FETAX VALIDATION AND SPECIES DIFFERENCES IN DEVELOPMENTAL TOXICITY OBSERVED WITH *XENOPUS LAEVIS* AND *PIMEPHALES PROMALES* EMBRYOS

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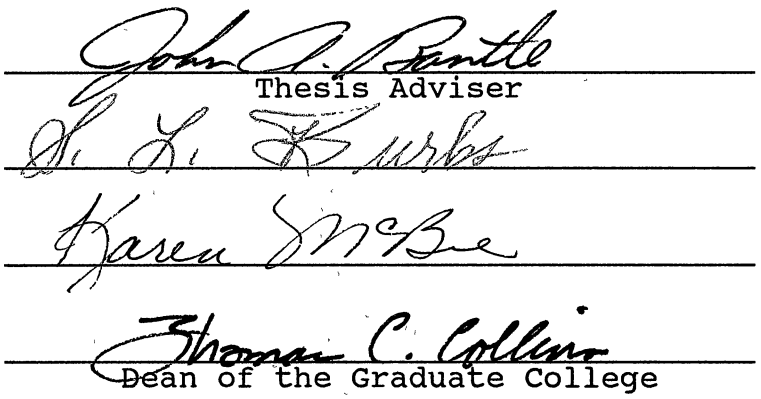

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PREFACE

The purpose of this study was twofold. One aspect was to continue validation of the Frog Embryo Teratogenesis Assay: Xenopus (FETAX). The second aspect was to develop a developmental toxicity species comparison test of early life stages between the African clawed frog (Xenopus laevis) and the fathead minnow (Pimephales promelas).

FETAX is a 96-hr whole embryo bioassay designed to evaluate pure compounds or mixtures. Fetotoxicity, teratogenicity, and effect on growth are the parameters explored. Validation enables FETAX to be useful as a rapid, routine test of developmental toxicity. In this study validation was performed by testing five compounds (ascorbic acid, sodium selenate, coumarin, serotonin, and 13-cis retinoic acid) and comparing the results with data from mammalian literature. The results of this portion of the study were consistent with mammalian data and support the use of FETAX for the screening of developmental toxicants.

The second aspect of this study, a species comparison test, was performed in order to compare Pimephales promelas and Xenopus laevis. This study of species sensitivity differences in developmental toxicity was performed using sodium acetate, caffeine, and 5-fluorouracil. An exposure

time of 120 hrs allowed for similar embryological events for each species to occur during the test. The relative amount of uptake of chemical by each species was determined by liquid scintillation spectrometry of whole fish and frog residue.

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CHAPTER I

INTRODUCTION

Overview

FETAX, the Frog Embryo Teratogenicity Assay: (Xenopus), has wide applications in assessing human health hazards and is a useful approach to studying environmental toxicology. The impact of anthropogenic contamination and the number of teratogens present in the human environment has necessitated the development of routine teratogenicity testing that is rapid and inexpensive. FETAX was initially developed by Dumont et al. (1983) to evaluate the potential developmental toxicity of chemicals to the frog embryo. FETAX has been used with environmental mixtures (Dumont et al., 1983; Bantle et al.,1989a), heavy metals (Dawson et al., 1985; Dawson et al., 1988a), and pharmaceuticals and pure compounds (Courchesne and Bantle, 1985; Dawson and Bantle, 1987a; Sabourin and Faulk, 1987; Fort et al., 1988; Dawson et al., 1988b; Dawson et al., 1989).

FETAX is a 96-hr whole embryo bioassay designed to evaluate compounds or mixtures for their fetotoxicity, teratogenicity, and effect on growth. The data generated are relevant to human health (Smith et al., 1983) and to ecotoxicology.

Validation of FETAX is performed by comparing mammalian data with the results obtained from FETAX so that FETAX can become a fully developed and reliable teratogenesis screening assay. FETAX has a predictive accuracy of greater than 85% when tested against a variety of known mammalian teratogens and nonteratogens (Bantle et al., 1989b; Sabourin and Faulk, 1987; Dawson and Bantle, 1987a; Courchesne and Bantle, 1985). The predictive accuracy is increased to approximately 92% when metabolic activation system tests are included.

A species comparison between the African clawed frog (Xenopus laevis) and the fathead minnow (Pimephales promelas) is an additional aspect which was explored in this investigation. Because of the number of tests being performed with aquatic organisms, an interspecies comparison study is necessary to investigate the sensitivity of different organisms. In addition, the fathead minnow is a standard aquatic test organism and makes an excellent basis of comparison.

