1988, Schultz and Ranney 1988, Tosato et al. 1990). Models are useful in predicting possible dangers to organisms in the environment. However, they are limited in predictability because of the lack of information on interactions and biotransformation of the chemicals. The proposed experiments will attempt to generate a model that includes these types of effects for α-chaconine and α-solanine (Figure I-1 and I-2).

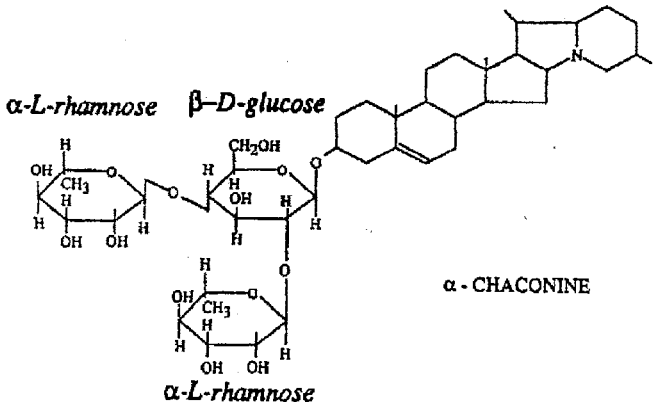

Figure I-1. α-Chaconine Structure

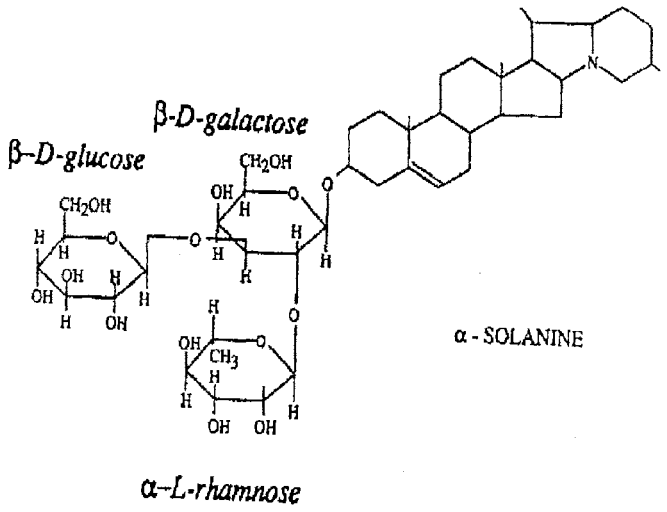

Figure I-2. α-Solanine Structure

Distribution of Alkaloids in Potatoes

The location of GAs within the potato and the concentrations of each can influence toxicity. GA content is high in the meristematic regions including the foliage, blossoms, and sprouts, followed by the peel and the tuber flesh (Table I-1). Within the tuber, the greatest concentrations of GAs are found in the layer under the peel and the tuber flesh. Wood and Young (1974) determined the distribution of the total GAs in the potato (Table I-1).

Table I-1. Distribution of the Total Glycoalkaloids in the Potato Plant

	Total GA (mg/100 g fresh weight)
Plant	
Sprouts	200-400
Flowers	300-500
Leaves	40-100
Normal Tuber Tissue	
Skin, 2-3% of tuber	30-60
Peel, 10-15% of tuber	15-30
Peels and eye, 1/8-in (3-mm) disk	30-50
Peels from bitter tubers	150-220
Flesh	1.2-5
Whole tuber	7.5
Bitter tuber	25-80

Wood and Young 1974.

Knowledge of GA locations within the potato is important in estimating the exposure to the general public and in identifying of potatoes that may be toxic. Given a rough estimate of exposure, a model could estimate possible toxic or teratogenic hazards to which an organism might be exposed. The first part of any model should explain the possible threats to which an organism might be exposed. If the dose is proportional to the effect, estimations on effects can be made.

Toxicity of Plant Alkaloids

Accidental consumption of potatoes containing high amounts of GAs has

caused severe illness and has occasionally caused death. Jadhav et al. (1981). reviewed several cases of reported potato toxicity. Of these reports, most cases of sickness began with headache and vomiting along with diarrhea and abdominal pain. Neurological symptoms were also reported with the cases, although very few deaths occurred. Where measured, the GA content of the potatoes ranged from 33.3 mg/100g to 43 mg/100 g. In a recent review of the literature, Slanina (1990) reported over 2,000 cases of GA poisoning, which led to approximately 30 deaths. Slanina (1990) also reported that the lowest dose to induce toxic symptoms was approximately 2 mg/kg. The mechanisms of action is unclear. Jelinek et al. (1976) demonstrated that solanine is toxic to chick embryos at doses of 1 μg/kg, while Sharma et al. (1978) reported no teratogenic effects in hamsters. However, both rabbits and swine exhibited malformations consisting of problems in neurulation and anencephaly. Sharma et al. (1978) stated that further experiments with susceptible species need to be performed to investigate the mechanism of action of the toxins. Slanina (1990) also stated the need for further testing of GAs to elucidate their mechanisms of toxicity.

Rationale for Procedures

The Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) is a 96-h bioassay designed to determine the relative developmental hazard of compounds. FETAX was formerly described by Dumont et al. (1983). FETAX has been used in several laboratories in studies of developmental toxicity of pure compounds as well as environmental samples (Dumont et al. 1983; Courchesne and Bantle 1985; Dawson et al. 1985; Sabourin and Faulk 1987; Fort et al. 1989) and has yielded a

≥90% predictive accuracy rate when tested with selected chemicals of known developmental toxicity in mammals. FETAX yields data seventeen days sooner than the shortest mammalian assay and is significantly less expensive. The relative toxicities of several plant alkaloids (Friedman et al., 1991; Friedman et al., 1992) have been tested with FETAX. Structure activity relationships have also been initially evaluated based on these experiments. FETAX showed those aglycones are much less toxic to frog embryos than the corresponding glycosides. Friedman et al. (1992) suggested the reduced toxicity could be due to differences in structure or water solubility. Analysis of these compounds using FETAX provided investigators with a rapid method for developing models for describing the potential developmental toxicity of plant alkaloids which would not be possible with other systems.

Several compounds have also been tested with Aroclor 1254 and Isoniazid induced microsomes with and without showing indications of biotransformation (Fort and Bantle 1990, Fort et al. 1991). It is possible that biotransformation may play an important role in glycoalkaloid toxicity and FETAX has been proven effective in identifying these types of roles. It is also possible that some of the toxic properties of the alkaloids may act synergistically to cause additional developmental toxicity. FETAX lends itself to these types of experiments because the end points of FETAX and many components of common models can be broken down into several parts. Both α-C and α-S contain three carbohydrate subunits that can be removed by hydrolysis reactions. These products may have an effect on the toxicity of the potato alkaloids. The number of carbohydrate subunits and their placement were correlated to the toxicity of the compounds. The use of Structure Activity Relationship (SAR) to analyze structure toxicity relationships has been performed

for several type of compounds (Schultz et al. 1989a,b, Schultz et al. 1990, Dawson et al. 1990a). FETAX has been useful in determining structure-activity relationships. Schultz et al. (1988) found that altering the structure of semicarbazide altered the severity of osteolathyrism observed with *Xenopus* embryos.

Quantitative-Structure-Activity Relationships (QSARs) have also been examined for use in risk assessment. QSARs are a mathematical expression that relate the variations of biological activity to the variation (differences) of a series of structurally similar compounds. To date, the scope of QSARs has broadened to include not only quantitative data, such as amounts of products formed in biological systems, but to predict any type of data (effect) related to both toxicity and exposure of chemicals (Tosato et al. 1990). QSARs may represent a 'philosophy' for the systematic analysis of environmental chemicals (Tosato et al. 1990). Accordingly, it may provide a strategy for the screening of large numbers of chemicals and for the ranking of them according to potential hazards. It is expected that QSARs will be able to predict the biological activity for untested compounds, provide comparative toxicity information on similar compounds, indicate which subunits cause a given effect, and help elucidate the mechanism of biological activity. One requirement for QSAR is that the compounds be structurally similar and act according to the same biological mechanism of action. The potato GA α-C and α-S are very similar in structure and differ only in the carbohydrate side chain. It has been suggested that the mechanisms of action are apparently similar for the toxic GA because of the similarities in malformations caused by both compounds in FETAX (Friedman et al. 1991). The experiments proposed are on the hydrolysis products of α-C and α-S. The purpose of this study is to determine the importance

of carbohydrate side chains to the developmental toxicity of GAs found in potatoes. Also these experiments should provide information on the important factors governing the toxicity of GAs.

Testing for GA Interactions. Depending on the potato variety, a potato has been reported to contains GA levels consisting of α-C and α-S in ratios of 74:26 to 40:60, respectively (Sharma and Salunkhe 1989). Because a potato contains a mixture of the alkaloids, the GAs should be tested together to estimate potential interactions that might occur in a real world situation and to produce more accurate estimates of possible developmental toxicity. Dawson and Wilke (1991a) demonstrated that FETAX is useful in determining the toxicity of mixtures. Rayburn et al. (1991a) and Rayburn et al. (1991b) showed that FETAX could identify synergism and antagonism on developmental toxicity caused by low levels of solvents such as dimethylsulfoxide, acetone, and triethylene glycol. Identifying interactions is important because calculations of combined toxicities most often only assume an additive or less than additive effect. In a study on the lysis of phospholipid/sterol liposomes by Roddick et al. (1988), α-C and α-S exhibited synergism that was specific and did not occur if one of the GAs was substituted by a related compound such as tomatine or $ß_2$-chaconine. The work of Roddick and Runnenberg (1987) however, employed the use of synthetic lipid membranes, while Roddick et al. (1988) used isolated cells from rabbit (erythrocytes), beet, and fungal protoplasts. The latter work showed a synergistic activity on the lysis of actual cells. However, the work was not carried out on whole animals, and therefore, whole animal testing should be examined. The synergism observed with the lipid membranes may also indicate synergism on developmental toxicity or no synergism at all on the three end points of FETAX. The purpose of these experiments is to

determine if any interactions exist when a whole embryo is used. These experiments should show what, if any, types of interactions will occur and how a model should be adapted to predict toxicity. Applying the information gained about interactions to a working model of GA developmental toxicity should increase the predictive accuracy of the model. Models that do not include the possibility of interactions are weaker than models that do include these possibilities.

GAs that are stored in the body might be more readily metabolized by enzymes in the liver at times of increasing metabolic stress, such as starvation or debilitating illness (Claringbold et al., 1982). Friedman et al. (1991) showed that when Aroclor 1254-induced microsomes were used in the metabolic activation system for FETAX, toxicity decreased. The study left several unanswered questions as to the cause of this decreased toxicity. After studying the structure activity and possible interactions, the possibility that the compounds may be biotransformed cannot be overlooked. The metabolic activation experiments are designed to determine whether cytochrome P450 biotransformation decreases or increases toxicity. Friedman et al. (1991) determined that the CO-gassed microsomes, that were supposed to bind and inactivate cytochrome P450, displayed the same activity as normal microsomes. This result could suggest a different biotransformation route other than the route of the mixed function oxidase (MFO) system. Instead the flavin monooxygenase system is causing the reduction of the developmental toxicity. By testing two different inducers of cytochrome P450 enzymes (Aroclor 1254 and isoniazid) with different strategies for removal of specific enzymatic pathways, we hope to define the specific pathways for GA biotransformation. These pathways will help make predictive models of developmental toxicity more accurate.

Hypothesis

Gylcoalkaloid's toxicity is dependent on the number of carbohydrate groups attached, on other glycoalkaloids present and on the production of metabolites by cytochrome P450. Each part of the hypothesis will be broken down into sub-hypotheses as follows:

1. The number of carbohydrate units does not change toxicity.
2. Chaconine and solanine act additively on toxicity.
3. Mammalian microsomal enzymes do not change the developmental toxicity of the alkaloids.

Structure Activity Relationships

If the carbohydrate subunits play an important role in the altering the developmental toxicity of the GAs they could alter the developmental toxicity in several ways. One way is if the number of carbohydrate subunits could increase or decrease the developmental toxicity of the GAs. If this change in developmental was the case, we would expect that the trioside would be more toxic than the disides, which would be equal, and the monoside would be the least toxic, or a reverse of the developmental toxicity could be true. If the orientation of the sugar groups was most important factor then we would expect the disides to have different toxicities. If the carbohydrate was not affecting toxicity, we would either expect all of them to have the same toxicity or we would expect them to have a random toxicity with no correlation to the number of sugar groups.