Xenopus laevis was chosen as a test animal because of the reasons stated above, while the fathead minnow was chosen because it is used in many laboratories as an assay fish for determining the acute and chronic toxicity of complex environmental mixtures (Dawson et al., 1988a) and pure compounds (Benoit et al., 1982; Holcombe et al., 1982; Birge at al., 1985; and Norberg and Mount 1985).

The use of fish and amphibian embryos together as test organisms offer several advantages when evaluating the teratogenicity of chemicals or environmental mixtures. The primary focus of this part of the investigation was to establish the sensitivity differences of each species given the same length of exposure and relative embryological stages, and to determine the relative amount of chemical uptake by each species.

Specific Objectives

Continued validation of FETAX with known mammalian teratogens and nonteratogens is important to its success as a screening assay. Validation of FETAX depends upon the repeatability of results, interlaboratory data, and comparisons with compounds previously tested in mammals. The first part of this study investigated the teratogenicity of five pure compounds using FETAX. These compounds were ascorbic acid, sodium selenate, coumarin, serotonin, and 13-cis retinoic acid. Standard FETAX protocol was followed.

The purpose of the second part of this study was to compare the sensitivities of Xenopus laevis and Pimephales promelas to several chemical samples by designing a frog and fish embryo teratogenesis bioassay. The assay was designed so that similar embryological stages of each species were exposed for the same length of time. As a result, toxicant sensitivities between the two species

could then be determined by testing several chemical samples with radioactive labeling. The relative amount of uptake by each species was compared to the effects of the test materials upon growth, malformation, and mortality.

Compounds tested with the frog and fish assay included a mammalian nonteratogen (sodium acetate), moderate teratogen (caffeine) and strong teratogen (5-fluorouracil). FETAX 96-hr data was available for caffeine and 5-fluorouracil. Similar data was unavailable for fish. A range and at least two definitive tests were conducted for each of the compounds.

Experimental Design

Standard FETAX for Validation Purposes

As stated earlier, FETAX is a 96-hr static-renewal test useful for the detection of developmental toxicants. Initial versions of the assay were originally developed and applied by Greenhouse (1976), FETAX has been used to assess environmental mixtures, pure compounds, compounds with metabolic activation, and solvent interactions.

Repeatability of results and developmental relevance are an attractive feature of FETAX. The validation of FETAX depends upon obtaining results that are correlative of mammalian studies. Sabourin and Faulk (1987) found that teratogens caused similar malformations in mammals and in Xenopus. Dumont et al. (1983) found that hydrocephaly was induced by meclizine in both frogs and mammals, and

Courchesne and Bantle (1985) found that several genotoxic compounds caused the same general results in FETAX as in rodent embryos.

The protocol for standard FETAX allows for a 96-hr exposure period. In order to obtain viable embryos, frogs were stimulated to breed by injection of human chorionic gonadotropin. The eggs were collected, treated with cysteine to remove the jelly coat, and sorted according to developmental stage. The eggs were exposed to differing concentrations of each chemical and the test material was renewed every 24 hrs. At the end of the 96-hr period, mortality and malformation were recorded and head-tail lengths were measured. Teratogenic hazard was determined by dividing the LC50 (lethal concentration) by the EC50 (malformation) to obtain a TI (Teratogenic Index) value. Compounds with a TI greater than 1.5 are considered to have teratogenic potential.

Compounds tested with standard FETAX included coumarin, 13-cis retinoic acid, ascorbic acid, and serotonin. These compounds were chosen for their differing teratogenic values, availability, and for known mammalian developmental toxicity.

Frog and Fish Embryo Bioassay

The standardized protocol of FETAX was modified so that conditions were satisfactory for an embryological comparison between Xenopus laevis and Pimephales promelas.

The frog and fish assay closely followed standard FETAX except for the length of exposure time, use of a photoperiod, and the use of a modified FETAX solution (Dawson et al. 1988a). Both frogs and fish were exposed to sodium acetate, caffeine, and 5-fluorouracil and were treated under the same conditions.

The exposure period chosen for this investigation was five days or approximately 120 hrs. This is longer than the four day or 96-hr period of exposure for standard FETAX using frog embryos and shorter than the EPA embryo-larval survival and teratogenicity assay using fish embryos which has an eight days or 184 hr exposure period (USEPA, 1989). Xenopus laevis eggs 7 to 9 hrs old (large-cell to small-cell blastula stages) and Pimephales promelas eggs 8 to 10 hrs old (32-cell blastodisc to high blastula stage) were exposed at the start of the test. By 120 hours, both the frog tadpoles and most of the fish embryos were hatched, free-swimming, and had undergone major organogenesis. The developmental stages for both species are similar through this period of time. However, organogenesis rates and order of development differs among several organ systems and may account for malformation differences seen or other sensitivity differences.

Radiolabeling Assay Protocol

After completing the definitive tests, Xenopus laevis and Pimephales promelas embryos were tested with

radiolabeled ^{14}C sodium acetate, caffeine, and 5-fluorouracil in order to compare the relative amounts of uptake. Two control dishes with 10 embryos each were used for both frog and fish. Each radioisotope was tested in a dish containing 10 frog or 10 fish embryos. A preliminary test conducted with sodium acetate determined that an addition of 11×10^6 disintegrations per minute (dpm) to the dish gave sufficient radioactivity to be detected at the end of the test. Therefore, for the final definitive radiolabeled assay, 11×10^6 counts of each chemical were added to the appropriate dishes. 100 μl of SA (concentration of 0.05 mCi/ml); 50 μl of CAF (0.05 mCi/0.5 ml) and; 25 μl FLU (0.2 mCi/1 ml) were added to each dish.

The test was conducted statically without renewal. Radiolabeled chemicals were added once at the beginning of the test on day 0 to covered dishes containing frog embryos at small cell blastula stage and fish embryos at high blastula stage. Dead embryos were removed daily. At day 5, the test was ended.

Whole frog and fish larvae were sampled at the end of the test. The number of frogs (45 out of 50: 90%) and fish (42 out of 50: 84%) analyzed depended on mortality during the test and animals damaged or lost during processing.

Frog and fish larvae were carefully rinsed to remove adsorbed chemicals and were wet weighed before analysis. Samples were solubilized by placing each animal into a small tube, adding TS-1 tissue solubilizer and allowing the

samples to digest over two days. A neutralizer cocktail was then added, the contents of the tube mixed, and the samples were then analyzed using a Beckman LS-3100 Series Liquid Scintillation Counter. A quench curve determined that little or no quench was occurring.

Counts per minute (cpm) were determined for each sample consisting of an individual fish or frog. CPMs were divided by the mean wet weight of fish or frogs to give the counts per body weight (cpm/mg) for each species.

Statistical Analysis of Data

Probit analysis (Tallarida and Murray, 1980) determined the 96-hr LC50 (median lethal concentration), 96-hr EC50 (concentration inducing malformations in 50% of the surviving embryos) and 95% confidence intervals for each test compound. In order to compare and assess levels of teratogenic potential, a Teratogenic Index [TI=LC50/EC50(malformation)] was determined (Courchesne and Bantle, 1985; Dawson and Bantle, 1987a; Dawson et al., 1989; Fort et al., 1988; Bantle and Dawson, 1988; and Dawson et al., 1988b).

Head-tail length (growth) data was collected at the end of each definitive test using an IBM-compatible computer equipped with digitizing software (Jandel Scientific, Corte Madera, CA). The Minimum Concentration to Inhibit Growth (MCIG) was calculated using the t-Test for grouped observations ($p<0.05$).

Counts per minute (cpm) were determined for each sample consisting of an individual fish or frog. CPMs were divided by the mean wet weight of fish or frogs to give the counts per body weight (cpm/mg) for each species.

In order to make comparisons between frog and fish responses (i.e. how much greater one responded over the other), potency ratios were obtained for uptake counts, malformation, mortality and growth. The uptake counts, EC50s, and LC50s were compared between species using the t-test for grouped observations.

CHAPTER II

LITERATURE REVIEW

Introduction

This chapter covers basic concepts and research that apply to the second portion of the study--the frog and fish species comparison study. Species selection and abnormal development in frogs and fish are explained. The test designed for this study was a 5 dy (120 hr) bioassay and the reasons for this are discussed in the exposure time and toxicant sensitivity section. The use of isotopes and previous uptake studies employing radiolabeling compounds with fish and amphibians are also cited here. Detailed information regarding the validation of FETAX and the specific chemicals used in the tests can be found in Chapters III and IV.