Interactions of Plant Alkaloids

Synergism of α-chaconine and α-solanine has been examined by Roddick and Runenberg (1987) in relation to the lysis of phospholipid/sterol liposomes. They suggest that α-chaconine and α- solanine appear to be synergistic in the membrane-lytic action of these alkaloids. If these are important mechanisms for toxicity and teratogenicity, it would be reasonable to predict that α- chaconine and α-solanine would interact synergistically in the FETAX assay.

By using toxic unit analysis, we determined whether the mixtures were potentiated, concentration additive, response additive or antagonistic. Compounds that have the same mode of action have strictly additive (concentration additive) toxicity in combination. In contrast, chemicals that have different modes of action are expected to show less than concentration additive (response-additive). If the chemicals have a greater than additive effect, potentiation is seen. Therefore, modes of action of chemicals can be postulated from these results.

Metabolic Activation (MAS)

An exogenous metabolic activation system can be used in an attempt to determine what role rat liver microsomal enzymes perform in the toxicity of potato glycoalkaloids. If these enzymes do show an effect, a difference is expected between activities of the P450 enzymes and FMO enzymes.

Mechanisms other than the P-450 enzymes might be responsible for a reduced toxic effect when MAS is added. To determine if other mechanisms of action are occurring, several comparative controls could be performed along with each experiment, including unactivated (no microsomes added) controls (2 concentrations), activated (with microsomes) full test concentrations, P-450 inactivated

microsomes (CO-gassed microsomes), and flavin monooxygenase inactivated (heat-exposed) microsomes.

The experiments described above elucidated important aspects of plant alkaloid developmental toxicity. By examining the structure-activity relationships of alkaloids, investigators will be able to predict both which compounds are developmentally toxic and the functional groups that contribute to the observed toxicity. The metabolic activation experiments will help determine what factors are important in the detoxification of the glycoalkaloids seen in earlier studies. The interaction experiments begin to explore the possibility that plant alkaloids can interact synergistically to increase developmental toxicity. These experiments will elucidate the structures, mechanisms of action and possible biotransformation effects on the developmental toxicity of plant alkaloids. Therefore, the experiments proposed here will increase our knowledge of the developmental toxicity of plant glycoalkaloids.

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CHAPTER 2

THE ROLE OF THE CARBOHYDRATE SIDE CHAINS OF POTATO GLYCOALKALOIDS IN DEVELOPMENTAL TOXICITY

James R. Rayburn*, John A. Bantle*, and Mendel Friedman**

*Department of Zoology, Oklahoma State University, Stillwater, OK 74078

**Food Safety and Health Research Unit, USDA-ARS Western Regional Research Center, 800 Buchanan Street, Albany, CA 94710

Abstract

As part of a program to improve the safety of plant-derived foods such as potatoes, we examined the developmental toxicity of seven structurally-related individual compounds using the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). The role of the carbohydrate moiety of *Solanum* glycosides in influencing the developmental toxicology of these compounds was assessed. Comparative evaluations were carried out of the tri-glycosides α-chaconine and α-solanine, the diglycosides β_1- and β_2-chaconine and β_2-solanine, and the mono-glycosides γ-chaconine and γ-solanine. The results show that biological activity was influenced by the chemical structure of the carbohydrate, i.e. galactose, glucose, rhamnose, the number of carbohydrate groups making up the side chain attached to the 3-OH position of the aglycone solanidine, and the stereochemical orientation of the chaconine diglycosides. The developmental toxicity of these compounds in FETAX generally decreased following removal of the carbohydrates from the triglycosides.

Key Words: potatoes, glycoalkaloids, chaconines, solanines, relative potencies, food safety, frog embryos.

Introduction

Glycoalkaloids are potentially toxic secondary plant metabolites found in potatoes and tomatoes (Jadhav et al., 1991). Relatively high concentrations have been found in potatoes consumed by animals and humans (Bushway and Ponnampalm, 1981; Friedman and Dao, 1992; Osman, 1983; Ponnampalm and Mondy, 1983; Sinden et al., 1984). Levels are especially high in green and damaged potatoes and in immature tomatoes (Kozukue and Mizuro, 1990; Morris and Lee, 1984). Glycoalkaloids are produced both during the growth of the plant and during post-harvest storage.

Adverse effects following potato glycoalkaloid ingestion by animals and humans include anticholinesterase activity in the central nervous system, induction of hepatic ornithine decarboxylase (ODC), disruption of cell membranes, and possible teratogenicity (Caldwell et al., 1991; Renwick et al., 1984; Sharma et al., 1978). We previously examined the relative embryotoxicities of structurally different *Solanum* alkaloids in the Frog Embryo Teratogenesis and frog membrane potential assays (Blankemeyer et al., 1992; Friedman et al., 1991, Friedman et al., 1992). The purpose of this study was to better understand the structural features governing the developmental toxicity of these compounds. Our results show that glycoalkaloids are more toxic than corresponding aglycones lacking the carbohydrate groups. The two major potato glycoalkaloids, α-chaconine and α-solanine, are tri-saccharides or triosides; i.e. they have three carbohydrate groups attached to the 3-position of the aglycone (Figure II-1). One or more carbohydrate

groups can, in principle, be hydrolytically cleaved by enzymatic hydrolysis in potatoes (Bushway et al., 1988, Bushway et al., 1990), or after consumption of the glycoalkaloids, by enzyme-and/or acid-catalyzed digestion. Therefore, it was of interest to find out whether the hydrolysis products of these compounds, the so-called β- and γ-chaconines and solanines, with two or one carbohydrate each, behave similarly to the parent compounds in the embryo assays. The present study concerns the developmental toxicity of these glycoalkaloid hydrolysis products in FETAX.

Material and Methods

Test Materials. α-Chaconine and α-solanine were obtained from Sigma Chemical Co., St. Louis, MO, USA. β_1-, β_2-, and γ-chaconines and β_2- and γ-solanines were isolated and purified from incomplete hydrolysis mixtures of the parent compounds and characterized by high-pressure liquid chromatography and mass spectrometry (Friedman et al., 1993). Our efforts to isolate β_1-solanine were unsuccessful. All compounds produced a single peak on HPLC chromatograms.

FETAX Tests. The Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) was used to assess the developmental toxicity of the hydrolysis products of α-chaconine and α-solanine. The FETAX assay procedure followed the ASTM Standard Guide for the Conduct of FETAX (ASTM, 1991; Bantle et al., 1991). Two sets of 25 embryos each were placed into 60 mm covered glass Petri dishes with varying concentrations of the test compounds dissolved in FETAX solution which contained 10.8 mM NaCl, 1.2 mM $NaHCO_3$, 0.58 mM $MgSO_4$, 0.44 mM $CaSO_4$, 0.4 mM KCl, and 0.14 mM $CaCl_2$.

The same four concentrations (mg/L) of α-, β_1-, β_2-, and γ- chaconine were

tested with each clutch of embryos. There were four dishes of controls and two replicates per concentration. The same five concentrations (mg/L) of α-, β_2,- and γ -solanine were employed with each clutch of embryos. Embryos from the same clutch were used to reduce variation in each experiment. Two preliminary experiments were performed to verify concentrations and these results are from the final experiment.

Stock solutions of the chaconines were made by adding 5 mg of the compound to 500 mL of FETAX solution. The stock solutions of solanines were made by adding 10 mg of the compound to 500 mL of FETAX solution. Appropriate dilutions were made to achieve the final concentrations. To avoid possible solvent-test material interactions, we did not use additional co-solvents (Rayburn et al., 1991a; Rayburn et al. 1991b). The pH of the solanine solutions was reduced from 8.1 to 6.9 to achieve solubility. The FETAX solution (including all controls) used for that experiment was also adjusted to pH 6.9, which is within limits set by the ASTM Guide for FETAX (ASTM, 1991). Additional studies using HPLC to measure concentrations in solutions confirmed the previous concentrations.

The embryos were cultured at 24 ±1°C under static renewal conditions for 96-h. Solutions were renewed every 24 h of the four day test, and any dead embryos were removed. The acidities of the stock and control dishes were measured each day to verify that the pH was between 6.5-7.7. At 96-h, (stage 46) surviving embryos were fixed in 3% (w/v) formalin. Stage 46 embryos possess hind-limb buds and tightly coiled guts, but do not yet feed. Malformed survivors, dead embryos, and the developmental stage of surviving embryos were determined using a dissecting microscope (Nieuwkoop and Faber, 1975).

For each test, probit analysis (Tallarida and Murray, 1983) was used to determine the 96-h LC50 (median lethal concentration or the concentration causing 50% embryolethality), 96-h EC50 (malformation, or the concentration inducing gross terata in 50% of the surviving embryos), and a teratogenic index (TI) equal to 96-h LC50/ 96-h EC50.

As a measure of growth, head-tail length was measured by following the body contour using an IBM-compatible computer equipped with digitizing software (Jandell Scientific, Corte Madera, CA). For each test compound, the minimum concentration to inhibit growth (MCIG) was calculated using the T-test for grouped observations. Values were considered significant if $P<0.05$.

Results and Discussion

History and Rational. In previous studies (Friedman et al., 1991, Friedman et al., 1992), we showed that the teratogenicity and embryotoxicity of the glycosides of the steroidal aglycone, solanidine, are strongly dependent on the carbohydrate residues attached to the steroidal secondary 3-OH groups. Both the nature and order of attachment of the carbohydrate residues appear to influence biological activity. Thus, the relative potency of α-solanine, a trioside with three sugars--glucose, galactose, and rhamnose, is significantly lower than that of α-chaconine, a trioside in which two rhamnoses and one glucose molecule are attached to the same aglycone.

It was postulated that the carbohydrate residues influence biological activity by participating in binding to sugar molecules associated with receptor sites of cell membranes. Because some of the hydrolysis products of α-chaconine and of α-

solanine shown in Figure II-1 are found in plant tissues and could also be formed during normal digestion and metabolism of the parent compounds following ingestion, it was of interest to compare the relative potencies of potato glycoalkaloids with one, two, and three carbohydrate sugar residues attached to the 3-position of the aglycone solanidine.

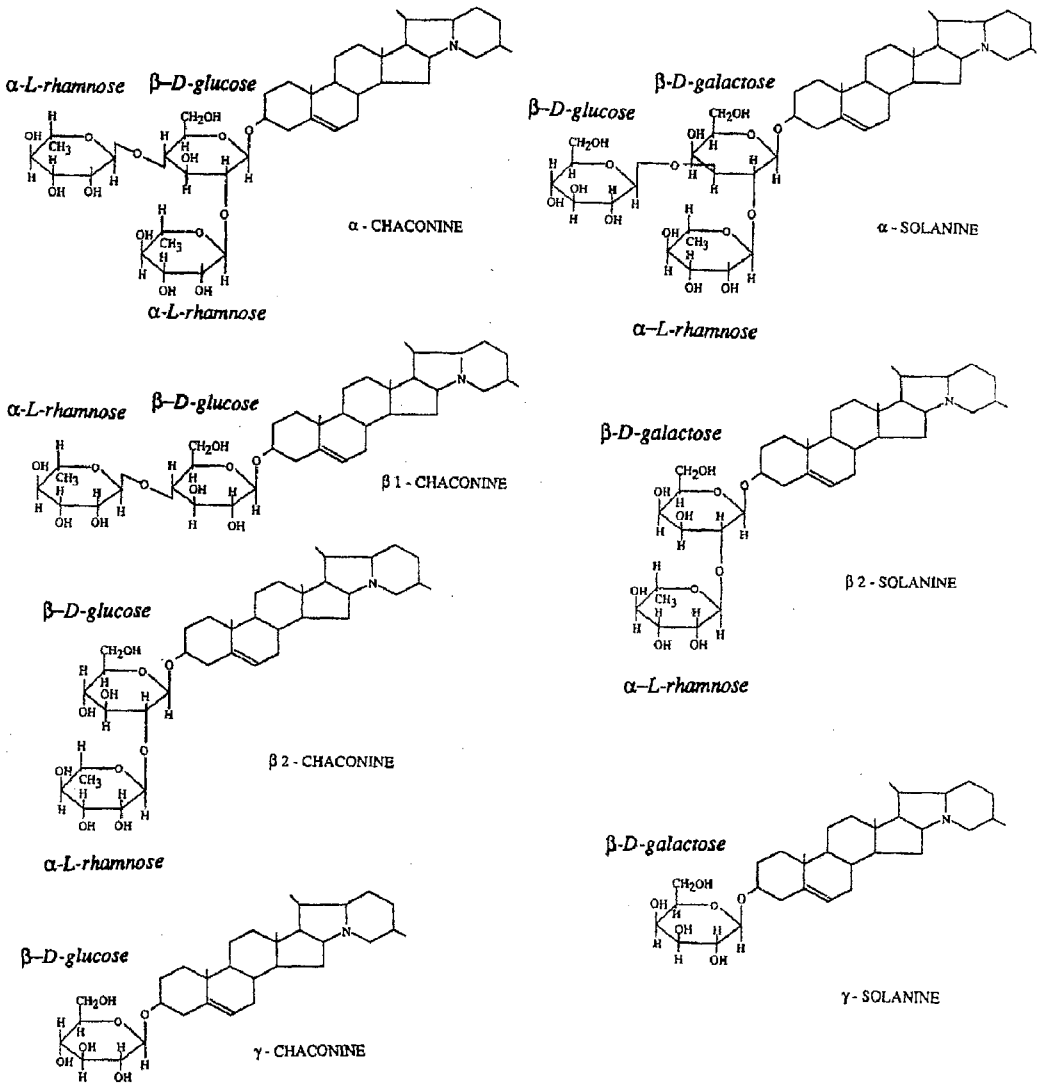

Figure II-1. Structures of glycoalkaloids evaluated by FETAX.