Species Selection

In order to conduct a species comparison assay and to determine sensitivity differences, as many test factors as possible must be kept constant. Resistance to a toxicant (thus the test results) can be affected by species, strain, previous exposure, age, size, health, and animal handling

procedures (Adelman and Smith, 1976). Xenopus laevis and Pimephales promelas were chosen as test animals because their similar developmental patterns could allow them to be exposed for the same length of time. They were also easily available and adaptable to an interspecies study.

The amphibian embryo has become a classical model for experimental embryology studies and much is known about its development (Nieuwkoop and Faber, 1975). The African clawed frog (Xenopus laevis) has been used successfully as a research animal for many decades. This species of frog is easy to rear and breed and most importantly, the eggs that are laid are useful for developmental toxicity studies. The Frog Embryo Teratogenesis Assay-Xenopus (FETAX) is a 96-hour in-vitro assay that allows for exposure of the frog embryo from the small cell blastula stage to the free-swimming tadpole stage at which the organism has undergone organogenesis.

The fathead minnow, Pimephales promelas, is an important forage fish and is a member of the Cyprinidae--the largest family of freshwater fishes in North America (Scott and Crossman, 1973). Fathead minnows have been tested for survival, growth, and reproductive responses but little work has been done with teratogenic assays. Birge et al.(1985) and McKim et al.(1977) have developed toxicity tests using the early life cycle stages of fish and amphibians. Benoit et al. (1982) found that early life stage toxicity tests with fathead minnows could

be used to predict long-term chronic toxicity. McKim et al. (1977) has shown that the embryo-larval life stages of fish are among the most sensitive tests and can be used in estimating the maximum acceptable toxicant concentration.

Abnormal Development in Frogs and Fish

There exist today over 600 known teratogenic agents in the environment that can cause developmental abnormalities in experimental animals (Shepard, 1980). A teratogen causes malformations which are abnormalities that occur during the development of an embryo. These can occur at sublethal doses of a toxicant because the normal embryo is a highly complex physical and chemical system that is dependent on and in equilibrium with its environment (Laale, 1981).

Embryological development depends upon genetic information that is time dependent and occurs in an orderly fashion. Developmental toxicants can affect the normal processes of cell division, interaction, migration, differentiation, and selective death. The process of malformation is complicated and the details of developmental inhibition in fish embryos by teratogens are not entirely known (Laale, 1971). In general, the normal development of a fish embryo can be affected by cellular contacts, movement of yolk, and molecular mechanisms (Laale, 1981). Similar mechanisms can occur in Xenopus embryos.

Amphibians and fish can be tested together in developmental toxicity assays because they exhibit similar trends in malformations when exposed to various agents. Vertebrate embryonic development has common origins and therefore one would expect that a developmental toxicant would have similar effects on homologous vertebrate structures if the organisms are exposed during the time these structures are being formed (Cameron, et al., 1985).

The malformations most often noted in fish include cephalic and vertebral system defects, dwarfing, ophthalmologic abnormalities, jaw disorders, and cardiac abnormalities. The skeletal system is commonly affected by inorganic and organic toxicants (Birge et al. 1983; Solomon and Faustman, 1987). The malformations occurring in Xenopus embryos have been cataloged and broken down into general categories. These include axial malformations, blistering and edema, eye abnormalities, extreme head abnormalities, head and face abnormalities, heart malformations, and gut abnormalities (Bantle et al., 1991).

Concentration Responses

FETAX determines teratogenic potential by comparing Teratogenic Index [TI=LC50/EC50(malformation)] values, embryo growth, and the type and severity of induced malformations. In general, TI values < 1.5 indicate low teratogenic potential and higher values indicate an increase in the potential hazard (Bantle and Dawson, 1988;

Courchesne and Bantle, 1985; Dawson and Bantle, 1987; Dawson et al., 1988b; Dawson et al., 1989; and Fort et al., 1988). With higher TI values, the mortality and malformation dose-response curves become separated and the potential for the production of deformed embryos in the absence of lethality increases (Dawson and Bantle, 1987a).

For each chemical tested in the study, an EC50 (malformation) and LC50 (mortality) were calculated and plotted as concentration-response data. Comparisons between different agents may depend upon the chemical structure of the agent and how closely they are related. In a study using Japanese medaka (Oryzias latipes), four model alkylating agents were tested on medaka embryos following a procedure similar to FETAX. Malformation and mortality dose-response curves did not vary significantly between agents having similar chemical patterns. However, they did vary between agents having different mechanisms of action. (Solomon and Faustman, 1987).