FETAX Assay. In a previous study (Friedman et al., 1992) we showed that the developmental toxicity data we obtained for α-chaconine, α-solanine, solanidine, and tomatine in FETAX generally paralleled reported data on the teratogenicity induced by these compounds in pregnant hamsters. Related studies suggested that FETAX predicts the developmental toxicity of teratogens in mammals with an accuracy of about 90% (Bantle et al. 1990). These considerations and the additional advantages FETAX has over other short-term tests suggest that it merits wide use as a developmental screen for compounds that are sufficiently soluble in FETAX solution. According to Dresser et al. (1992), these advantages include the following: (a) *Xenopus* development is already well known, (b) *Xenopus* embryos undergo fundamental developmental processes that are similar to those of mammals; (c) mating and ovulation can be induced at any time after sexual maturity; (d) embryos develop outside of the frog, facilitating observation of development and malformations; and (e) developmental endpoints can be determined within a relatively short 96-h test period.

Control Results. With α-chaconine controls, the number of dead embryos was 4% and the number of malformed embryos was 10.4%. The corresponding values for α-solanine were 8% and 5.5%, respectively.

Most of the mortality seen with the α- and β_1-chaconines occurred within 24 to 48 h. This data suggests that these compounds are interfering with very early development. After 48 h, there were very few deaths in the chaconine experiment. With the solanine compounds, more embryos died at 96-h than they did in the chaconine experiment.

In Figures II-2 and II-3, the higher the percent response at a given

concentration, the higher the developmental toxicity as evidenced by higher mortality, malformation, and growth inhibition. Generally, the percent mortality and malformation are directly proportional to the developmental toxicity. In contrast, the LC50 and EC50 values are inversely proportional to the developmental toxicity. Additional indications of teratogenic hazard are the severity of the malformation and whether growth of the embryos is inhibited at less than 30% of the MCIG value. Each endpoint must be considered separately when ranking developmental toxicity. Table II-1 compares developmental toxicity of all compounds evaluated in this study.

Table II-1. Developmental Toxicity Ranking of Chaconines and Solanines Based on 96-h LC50, EC50, MCIG, and TI Values.

Compound	molecular weight	clutch (embryos/ clutch)	LC50[a] (C.I.)[b]	EC50[c] (C.I.)	MCIG[d]	TI[e]
α-Chaconine	852.1	1 (200)	0.0022 (n.a.)[f]	0.0020 (n.a.)	<0.0047	1.1
β_1-Chaconine	705.9	1 (200)	0.0043 (0.0038-0.0049)	0.0025 (0.0021-0.0029)	0.0057	1.72
β_2-Chaconine	705.9	1 (200)	>0.014	0.0092 (0.0084-0.010)	0.0085	>1.52
γ-Chaconine	559.7	1 (200)	>0.018	0.01397 (0.0125-0.0156)	0.0071	>1.28
α-Solanine	868.0	2 (250)	0.0297 (0.025-0.035)	0.0131 (0.012-0.014)	<0.0092	2.26
β_2-Solanine	721.2	2 (250)	>0.0222	0.0187 (0.017-0.021)	>0.0222	>1.2
γ-Solanine	659.7	2 (250)	>0.024	0.0230 (0.022-0.024)	0.024	>1

[a] LC50 = concentration in μmol/L causing 50% embryolethality in 96 h.
[b] C.I. = 95% confidence interval.
[c] EC50 = concentration in μmol/L causing malformations in 50% of surviving animals in 96-h.
[d] MCIG = minimum concentration in to inhibit growth μmol/L.
[e] TI = (LC50/EC50[malformation]).
[f] n.a. = not available.

Chaconine Series. Figure II-2 shows the percentage of mortality versus the percentage of concentration for the four compounds in the chaconine series. The results indicate that α-chaconine and β_1-chaconine were both quite lethal to embryos, with α-chaconine requiring somewhat less material than β_1-chaconine to kill the same number of embryos. In contrast, β_2- and γ-chaconines induced very little mortality in the concentration range studied.

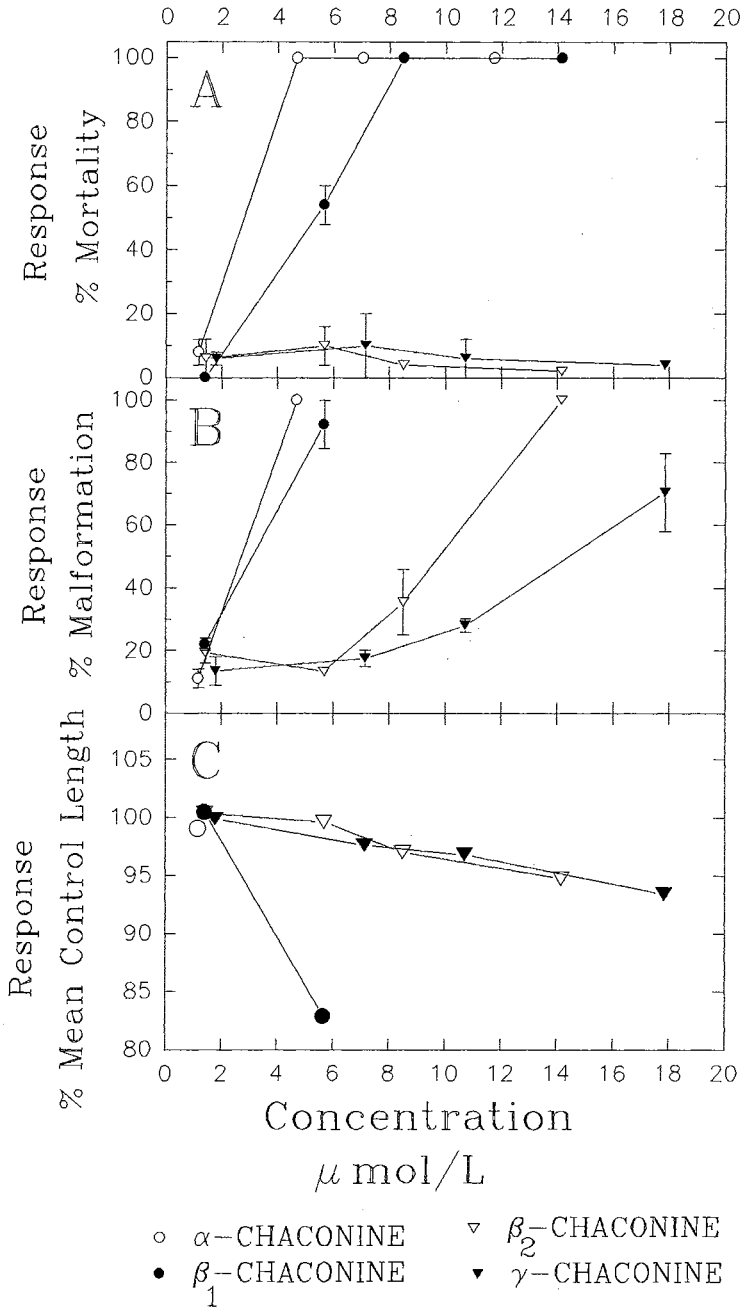

Figure II-2. Comparison of the developmental toxicity of chaconines in FETAX. Error bars indicate standard deviation. (n=2)

The data show that β_1-chaconine induced more malformations than β_2- and

γ-chaconines. α-Chaconine also caused severe malformations that were very similar to β_1-chaconine. However, all the embryos died before 96-h. Previous experiments using lower concentrations of α-chaconine also showed these malformations (Friedman et al., 1991). The malformation curves for α- and β_1-chaconines were steep and had few points due to high mortality at the second and third concentrations tested (Figure II-2). The embryos that died during the test frequently had anencephaly, as previously reported by Friedman et al. (1991). Microscopic inspection of surviving embryos revealed that most of the malformations induced by α- and β_1-chaconines were slight to moderate facial malformations. However, gross malformations were more common with α- and β_1-chaconines than with the β_2- and γ-pair. Most of the malformations seen with β_2- and γ-chaconines were facial and gut malformations, in the slight to moderate category. At the higher concentrations, there were in addition many embryos with darkly pigmented oral suckers.

Data from these experiments and previous studies (Friedman et al., 1991; Friedman et al., 1992) suggest a similar pattern of toxicity for growth. Thus, α- and β_1-chaconines had similar effects on the percentage of mean control length and β_2- and γ-chaconines had similar effects on growth that were much less pronounced than those of α- and β_1-chaconines.

Solanine Series. Mortalities of the embryos induced by α-solanine were concentration-dependent (Figure II-3). In contrast, mortalities induced by β_2- and γ -solanines increased with concentrations up to 12 mg/L and then decreased. The mortalities for both compounds peaked at about the same concentration. These bell-shaped concentration-response curves were repeated (data not shown).

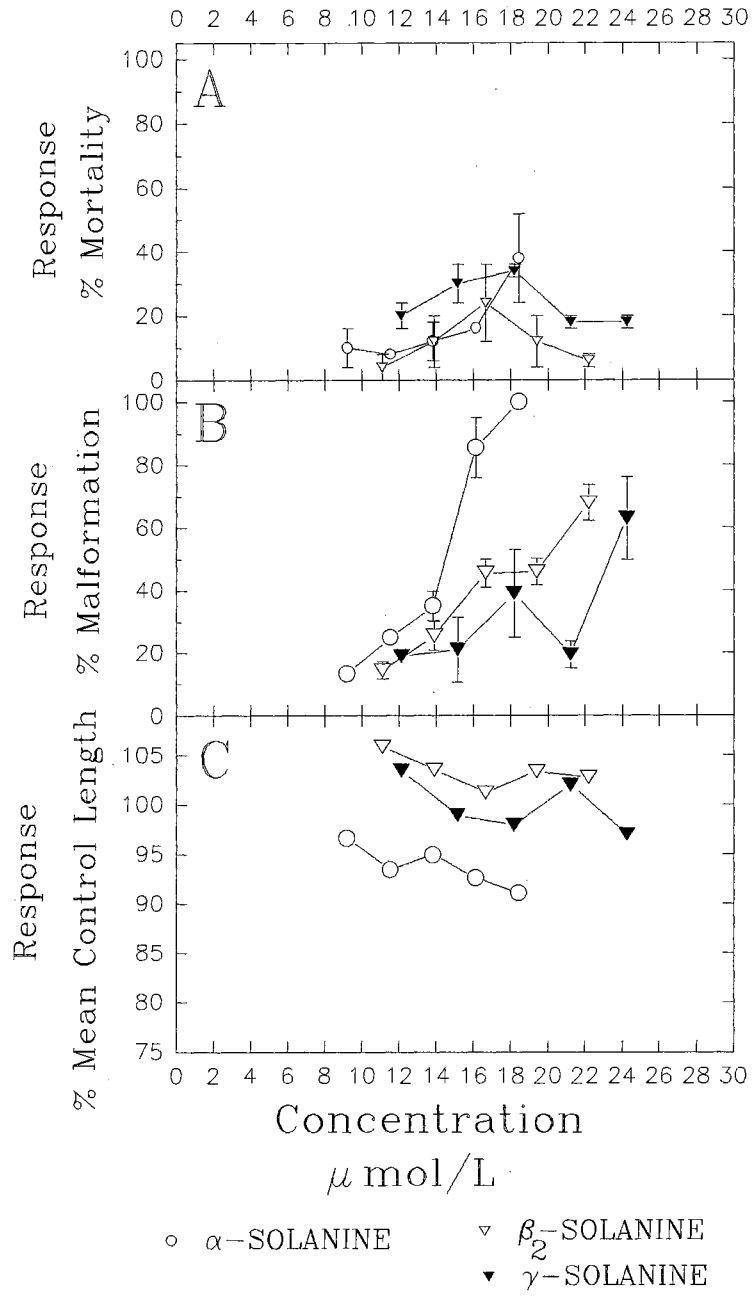

Figure II-3. Comparison of the developmental toxicity of solanines in FETAX. Error bars indicate the standard deviation.(n=2)

Figure II-3 shows that malformations induced by α-solanine increased nearly

linearly with concentration in the range 8 to 16 mg/L. Solubility problems prevented higher concentrations from being tested with the β_2- and γ- solanines. The number of malformations induced by β_2- and γ- solanines were similar to those from α-solanine up to a concentration of about 12 mg/L; at higher concentrations the malformation rate dropped below that of α-solanine. Most of the malformations seen with α-solanine were moderate loose or mid-gut coiling. Most of the malformations observed with β_2- and γ-solanines were slight to moderate gut coiling. However, embryos exhibited the same dark pigmented oral suckers observed with exposure to β_2-chaconine and γ-chaconine induced.

The growth curves of β_2- and γ-solanine were again similar while α-solanine inhibited growth markedly, significantly more than either β_2- or γ-solanine.

Role of Carbohydrates. Table II-1 lists the calculated 96-h LC50, EC50, MCIG, and TI values for the compounds evaluated in this study. This table allowed an analytical comparison of their developmental toxicities. As already mentioned for the EC50, LC50, and MCIG values, the lower the number of carbohydrate subunits the greater the potency of compounds. The results showed that removal of a rhamnose residue from α-chaconine to form β_1- chaconine increased the LC50 value by a factor of 2, lessened the effect on the EC50 and MCIG values, and increased the TI from 1.1 to 1.72. Removal of the other rhamnose group from α-chaconine to form β_2-chaconine (which differs from the β_1- isomer only in stereochemistry, these compounds have identical composition), increased the LC50 value by a factor of more than 6, the EC50 value by a factor of about 4.5, the MCIG value by a factor of 2, and in the TI value from 1.1 to more than 1.5. Removal of both rhamnose residues from α-chaconine to produce the mono-glycoside, glucosyl-solanidine (γ-chaconine), resulted in further decreases in overall toxicities as

measured by the cited parameters (Table II-1). Although the trends of developmental toxicity for the three solanines seem to be shifted in the same direction as for the chaconine series, the absolute values for LC50, EC50, and MCIG are higher, the percent difference between the solanines is smaller.

An earlier study (Friedman et al., 1991) found that the aglycone solanidine, formed on complete removal of the carbohydrate groups from either α-chaconine or α-solanine (Figure II-1), had a low level of toxicity. This finding and the current results suggest that the carbohydrate side chain of the glycoalkaloids are paramount in influencing biological activity. Not only the number but also the type of carbohydrate, i.e. galactose, glucose, rhamnose, as well as the order of attachment and the stereochemical orientation, e.g. β_1- and β_2-chaconines, affect the developmental toxicity of the glycoalkaloids. It is possible that removal of sugars may influence the transport of these compounds across cell membranes, i.e. toxicity differences may reflect transport phenomena as the side chains become smaller and less polar.

Acknowledgments

We thank Gary M. McDonald for his help in characterizing the test compounds and for conducting the HPLC assays and Prof. A.A. Bushway for helpful comments.

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CHAPTER 3.

SYNERGISM IN THE DEVELOPMENTAL TOXICITY CAUSED BY TWO POTATO GLYCOALKALOIDS USING *XENOPUS* EMBRYOS

James R. Rayburn[a], Mendel Friedman[b], and John A. Bantle[a*]

[a] Department of Zoology, Oklahoma State University, Stillwater, OK 74078 USA.

[b] Food Safety and Health Research Unit, USDA-ARS Western Regional Research Center, 800 Buchanan Street Albany, CA 94710 USA.

* Author to whom correspondence should be addressed.

Abstract

As part of a program to improve the safety of plant-derived foods such as potatoes, we examined the embryotoxicities of two major potato glycoalkaloids, α-chaconine and α-solanine, individually and in mixtures, using the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). The study of glycoalkaloid interactions is important because humans are exposed to complex glycoalkaloid mixtures through food consumption. This study was undertaken to determine whether glycoalkaloids interact with one another to affect developmental toxicity in *Xenopus* embryos. The 96-h LC50 and EC50 (malformation) for several mixtures were determined using standard FETAX assay procedures. The Toxic Unit (TU) Analysis method was used to calculate possible antagonism, synergism, or response addition of a number of mixtures ranging from 3:1 to 1:20 TU of α-chaconine to α-solanine. Some combinations exhibited strong synergism in the following measures of embryotoxicity: (a) 96-h LC50, defined as the median concentration causing 50% embryo lethality; (b) 96-h EC50, defined as the concentration causing 50% malformation of the surviving embryos; and (c) teratogenic index, TI, equal to LC50/EC50. The results showed that all mixtures caused synergistic responses for mortality and malformation. The results suggest that (a) the synergism observed for a specific mixture cannot be used to predict possible synergism of other mixtures with different ratios of the two glycoalkaloids; (b) toxicities observed for individual glycoalkaloids cannot be used to predict toxicities of mixtures; and (c) specific combinations found in different potato varieties need to be tested to assess the safety of a particular cultivar.

Introduction

Glycoalkaloids (GAs) are nitrogen-containing, potentially toxic secondary plant metabolites found in numerous plant species including potatoes and tomatoes. As part of a mutidisciplinary program of potato improvement, we previously evaluated the relative safety of potato and tomato glycoalkaloids in the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) to determine which structural features govern toxicity (Friedman et al., 1991; Friedman et al., 1992). Our results showed that the number and nature of the sugars attached to the alkaloids appear to govern relative developmental toxicity. These studies have helped us to understand how the glycoalkaloids exert their biological and toxicological effects in animal cells, but more study is needed to ensure the continued safety of these foods.

GAs are toxic to humans and humans seem to be more sensitive to them than are animals (Slanina, 1990). One report that indicates that there is a positive correlation between the consumption of blighted potatoes and the incidence of anencephaly and spina bifida in humans (Renwick, 1982).

Depending on the potato variety, potatoes contain glycoalkaloid (GA) levels consisting of α-chaconine (α-C) and α-solanine (α-S) at concentration ratios of 74:26 to 40:60, (α-C:α-S) (Sharma and Salunkhe, 1989). Therefore, people who consume potatoes are not ingesting only one glycoalkaloid, but rather a mixture of at least two alkaloids, mostly α-C and α-S in different ratios depending on the variety of potatoes. Therefore, additional studies are needed to determine whether the GAs act additively, synergistically, or antagonistically when consumed as mixtures in differing ratios.

Dawson and Wilke (1991a,b) demonstrated in earlier studies that FETAX is

a useful assay for determining the developmental toxicity of mixtures of two or more compounds. Rayburn et al. (1991a,b) showed that FETAX could identify synergism and antagonism caused by low levels of solvents such as dimethylsulfoxide, acetone, and triethylene glycol. Bantle et al. (1990) showed FETAX could determine mammalian teratogens. In related studies, Roddick et al. (1988) demonstrated synergism between α-C and α-S in the lysis of phospholipid/steroid liposomes, and Fewell and Roddick (1993) showed that α-C and α-S act synergistically against fungi that infect potatoes. The synergistic toxicity observed in mixtures of the two compounds operate at levels that exhibit little or no effect with the individual glycoalkaloids. The synergism seen with the lysis of lipid membranes suggests possible synergistic effects of the two compounds on developmental toxicity.

The objective of this study was to use FETAX to measure possible synergism in the developmental toxicity of α-C and α-S. A second objective was to determine whether we could use the information gained on the interactions of the glycoalkaloids on the developmental toxicity of frog embryos to construct a working model that would be able to predict toxicities of mixture combinations not previously tested.

Materials and Methods

FETAX Procedures. The FETAX assay procedures followed the ASTM Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) (ASTM, 1991) including animal care and maintenance. All deviations from standard procedure are given below.

Materials. α-C and α-S were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Test solutions. Stock solutions of α-C and α-S were made at approximately two times their 96-h LC50 to ensure that 100 percent of the effects would be seen with the mixtures. The estimated 96-h LC50 was from previous experiments (data not published). Four binary combinations of α-C and α-S were prepared: 3:1, 1:1, 1:3, and 1:20. These numbers refer to TUs, with 1 TU equal to the 96-h LC50 or the 96-h EC50. These numbers are also original estimates of the TU ratios, the actual TU ratios are individually calculated. In all graphs, and tables these ratios are used to identify samples. The pH of test solutions were monitored for each experiment and determined to be between 6.5 and 7.5 for all experiments.

The TU combinations selected were able to show synergism or antagonism caused by either or both compounds. Synergistic relationships of the compounds can be shown by comparing the 96-h LC50 values of the mixtures in TUs and by placing the 96-h LC50 values of the mixtures on a graph such as Figure III-1. By using TU analysis, the determination can be made whether the mixtures are synergistic, concentration additive, response-additive. or antagonistic.

One range-finder and two definitive experiments were performed with each of the four mixtures and the individual compounds. Each experiment was performed with the same embryo clutch to minimize variation; each experiment required 1900 embryos. Following standard FETAX procedures, six concentrations of each mixture were tested so that a linear range from 0-100% of test embryos were killed or malformed. In addition, five concentrations of each individual component alone were tested to serve as positive controls, i.e. 1:0 and 0:1 solutions. Two replicates were performed for each concentration at 25 embryos per replicate.

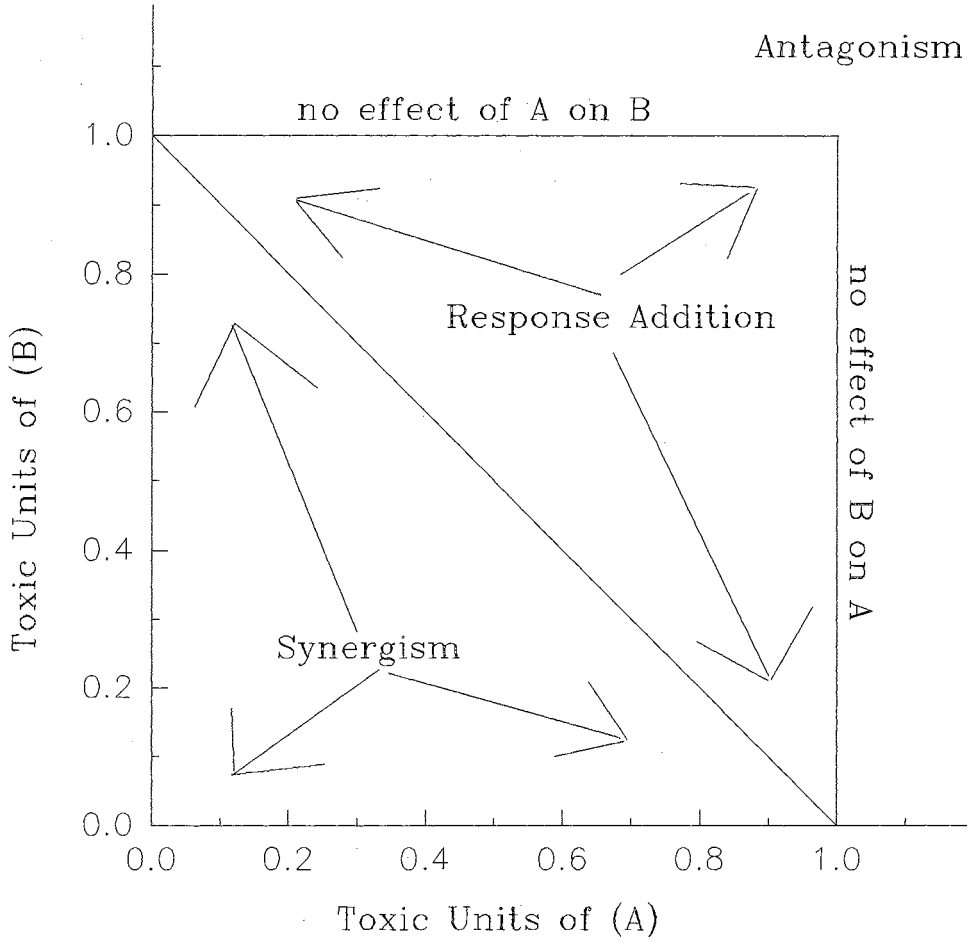

Figure III-1. A typical isobole diagram.