Developmental toxicity may also be assessed by considering the Minimum Concentration to Inhibit Growth (MCIG expressed as % compound LC50) (Courchesne and Bantle, 1985; Dawson and Bantle, 1987a; Dawson et al., 1989). Rates of growth inhibition (i.e. slope) and overall reduction in embryo growth vary with the severity of the teratogen. Dawson et al. (1989) suggest that compounds with significant teratogenic potential generally inhibit

growth at concentrations < 30% of the respective LC50 values.

Growth was the most sensitive indicator of stress in a study of the effects of phenolics on the early life stages of the fathead minnow (Holcombe et al, 1982). Norberg and Mount (1985) also found growth to be a more sensitive indicator in tests with fathead minnows.

Exposure Time and Toxicant Sensitivity

One question addressed in this investigation is how exposure time affects toxicant sensitivity and species differences. In a study of the effects of mercury with six fish species, Birge et al.,(1979) found that the frequencies of malformations increased with longer exposure times of egg to hatching. All fish were exposed through hatching plus four days. This exposure period varied as the average hatching time was three days for sunfish, four days for bass and goldfish, six days for catfish, and 24 days for trout.

The exposure period of egg to hatching plus four days is used in the EPA embryo-larval survival and teratogenicity test (USEPA, 1989). Birge et al. (1985) found that this amount of time appeared adequate to determine the sensitivity of the test organism to the sample and also was short enough to preclude the need for feeding. As can be seen, however, the egg to hatching

times can vary widely between species and not all fish or amphibians hatch at the same stage of development.

Different exposure times were used in a study of the effluents of Tar Creek and the Neosho River, Oklahoma, (Dawson et al, 1988a) with fathead minnows and Xenopus embryos exposed to metal-contaminated sediment extracts and zinc sulfate. Minnows were exposed for six days starting with the gastrula stage and continuing through hatching. Xenopus embryos were exposed starting with the blastula stage for four days. The types of malformations seen in both test species were similar, the TI values [Teratogenic Index=LC50/EC50 (malformation)] indicated that the test sample was teratogenic to both species, and minnows were found to be more sensitive.

Balon (1984) explains fish development as a series of rapid changes called thresholds, which separate one steady state from the next. He states "Hatching is rarely a developmental threshold. The transition to exogenous feeding, rather than hatching, is the decisive threshold of ultimate survival value." Hatching is often proclaimed to be the end of the embryo period but Balon states that hatching can be affected by external events which can be separate from development. Experimental evidence by Yamagami (1981) supports Balon's view.

Obviously not all organisms will develop at the same pace and thus if one wants the exposure period to cover the same development stages, different time periods may be

necessary. Mancini (1983) suggested a method for calculating the effects of time variable exposure on aquatic organisms. He observed that organisms die at different times and that there is a distribution of sensitivity among the organism population. This sensitivity difference can be attributed to chemical's rate of entry into the organism, the rate of detoxification, or the concentration in the individual organism which causes mortality. This was one reason why radiolabeled compounds were incorporated into this study--to account for the chemical uptake into the organism.

Use of Isotopes

Isotopes have been used widely in biology and agriculture. The selection of isotopes depends largely upon what is available. When choosing an isotope the effect of radiation upon the organism being studied must be taken into account. The carbon isotope is a soft beta emitter and should not produce radiation effects when used in small amounts. When quantifying the radioactive material, liquid scintillation counting is satisfactory for low energy emitters (Peterle, 1966).

Sodium acetate CAS# 6131-90-4 (SA), caffeine CAS# 58-08-2 (CAF), and 5-fluorouracil CAS# 51-21-8 (FLU) were chosen as test compounds for the species comparison study based on their developmental toxicity, cost, and available mammalian data. Test compounds were obtained for initial

testing from Sigma (St. Louis, MO). Radiolabeled [^{14}C] SA was obtained from ICN Biomedicals, Inc. and had a radiochemical purity of > 98% (specific activity: 7.1 mCi/mmol). Caffeine, [1-methyl-^{14}C] was obtained from NEN Research Products with a radiochemical purity of 97% (specific activity: 55.7 mCi/mmol). 5-fluorouracil-2-^{14}C was obtained from Sigma Chemical Company with a radiochemical purity of >98% (specific activity: 22 mCi/mmol).

Uptake Studies with Radiolabeled Chemicals

Several studies have attempted to explain toxicological differences between species using rate of uptake, bioconcentration, and toxicant distribution and elimination as the basis for comparison. Previous studies have used radiolabeled chemicals and liquid scintillation counting to evaluate the rate of uptake, bioconcentration, distribution, and elimination in aquatic organisms (Werner and Kimerle, 1982; Muir et al., 1985; and Ingebrigsten, 1988) and in fathead minnows (Mayes, 1985).