The diagram is for the determination of synergism, concentration addition, response addition, no interaction, and antagonism. Each chemical has been converted to toxic units along the x and y axes. Points that fell below the diagonal concentration addition line were interpreted to indicate synergism. Points that fell above the diagonal concentration addition line but below the no effect line to indicated response addition. Points that fell above the no effect line were considered to indicate antagonism.

Compounds that have the same mode of action should have strictly additive (concentration additive) toxicity in combination (Dawson and Wilke, 1991a). In

contrast, chemicals that have different modes of action are expected to show less than additive toxicity (response-additive). If the chemicals affect each other in unpredictable ways, enhancement (synergism) or a decrease (antagonism) in predicted limits of response can be seen. It should be remembered that a greater than additive effect will be represented as a lower LC50 for that mixture. Also, a lower or decreased effect will fall outside the no effect lines (Figure III-1). Therefore, modes of action of chemicals can be postulated from these results.

Statistical Analyses. The 96-h LC50 and EC50 (malformation) were determined by using probit analysis Litchfield-Wilcoxin, or by using the Spearman-Karber trimmed method (Tallarida and Murray, 1990). The Bonferroni T-test was used to determine the No Observable Effect Concentration (NOEC) and Lowest Observable Effect Concentration (LOEC), when possible. The Minimum Concentration to Inhibit Growth (MCIG) was determined by the grouped T-test. Probit analysis was used to generate the 96-h LC50 and the 96-h EC50 (malformation). In this analysis, the numbers were converted to PROBIT numbers and the data were fitted to a straight line. A linear regression analysis was then used to produce an equation that would calculate a predicted response for any concentration, or a concentration for any response wanted. Operationally, the equation was solved for a predicted response of 50%, and the 95% confidence intervals were calculated.

Response addition. The equation obtained from the PROBIT analysis makes it possible to calculate a predicted 96-h LC50 or 96-h EC50 assuming an additive response for mortality or malformation for any mixture of two compounds for which the 96-h LC50 or EC50 are known. To calculate the response addition for the 96-h LC50 and the 96-h EC50 requires knowing the concentration ratios of the two

compounds and the equation for each compound separately. The estimated LC50 or EC50 for the mixture was then converted to TU and placed on the TU graph for comparison to the actual data. (Figure III-1

Results

Control mortality was 5% (10/200) and the control malformation was 5.8% (11/190) for the two definitive experiments. Most of the control malformation observed was abnormal gut coiling. Abnormalities seen with α-C and α-S at low concentrations were mostly abnormal gut coiling and some facial abnormalities. Concentrations that produced 40% mortality or higher caused severe facial and head malformations in surviving embryos. Types of head malformations included reduced brain size as well as several cases of brain development failure . These malformations are typical of α-C and α-S (Friedman et al. 1991) and were observed in experiments with the individual compounds and all of the mixtures. There were no new, different, or lack of any of these malformations in the mixtures.

Experiment 1

Determination of TU for mixtures analysis. Table III-1 shows the 96-h LC50 and the 96-h EC50 results from the first definitive experiment with the mixture study. The 96-h LC50 for α-C was 1.9 mg/L. This number was defined as 1 TU for α-C for the mortality (LC50) data. The 96-h EC50 for α-C was 1.42 mg/L and was defined as 1 TU for α-C for the malformation data. The 96-h LC50 and EC50 for α-S were defined similarly and were 17.74 mg/L for mortality and 13.57 mg/L for malformation.

Table III-1. Joint Action of α-Chaconine and α-Solanine on Xenopus Embryo Developmental Toxicity for Experiment 1.

Mixture Ratio (α-C:α-S)[a]	LC50 (mg/L)		EC50 (mg/L)	
	α-C	α-S	α-C	α-S
1:0	1.9 (1.70-2.18)[b]	--	1.42 (1.34-1.5)	--
3:1	1.07 (0.69-1.64)	1.01 (0.71-1.44)	0.70 (0.58-0.84)	0.58 (0.48-0.70)
1:1	0.88 (0.68-1.14)	2.20 (1.71-2.83)	0.48 (0.41-0.56)	1.20 (1.02-1.41)
1:3	0.44 (0.34-0.56)	3.3 (2.6-4.2)	0.32 (0.27-0.39)	2.41 (2.00-2.91)
1:20	0.36 (0.24-0.53)	17.87 (11.9-26.7)	0.07 (0.06-0.09)	3.55 (2.79-4.52)
0:1	--	17.74 (16.8-18.7)	--	13.57 (12.6-14.5)

[a] α-C = α-Chaconine ; α-S = α-Solanine.

[b] 95% Confidence Intervals.

Mortality Interactions. For the mixture identified as 3:1, 1.07 mg/L of α-C and 1.01 mg/L of α-S were required to kill 50% of the embryos (96-h LC50). This represented 0.56 (1.07/1.9) TUs of α-C and 0.06 (1.01/17.74) TUs of α-S (Table III-2). The 3:1 mixture had a TU value of 0.62 when totaled. The 1:1, 1:3, and

1:20 summed mixture ratios were also calculated, giving total TU's of 0.58, 0.41, and 1.19 and , respectively. An isobole diagram (Figure III-2) indicates that three of the four mixtures were synergistic and the fourth was either concentration additive or the α-C had no effect with on α-S toxicity. The exception was the 1:20 mixture. The open squares in Figure III-2 represent the response additive addition.

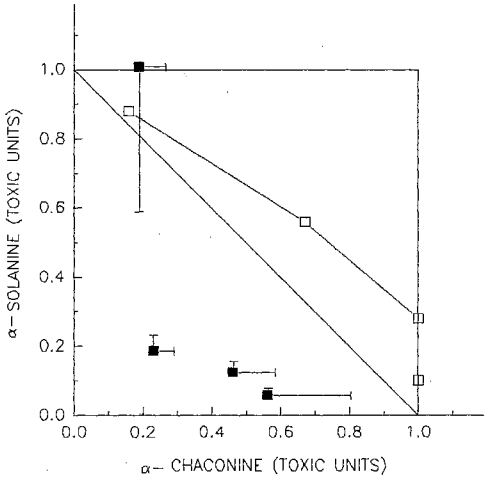

Figure III-2. Isobole diagram for the joint action of α-Chaconine and α-Solanine in Experiment 1 on the mortality of Xenopus embryos. Data points with 95% confidence intervals for α-Chaconine and α-Solanine alone, are plotted in toxic units on the x and y axes, respectively. The toxic unit values for mortality are plotted for the 3:1, 1:1, 1:3, and 1:20 mixtures. All data points, except for the 1:20, are in the synergism section of the graph. The 1:20 point lies on the no effect line. □ Predicted response addition LC50 of the mixtures in toxic units. ■ LC50 of the mixtures in toxic units.

Table III-2. Toxic Unit Analysis of α-Chaconine and α-Solanine Developmental Toxicity for Experiment 1.

Mixture Ratio (α-C:α-S)[a]	LC50 (TU)			EC50 (TU)		
	α-C	α-S	Mixture (Total TU)	α-C	α-S	Mixture (Total TU)
1:0	1.00	--	--	1.00	--	--
3:1	0.56	0.06	0.62	0.49	0.04	0.53
1:1	0.46	0.12	0.58	0.34	0.09	0.43
1:3	0.23	0.18	0.41	0.23	0.18	0.41
1:20	0.18	1.01	1.19	0.05	0.26	0.31
0:1	--	1.00	--	--	1.00	--

[a] α-C = α-Chaconine ; α-S = α-Solanine.

Malformation Interactions. For the mixture labeled 3:1, 1.07 mg/L of α-C and 0.58 mg/L of α-S were required to induce 50% of the surviving embryos to be malformed. These concentrations represent 0.49 (0.7/1.42) TU of α-C and 0.04 (0.58/13.57) TU of α-S (Table III-2). These numbers combined indicate the mixture contained 0.53 TU (Table III-2). The TUs of the three other mixtures, 1:1, 1:3, and 1:20, were calculated to be 0.43, 0.41, and 0.31, respectively. All mixtures showed synergism in the case of malformation (Figure III-3). The hollow circles represented the predicted response addition for malformation. The difference between actual and predicted response addition indicated even more synergism than concentration addition would indicate.

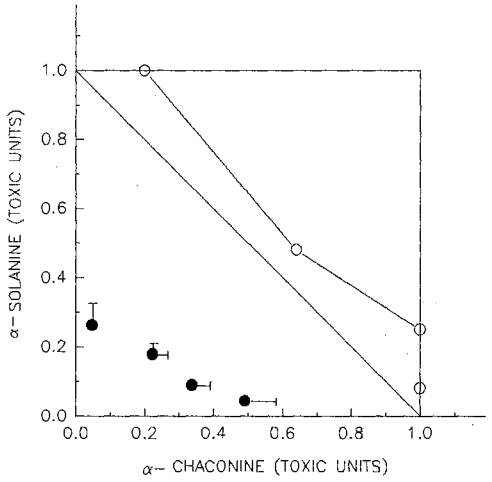

Figure III-3. Isobole diagram for the joint action of α-Chaconine and α-Solanine in Experiment 1 on malformation of Xenopus embryos. Data points with 95% confidence intervals for α-Chaconine and α-Solanine alone are plotted in toxic units on the x and y axes, respectively. The toxic unit values for malformation are plotted for the 3:1, 1:1, 1:3, and 1:20 mixtures. All data points are in the lower left hand quadrant part of the graph indicating synergism. ○ Predicted response addition EC50 of the mixtures in toxic units. ● EC50 of the mixtures in toxic units.

Experiment 2

Determination of TU for mixtures analysis. Table III-3 showed the 96-h LC50, EC50 results from a second experiment performed in exactly the same manner with a different clutch of eggs. All calculations were done as stated earlier, and the experiments were kept separate due to variation between clutches of embryos. Therefore, 3.29 mg/L of α-C represented 1 TU in the second experiment. Note that the LC50 of α-C was about 1.7 times greater than in the first experiment. The LC50 for α-S also changed from 17.74 (Table III-1) to 13.44 (Table III-3), which was approximately 75% of the toxicity in experiment 1. Although the 96-h

LC50 changed between the two experiments, the TU graphs were remarkably similar to one another. The mortality results presented in Figure III-2 (Experiment 1) were very similar to Figure III-4 (Experiment 2). Similar results on malformation were also seen when Figure III-3 (Experiment 1) was compared with Figure III-5 (Experiment 2).

Table III-3. Joint Action of α-Chaconine and α-Solanine on Xenopus Embryo Developmental Toxicity for Experiment 2.

Mixture Ratio (α-C:α-S)[a]	LC50 (mg/L)		EC50 (mg/L)	
	α-C	α-S	α-C	α-S
1:0	3.29 (2.79-3.88)	--	2.16 (1.84-2.55)	--
3:1	0.90 (0.75-1.08)	0.75 (0.63-0.90)	0.59 (0.20-1.76)	0.49 (0.16-1.47)
1:1	0.56 (0.40-0.77)	1.39 (1.00-1.92)	0.32 (0.24-0.43)	0.81 (0.61-1.1)
1:3	0.49 (0.37-0.65)	3.67 (2.77-4.84)	0.21 (0.15-0.28)	1.55 (1.16-2.06)
1:20	0.23 (0.16-0.33)	11.65 (8.2-16.6)	0.048 (0.035-0.065)	2.38 (1.7-3.3)
0:1	--	13.44 (12.0-15.1)	--	9.54 (8.4-10.8)

[a] α-C = α-Chaconine ; α-S = α-Solanine.

[b] 95% Confidence Intervals.

Mortality Interactions. The mortality TUs for the mixtures labeled 3:1, 1:1, 1:3, and 1:20 in the second experiment were 0.33, 0.27, 0.42, 0.94, respectively. These numbers were very similar but slightly lower than the first experiment. In particular, the TUs for mixture 1:20 were 1.19 for the first experiment and 0.94 for the second. Both numbers were significantly higher than all other TUs reported for all other mixtures in both experiments. Being close to one these TU values would indicate that this mixture ratio was synergistic on mortality as were the other mixtures imply.

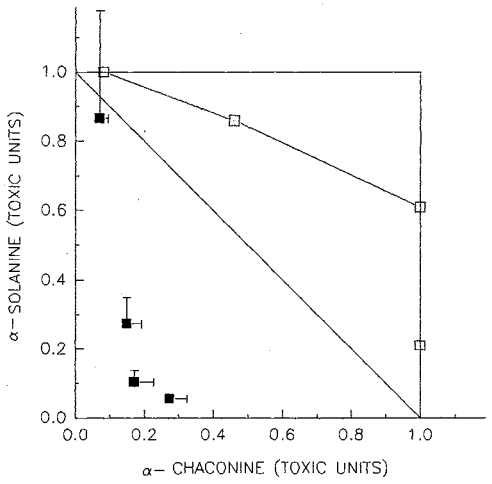

Figure III-4. Isobole diagram for the joint action of α-Chaconine and α-Solanine in Experiment 2 on the mortality of Xenopus embryos. Data points with 95% confidence intervals for α-Chaconine and α-Solanine alone were plotted in toxic units on the x and y axes, respectively. The toxic unit values for mortality were plotted for the 3:1, 1:1, 1:3, and 1:20 mixtures. All data points except for the 1:20 are in the synergism section of the graph. The 1:20 point lies on the no effect line. □ *Predicted response addition LC50 of the mixtures in toxic units.* ■ *LC50 of the mixtures in toxic units.*

Table III-4. Toxic Unit Analysis of α-Chaconine and α-Solanine Developmental Toxicity for Experiment 2.

Mixture Ratio (α-C:α-S)[a]	LC50 (TU)			EC50 (TU)		
	α-C	α-S	Mixture (Total TU)	α-C	α-S	Mixture (Total TU)
1:0	1.00	--	--	1.00	--	--
3:1	0.27	0.06	0.33	0.27	0.05	0.32
1:1	0.17	0.10	0.27	0.15	0.08	0.23
1:3	0.15	0.27	0.42	0.10	0.16	0.26
1:20	0.07	0.87	0.94	0.02	0.25	0.27
0:1	--	1.00	--	--	1.00	--

[a] α-C = α-Chaconine ; α-S = α-Solanine.

Malformation Interactions. The malformation TUs for the mixtures 3:1, 1:1, 1:3, and 1:20 in the second experiment were 0.32, 0.23, 0.26, and 0.27, respectively. Again, numbers were lower in the second experiment, but both experiments showed marked synergism for all mixtures tested. Because each experiment had different LC50 and EC50 values for each of the alkaloids, the actual TU ratios varied. Table III-5 shows the ratios of the alkaloids in actual concentrations and in TU values for each endpoint and experiment. Note that the actual concentration ratios changed very little between experiments. However, note that the TU ratios did change. The ratios on the far left of the graph indicated the ratios that were expected based on previous experiments. Note the actual TU ratios ranged from 9.9:1 to 1:5.3 for mortality for the first experiment and ranged from 4.9:1 to 1:12.4 for the second. This ratio change was a significant increase in α-S TU as compared to α-C TU. This increase was caused by the decrease in toxicity of

α-C and an increase in α-S toxicity.

Table III-5. Table Of Predicted And Actual Mixture Ratios Both In Actual Concentration And In Toxic Units For Both Experiments.

Attempted TU Ratios	Actual Concentration Ratio (mg/L)	Actual LC50 TU ratios	Actual EC50 TU ratios
Experiment 1 (α-C:α-S)			
1 : 0	1 : 0	1 : 0	1 : 0
3 : 1	1.1 : 1	9.9 : 1	11.5 : 1
1 : 1	1 : 2.5	3.7 : 1	3.8 : 1
1 : 3	1 : 7.5	1.2 : 1	1.3 : 1
1 : 20	1 : 49.6	1 : 5.3	1 : 5.3
0 : 1	0 : 1	0 : 1	0 : 1
Experiment 2			
1 : 0	1 : 0	1 : 0	1 : 0
3 : 1	1.2 : 1	4.9 : 1	5.3 : 1
1 : 1	1 : 2.5	1.6 : 1	1.7 : 1
1 : 3	1 : 7.5	1 : 1.8	1 : 1.7
1 : 20	1 : 50.6	1 : 12.4	1 : 11.2
0 : 1	0 : 1	0 : 1	0 : 1

α-C = α-Chaconine ; α-S = α-Solanine.

TU = Toxic Units.

EC50 = Malformation.

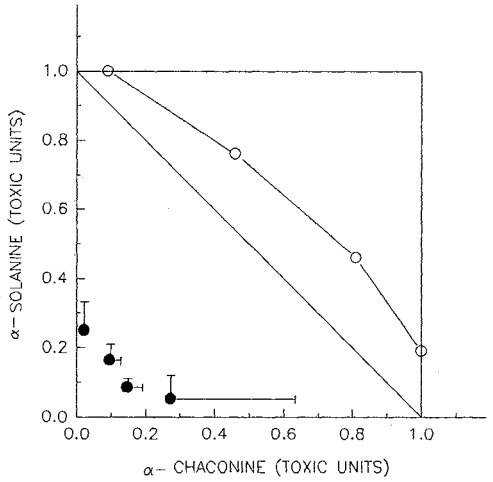

Figure III-5. Isobole diagram for the joint action of α-Chaconine and α-Solanine in Experiment 2 on malformation of Xenopus embryos. Data points with 95% confidence intervals for α-Chaconine and α-Solanine alone were plotted in toxic units on the x and y axes, respectively. The toxic unit values for malformation were plotted for the 3:1, 1:1, 1:3, and 1:20 mixtures. All data points in the lower left hand quadrant part of the graph indicate synergism. ○ Predicted response addition EC50 of the mixtures in toxic units. ● EC50 of the mixtures in toxic units.

Teratogenic Index Comparisons. Table III-6 shows the teratogenic indices (TI) for each of the individual compounds and of the mixtures. The TI is the 96-h LC50/96-h EC50. This ratio gives an estimate of the teratogenic hazard posed by a chemical or mixture. Note that the TIs for most mixtures are 1.8 or less with three exceptions: the 1:20 in mixture experiment 1; 1:20 mixture in experiment 2; 1:3 mixture in experiment 2. The 1:20 mixture had TIs that were 5.1 for experiment 1 and 4.8 for experiment 2. The 1:3 mixture in experiment 2 had a TI of 2.3, only slightly higher than 1.8. Also, in experiment 1 the TI was only 1.4. Therefore, the 1:3 mixture may or may not indicate an significant change in the TI.

Table III-6. Teratogenic Indices Comparisons For The Mixtures Tested For Both Experiments.

Mixture (α-C:α-S)	Experiment I TI	Experiment II TI
1:0	1.3	1.5
3:1	1.6	1.5
1:1	1.8	1.7
1:3	1.4	2.3
1:20	5.1	4.8
0:1	1.3	1.4

TI = Teratogenic Index (96-h LC50/96-h EC50).

Discussion

Mixtures of α-C and α-S acted synergistically on both mortality and malformation for FETAX in this study. This is the first time FETAX has indicated synergism using TU analysis (Dawson and Wilke, 1991a; Dawson and Wilke, 1991b). Thus not only is FETAX useful in determining the potential developmental toxicity of GAs it can also be used evaluate the interactions of GAs as well.

This work also demonstrated the use of calculating the response addition values for the mixtures and plotting them on the TU graph, not done in previous interaction studies (Dawson and Wilke (1991a,b)). The reason for this calculation is that theoretically it is possible that some response (independent) additions may be equal to or less than the concentration addition line depending on the slope of the

concentration-response curves (Pöch et al., 1990). This possibility would mean that if these values are not calculated the wrong conclusions could be drawn from these graphs.

In these experiments, α-C and α-S appear to act synergistically. Our findings suggest that it may not be possible to predict the developmental toxicity of a mixture of the two glycoalkaloids from the results of the individual compounds or of other mixtures. Human toxicity data are based on case studies where humans have been accidentally exposed to high GA concentrations through food consumption, thus human toxicity data are based on a complex mixture of several different alkaloids, animal studies generally use purified compounds and would not indicate if synergism was occurring. The synergism observed in this study could explain why humans are considered a sensitive species to GA poisoning when if fact they may not be.

The data in Table III-6 showed that it is possible for mixtures to vary in their teratogenicity depending on the ratio of the chemicals found in the mixture. This data suggests that two chemicals may interact synergistically or be concentration additive at one concentration ratio and the interaction differ at other concentration ratios.

In conclusion, the observed synergism between α-C and α-S in FETAX demonstrates a need for additional studies on the possible interactions between glycoalkaloids as well as molecular studies to understand how these interactions take place. These experiments are repeatable, and these results have shown that FETAX is useful in determining the developmental toxicity of complex mixtures. It is concluded that α-C and α-S act synergistically on the developmental toxicity in FETAX and that response addition should accompany TU analysis. Finally, this

study infers that safety limits for potato-containing foods based on individual compounds may not be justified. Glycoalkaloid risk assessment studies must take chemical interaction into account to accurately predict human hazard. The situation is even more complicated, because in addition to potatoes, other widely consumed foods, including tomatoes and eggplant, contain glycoalkaloids. Thus, if one were to consume a meal consisting of these three foods, one would ingest a minimum of five structurally different glycoalkaloids (α-C and α-S from potatoes, α-tomatine from tomatoes, and solamargine and solasonine from eggplant). The assessment of possible interactions among these glycoalkaloids is a challenging problem.

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CHAPTER 4

GLUCOSE-6-PHOSPHATE AND NADP PROTECTION OF THE DEVELOPMENTAL TOXICITY CAUSED BY α-CHACONINE

James R. Rayburn[a], John A. Bantle[a*], Charles W. Qualls Jr.[b], Mendel Friedman[c]

[a] Oklahoma State University, Department of Zoology, Stillwater, OK 74078.

[b] Oklahoma State University, Department of Veterinary Pathology, Stillwater, OK 74078.

[c] Food Safety and Health Research Unit, USDA-ARS Western Regional Research Center, 800 Buchanan Street Albany, CA 94710.

* Author to whom correspondence should be addressed.

Abstract

As part of a program to improve the safety of plant-derived foods such as potatoes, we examined the possible reasons why a reduction in toxicity was seen with glycoalkaloids when a metabolic activation system was used in conjunction with the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). The metabolic activation system was examined in two experiments. A concentration series of α-chaconine was tested with rat liver microsomes with and without a generator system (oxidized nicotinamide adenine dinucleotide (NADP), reduced nicotinamide adenine dinucleotide (NADPH), Glucose-6-phosphate, Glucose-6-phosphate dehydrogenase). The generator system and its components were tested at a single high concentration of α-chaconine to determine if they were affecting toxicity. It was discovered that the protective effect of the metabolic activation system was not due to microsomal enzyme systems, but rather to two components of the NADPH generator system, NADP and glucose-6-phosphate, which were added to rat liver microsomes to allow cytochrome P450 activity. Glucose-6-phosphate seems to be more protective than NADP. These results show that it is possible to reduce developmental toxicity of *Xenopus* embryos by adding glucose-6-phosphate. It also shows that the use of FETAX with an metabolic activation system must be performed with appropriate controls that take into account the possible interactions with independent components of the system.

Introduction

In a previous study Friedman et al. (1991) observed that the concentration-response curves for mortality, malformation, and growth shifted to the right, i.e. larger concentrations were required before a response was observed, when the Frog Embryo teratogenesis Assay-*Xenopus* (FETAX) of the potato glycoalkaloid α-chaconine was carried out in the presence of a metabolic activation system (MAS). This work included both α-solanine and α-chaconine.

The metabolic activation system was used in FETAX to identify possible human health hazards because *Xenopus* embryos have limited biotransformation enzymes. The MAS consists of rat liver microsomes, antibiotics to reduce bacterial contamination, and a generator system containing oxidized nicotinamide adenine dinucleotide (NADP), reduced nicotinamide adenine dinucleotide (NADPH), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase. Results from the earlier study implied that the MAS somehow protected the frog embryos against the glycoalkaloid's developmental toxicity. Friedman et al. (1991) noted that CO gassed microsome controls did not respond as predicted. The microsomal cytochrome P450 were supposedly inactivated by CO gas. It was expected that CO gassed microsomes would show the developmental toxicity seen with the glycoalkaloids without MAS, however, that increase in developmental toxicity did not occur. It was hypothesized that protein binding of the GA was the cause of the protection of the embryos. Another possibility was that the flavin-containing monooxygenase system, which is unaffected by CO inactivation and which is present in rat liver microsomes, was involved in the detoxification of GAs (Friedman et al. 1991).

The objective of this study was to determine more specifically which compound(s) was / were responsible for lessening the developmental toxic effects when the MAS system was utilized. To this end, tests were conducted on the toxicity of α-chaconine with MAS; on the toxicity of α-chaconine without MAS; and on the toxicity of α-chaconine with microsomes and antibiotics but with no generator system. Tests were also run with the individual components of the generator system in the absence of microsomes.

Material And Methods

Test Materials. α-Chaconine, NADP, NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Frog Embryo Teratogenesis Assay-Xenopus (FETAX) Procedures. Early embryological stages of the *Xenopus* were exposed through 96-h with various chemical concentrations and treatments to determine the developmental toxicity effects of these treatments. All procedures for the conduct of FETAX are described in the Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) (ASTM 1991) and Bantle et al. (1991).

Stock solutions were made and the pH was monitored for each experiment. The pH was between 6.5 and 7.5 for all experiments.

Metabolic Activation System. 150-250 gram male Sprague-Dawley rats were injected with 500 mg/kg body weight of Aroclor 1254. Five days, later the Aroclor administration rats were killed by cervical dislocation. The liver was

perfused and microsomes were prepared as stated in the Atlas of Abnormalities (Bantle et al., 1992) and Fort et al. (1988, 1989).

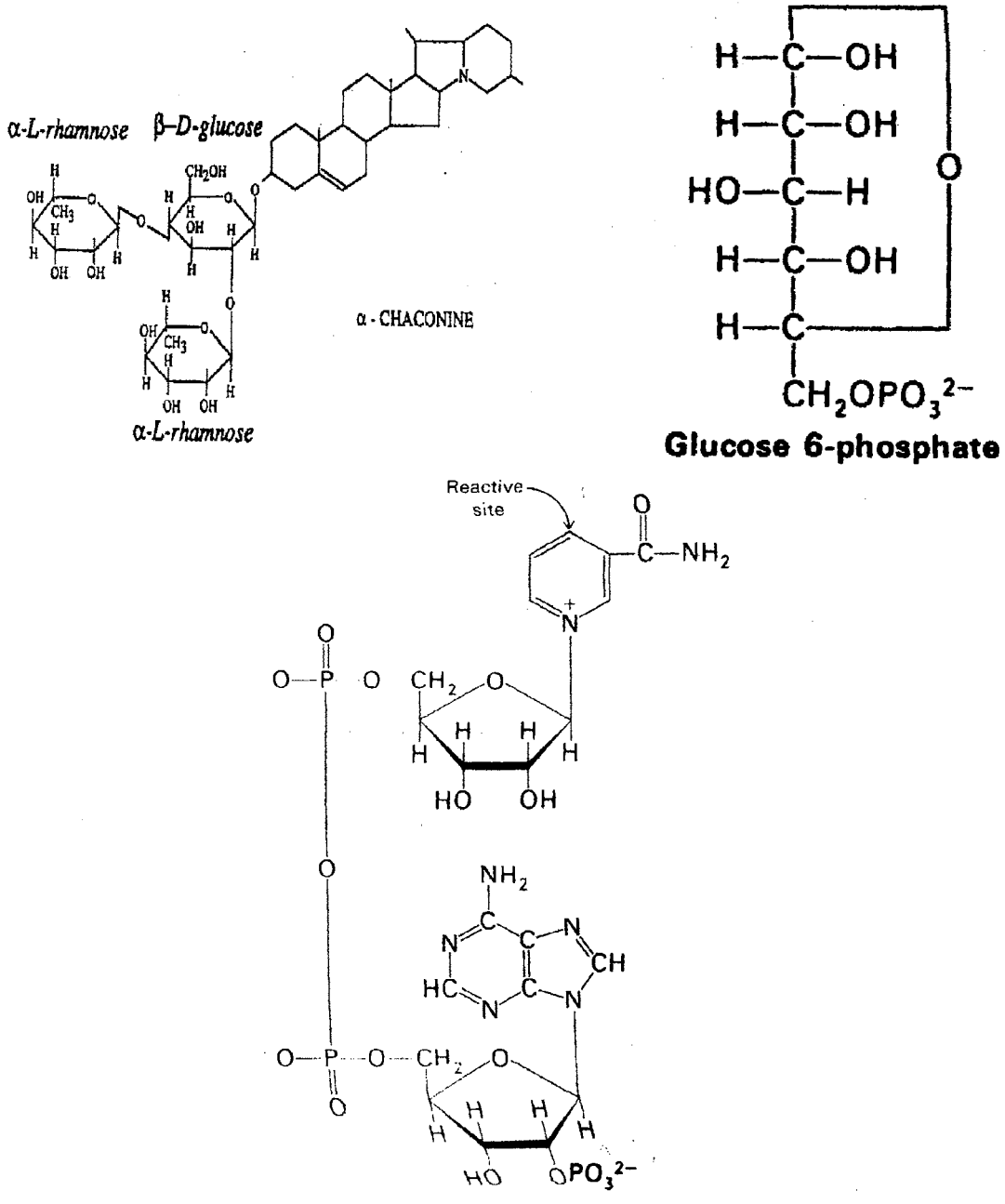

Figure IV-1. Structures of α-chaconine, glucose-6-phosphate and NADP.

Microsomes were analyzed for aminopyrine demethylase activity by procedure modified from Nash (1955) and Lucier et al. (1971), which was described by Bantle et al. (1991). Microsomal protein was measured using a BioRad kit for protein with bovine serum albumin as a standard (Bradford 1976).

Each metabolically activated treatment in the FETAX assay received microsomes equivalent to 0.04 U/ml aminopyrine demethylase activity, a NADPH generating system and antibiotics (100 U penicillin/ml and 100 U streptomycin/ml). The NADPH generating system consisted of 3.5 mM glucose-6-phosphate, 0.31 U glucose-6-phosphate dehydrogenase/ml, 0.1 mM NADP and 7 μM NADPH.

Results

The structures of α-C and glucose-6-phosphate are shown in Figure IV-1.

Concentration-response curves for α-C and MAS-activated α-C for mortality and malformation show at least a two-fold reduction in developmental toxicity with the inclusion of MAS (Figure IV-2). Figure IV-3 shows a concentration response curve for the same concentration series as Figure IV-2. Rat liver microsomes are present at the same protein concentration as those added in Figure IV-2 but with no generator added. Antibiotics have also been added to reduce bacterial contamination, therefore figure IV-3 shows the affect of the microsomal protein on α-C developmental toxicity. As can be seen, there is no protection against α-C toxicity connected with the microsomes or antibiotics. These results would suggest protein binding is not important in preventing the toxicity of α -C, because if protein binding was causing the decrease in developmental toxicity, there should be a reduction of toxicity in these treatments.

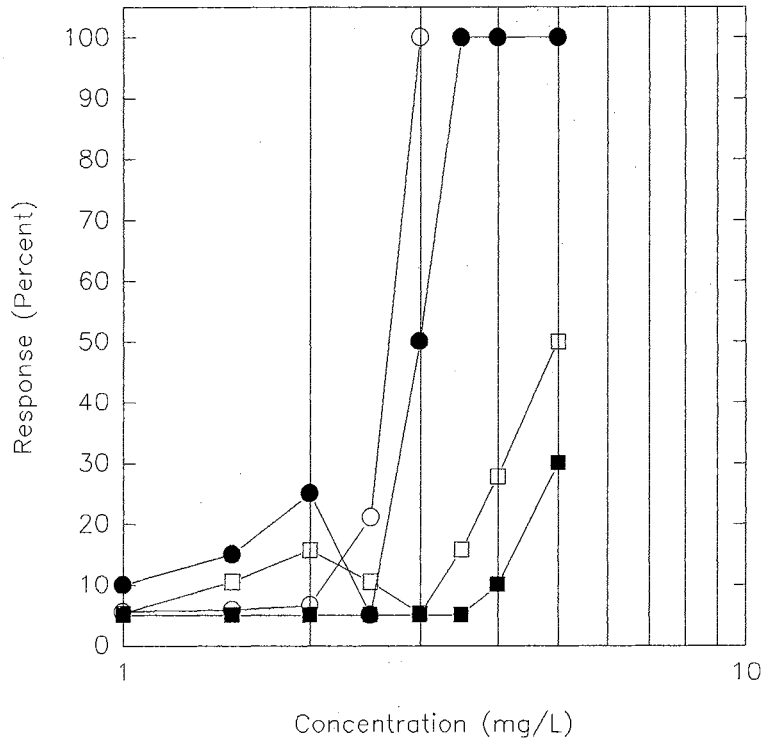

Figure IV-2. A concentration response graph comparing α-chaconine with and without a metabolic activation system.

The x-axis represents the concentration of α-chaconine. ○ represents concentration-response data for malformation in surviving embryos following exposure to α-chaconine. ● represents α-chaconine mortality response. □ represents concentration-response data for malformation in embryos exposed to α-chaconine plus the metabolic activation system. ■ represents α-chaconine plus metabolic activation system mortality response. Addition of the MAS system resulted in a decrease in the developmental toxicity of α-chaconine.

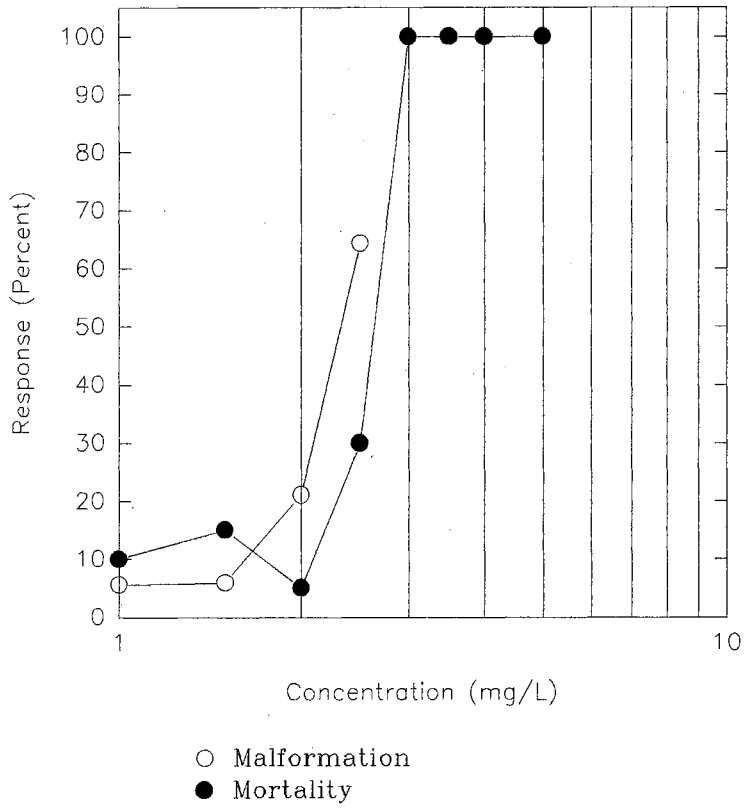

Figure IV-3. Protein binding effects on developmental toxicity. A concentration response graph that show the results of α-chaconine with rat liver microsomes present but no glucose-6-phosphate, NADP, NADPH or glucose-6-phosphate dehydrogenase. ○ represents α-chaconine malformation of surviving embryos response. ● represents α-chaconine mortality response.

From the previous data, 4 mg/L of α-C should yield 100% mortality without protection; with protection 4 mg/L α-C should yield less than 30% mortality. Therefore, this concentration was chosen to examine the remaining components of the MAS system. The control data show only 4.9% malformation

and 10.3% mortality for all of the following experiments. The 4 mg/L α-C showed 100% malformation with 99.4% mortality (Table IV-1). Only 1 embryo out of 160 survived exposure to 4 mg/L. Most of the embryo death occurred by 48-h of incubation.

Table IV-1. Effects of FETAX Generator Components on the Developmental Toxicity of α-Chaconine

Treatment	Total # Embryos Tested	N (# replicates)	Malformation %	Std. error	Mortality %	Std. error
Controls	320	16	4.9	1.12	10.3	1.55
4 mg/L α-chaconine	160	8	100.0	0	99.4	0.58
4 mg/L α-chaconine + glucose-6-phosphate + glucose-6-phospahte dehydrogenase +NADP + NADPH	80	4	10.5	5.08	28.8	10.66
4 mg/L α-chaconine + glucose-6-phosphate + NADP + NADPH	80	4	11.1	5.75	21.3	10.22
4 mg/L α-chaconine + glucose-6-phospahte dehydrogenase	40	2.	N.A.		100.0	0
4 mg/L α-C + glucose-6-phosphate	80	4	26.2	3.52	23.8	4.10
4 mg/L α-C + NADP	80	4	32.5	9.04	50.0	14.79
4 mg/L α-C + NADPH	80	4	N.A.		100.0	0

N.A. = not applicable because of 100% mortality

The effect of the NADPH generator system on α-C toxicity was tested without the inclusion of microsomes or antibiotics. This data showed a marked reduction of developmental toxicity by 70.6% and a reduction in malformation of

89.5% (Table IV-1). This reduction would indicate that the microsomes are not necessary to cause the reduction in developmental toxicity seen in Figure IV-2.

Glucose-6-phosphate dehydrogenase (G6PD), was removed. The only remaining components of the generator system were the glucose-6-phosphate, NADP, and NADPH. The mixture of glucose-6-phosphate, NADP, and NADPH reduced the toxicity of α-C by 78.1% and reduced malformation by 88.9%. The data shows that G6PD was not necessary to cause the reduction in α-C developmental toxicity. Glucose-6-phosphate dehydrogenase tested by itself with 4 mg/L α-C showed no decrease in developmental toxicity caused by α-C.

Glucose-6-phosphate was tested by itself with 4 mg/L α-C and showed a decrease in developmental toxicity of 75.6% and a decrease in malformation of 73.8%. NADP tested alone also produced an apparent reduction of developmental toxicity, caused 4 mg/L α-C toxicity by 49.4%, and reduced malformations by 67.5%. NADPH alone has shown no protection against 4 mg/L α-C toxicity at the concentration tested with FETAX.

Discussion

The metabolic activation system (MAS) has been used to determine if chemicals are bioactivated or inactivated by biotransformation enzymes found in rat liver microsomes. The Aroclor 1254 treatment was used because it causes an induction of a broad spectrum of cytochrome P450 enzymes. Cytochrome P450 enzymes require NADPH as a necessary co-factor. The NADPH is toxic to *Xenopus* embryos at high concentrations. Therefore, a NADPH generating system

was used that converted the less toxic NADP to NADPH by the glucose-6-phosphate dehydrogenase degradation of glucose-6-phosphate.

These data show that glucose-6-phosphate, and to a lesser extent, NADP, are capable of causing an apparent reduction of developmental toxicity in *Xenopus* embryos. The mechanism of action by which glucose-6-phosphate and NADP reduces α-C developmental toxicity needs to be determined. Since NADPH was the smallest component of the generator system, perhaps not enough of the NADPH was available to show a significant protective effect. The data suggests that glucose-6-phosphate alone could be responsible for the reduction of developmental toxicity observed with the full MAS system. In addition, the enzyme G6PD was not required to cause a reduction of the developmental toxicity of α-C. G6PD was also tested by itself, and it had no effect on developmental toxicity.

To set our findings in perspective, we will briefly review some of the reported biological effects of glucose and related carbohydrates and speculate on possible mechanisms of protection. Russell et al. (1992, 1993) showed that glucose at concentrations greater than 10 mM, reduced the number of viable cells in the bacterium *Prevotella rumiciola.* These findings suggest that glucose can be toxic to cells.

Glucose is also reported to (a) slow transport of myo-inositol in cultured human endothelial cells (Okuda et al. 1991); (b) prevent methotrexate-induced gastrointestinal toxicity (Badr and Chen, 1987); and (c) play a key role in the teratogenesis of human fetuses of diabetic mothers (Giavini et al. 1991)

Glucose 6-phosphate could exert its beneficial effect by competing with the carbohydrate groups of α-C (Figure 1) for receptor sites of cell membranes in the frog embryos. We believe this to be likely since in earlier studies we showed that (a)

both the nature and number of the sugars of the carbohydrate sidechain are important in influencing biological activity (Rayburn et al., 1994); and (b) the fundamental mechanism governing teratogenicity may be disruption of cell membranes and changes in ion fluxes and the interstitial currents of the membranes (Blankemeyer et al., 1992).

The following considerations reinforce our proposed mechanism. According to Stryer (1988), glucose enters most cells by a specific transport protein where it is phosphorylated by ATP to form glucose 6-phosphate. This event is catalyzed by hexokinase. The molecule then participates in numerous glycolysis and gluconeogenesis pathways *in vivo*. Boorer et al. (1992) report that a transcribed H^+/hexose cotransporter elicited depolarization of the *Xenopus* oocyte membrane potential. These events imply that glucose 6-phosphate may indeed be exerting its protective effect by binding to membrane receptor sites, thus preventing the carbohydrate groups of the glycoalkaloid from binding to the same receptors and disrupting the cell membranes

An alternative explanation Glucose-6-phosphate and NADP could be interacting with the GA itself, perhaps forming an association with the key parts of the α-C structure. If the ionic state of the α-C structure is altered, it is possible that this change could alter developmental toxicity. It is well known that properties, such as salt content, and pH, alter the toxicities of compounds (Rand, 1985). It may be possible that glucose-6-phosphate may be decreasing the bioavailability of α-C by interfering with transport.

Another possibility is that binding to receptor sites by α-C is prevented by salt formation between its tertiary nitrogen atom and the phosphate groups of the glucose 6-phosphate within the membrane. This suggestion implies, as we

previously proposed (Friedman et al., 1992), that the unshared electron pair of the tertiary nitrogen atom of α-C also participates in binding to cell receptor sites via hydrogen-bonding and acid-base equilibria. Additional studies are needed to confirm these possibilities.

Another metabolic process that could affect α-C developmental toxicity is similar to how fructose prevents nitrofurantion-induced cytotoxicity in isolated hepatocytes (Silva et al., 1991). Fructose, in this case prevents nitrofurantion-induced oxidative stress by preventing the ATP-dependent glutathione disulfide efflux by depleting ATP, thus enabling NADPH and glutathione reductase to reduce the glutathione disulfide back to glutathione.

In conclusion, although the mechanism of how glucose-6-phosphate and NADP reduces α-C developmental toxicity in *Xenopus* embryos is not known, it is certain that glucose-6-phosphate and NADP reduce developmental toxicity of α-C.. This study presents possible problems researchers may experience when using a MAS in an *in vitro* assay such as FETAX. These data suggest that additional controls consisting of elements of the generator system need to be performed in addition to CO-gassed MAS and cyclophosphamide positive controls.

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CHAPTER 5

CONCLUSIONS

The primary objective of this research project was to assess the developmental toxicity of the glycoalkaloids α-chaconine and α-solanine. The assessment of developmental toxicity included investigating how structural changes affected developmental toxicity, how the glycoalkaloids act when they combine with one another and how a metabolic activation system apparently reduced glycoalkaloid toxicity. In an effort to determine how structural changes affected the developmental toxicity of these alkaloids, their hydrolysis products, which were produced by removing the sugar groups on steroidal ring and were isolated, were then tested in FETAX. Also the several mixtures of the alkaloids were tested in an attempt to determine what kinds of interactions may be occurring with the glycoalkaloids. Finally, the metabolic activation system was evaluated to determine what caused the reduction in developmental toxicity seen earlier with α-chaconine and α-solanine. This multi-purpose evaluation of glycoalkaloid toxicity revealed several important parameters that alter glycoalkaloid developmental toxicity. This work has also shown the versatilely of FETAX in determining the developmental toxicity of compounds.

The developmental toxicity of α-chaconine and α-solanine were tested in FETAX as well as their hydrolysis products in attempt to determine the importance

of the carbohydrate subunits. α-Chaconine and α-solanine induced developmental toxicity was reduced as sugar groups were removed. Also the orientations of the sugar groups were important to toxicity as shown by the different toxicity of the β_1 and B_2 chaconines. Therefore, the carbohydrate part of the α-chaconine and α-solanine seem to play an important role in the developmental toxicity of these compounds. It is not known if this reduction is because of a change of the molecule at the active site or if it is because of a change in transport of the α-chaconine and α -solanine to their site of action. These data show that FETAX can be used to identify structure activity relationships of chemicals.

To determine if α-chaconine and α-solanine act synergistically or antagonistically, several mixtures of these chemicals were tested. Mixture testing is important in food chemistry because when humans are exposed to α-chaconine and α-solanine, it is most often through potatoes and they are exposed to both together, rather than individually. FETAX demonstrated its usefulness in determining the synergism that occurs between these two compounds. FETAX has also demonstrated that the developmental toxicity due to one mixture ratio may not be predicted by another mixture. Therefore, to determine the developmental toxicity of a particular mixture of α-chaconine and α-solanine, that mixture must be tested. These results have shown that α-chaconine and α-solanine act synergistically. These results show the importance of testing for interactions as well as the usefulness of FETAX in determining if compounds act synergistically.

To investigate why a rat liver microsomal enzymes seem to reduce the developmental toxicity of α-chaconine and α-solanine, but CO-gased microsomes did not function in increasing the α-chaconine and α-solanine developmental toxicity, several experiments were performed to determine why there was an

apparent discrepancy. These experiments were performed and it was noted that the generator system alone seems to reduce the development toxicity. The experiments that followed showed that glucose-6-phosphate and NADP allow reduced developmental toxicity. For this reason, when using a metabolic activation system, controls using the generator alone with the test chemical need to be tested. This addition would mean for a standard metabolic activation test there should be the following controls: (a) FETAX solution controls; (b) metabolic activation system controls; (c) cyclophosphamide positive controls; (d) cyclophosphamide negative controls; (e) test chemical negative controls; (f) test chemical with generator system. With these controls, it should be possible to determine metabolic activation with greater accuracy.

These results have shown that FETAX is useful in identifying possible mechanisms of action of compounds and complex mixtures. Although exact mechanisms of action have not been detailed, these results point to some very interesting directions in determining mechanism of action for the toxicity of GAs. Sugar groups seem to affect the toxicity of the glycoalkaloids. Removal of them decreased the developmental toxicity, and then when glucose-6-phosphate was added again, developmental toxicity decreased. With these results, it is possible to plan experiments to find out how glucose-6-phsophate in solution is reducing the developmental toxicity. FETAX has generated considerable information on the developmental toxicity of α-chaconine and α-solanine that has shown that FETAX is useful in accessing food toxicity.

VITA 2

James Robison Rayburn

Candidate for the Degree of

Doctor of Philosophy

Thesis: AN EVALUATION OF THE DEVELOPMENTAL TOXICITY OF POTATO GLYCOALKALOIDS USING THE FROG EMBRYO TERATOGENESIS ASSAY-XENOPUS

Major Field: Zoology

Biographical:

Personal data: Born in Alexandria, LA, December 17, 1965, the son of Dr. A.L. and Flossie Rayburn.

Education: Graduated from Buckeye High School, Buckeye, LA, in May of 1983. Graduated from Louisiana College, Pineville LA with a Bachelor of Science degree in Biology-Chemistry, with a minor in Math in May 1987. Received Master of Science degree in Zoology from Oklahoma Sate University, Stillwater, Oklahoma, in May 1990. Completed the requirements for the Doctor of Philosophy degree with a major in Zoology at Oklahoma State University in May 1994.

Professional Experience: Teaching Assistant, Department of Zoology, Oklahoma State University, January 1988- May 1994, and August 1993 to May 1994. Research Assistant for Dr. J.A. Bantle 1988 to 1994.

Professional Memberships: National Society of Environmental Toxicology and Chemistry; Ozark-Prairie Regional chapter of the Society of Environmental Toxicology and Chemistry.