Spacie and Hamelink (1982) described the rate of uptake in fish as it applied to the bioconcentration of organics. They found that several factors can influence uptake, including environmental and physiological factors that affect ventilation volume, metabolic rate, temperature, and fish behavior.

In a study of the distribution and elimination of ^{14}C-octachlorostyrene, Ingebrigtsen (1988) found differences in the tissue distribution pattern between two species of fish--cod (Gadus morhua) and rainbow trout (Salmo gairdneri). The isomers of 2,3,7,8-tetrachlorodibenzo-p-dioxin have been investigated in rainbow trout and fathead minnows with data concentrating on bioconcentration, metabolism, and bioaccumulation (Muir et al. 1985). In this study, lower rates of uptake were found at higher concentrations. The authors postulated that toxic effects exerted on the fish reduced the rates of respiration and metabolism, which resulted in decrease uptake rates.

Toxicological studies have been performed on rainbow trout, channel catfish, and bluegills using benomyl and carbendazim (Palawski and Knowles, 1986). Both fungicides were more toxic to catfish and less toxic to trout and bluegills. The study included acute toxicity tests, metabolism, and residue dynamic studies. It was concluded that the differential toxicity could not be explained by residue dynamic studies and it was likely that the selective toxicity to catfish was associated with events stemming from the pharmacodynamic phase of toxicant action.

In another test by Veith et al. (1979), adult fathead minnows, rainbow trout fingerlings, and juvenile green sunfish were exposed to organic chemicals. The bioconcentration factors were found to be temperature dependent in some cases.

Uptake studies with amphibians are scarce in the literature. However, one study dealt with the uptake of ^{14}C DDT [1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane] by wood frog embryos (Licht, 1985). Embryos at different stages and temperatures were exposed to radiolabeled DDT and it was found that the jelly capsules restricted the uptake of DDT.

In another species comparison study by Spehar (1983) bioconcentration factors of synthetic pyrethroids were determined for fathead minnows and snails. The residue analysis was conducted on a whole body basis with gas chromatography. Mean residue concentrations and bioconcentration factors were higher in fathead minnows than in snails, and minnows were more sensitive. The difference in bioaccumulation was attributed to differences in uptake, water temperature and lipid content.

CHAPTER III

ASSESSMENT OF THE DEVELOPMENTAL TOXICITY OF ASCORBIC ACID, SODIUM SELENATE, COUMARIN, SEROTONIN, AND 13-CIS RETINOIC ACID USING FETAX

Introduction

The Frog Embryo Teratogenesis Assay: Xenopus (FETAX) is a rapid, cost-effective alternative to mammalian teratogenesis assay systems and is helpful for identifying developmental toxicants (Dumont et al., 1982). Data may be used for prioritizing samples for further tests which currently use mammals.

FETAX is a 96-hr static-renewal assay capable of determining the developmental toxicity of pure compounds (Courchesne and Bantle, 1985; Dawson and Bantle, 1987a; Dawson et al., 1989) and complex mixtures (Dawson et al., 1985; Dawson et al., 1988a; Bantle et al., 1989). In addition to FETAX, a successful metabolic activation system (MAS) has been developed (Fort et al., 1988; Bantle and Dawson, 1988) and applied to the study of toxicological mechanisms of teratogenesis (Dawson et al., 1988b; Fort and Bantle, 1990). In this report, the developmental toxicity

of five compounds is evaluated using FETAX and the results are compared to mammalian literature.

Materials and Methods

Animal care, frog breeding and embryo collection were performed according to Dawson and Bantle (1987a). Sets of 20 or 25 embryos were placed in 60-mm covered glass Petri dishes with a range of concentrations appropriate to each test compound (Sigma Chemical Co., St. Louis, MO) dissolved in FETAX solution (Dawson and Bantle, 1987a). For each compound 8 to 16 concentrations were tested in duplicate. Embryos were exposed to FETAX in four separate control dishes of 20 or 25 embryos each. Treatment and control dishes contained a total of 10 ml of solution for 25 eggs exposed and 8 ml for 20 eggs exposed.

Dimethyl sulfoxide (DMSO) was used as a solvent for coumarin at a concentration less than 1.1% v/v which has been found not to cause any adverse effects in FETAX. The possibility of interactions between DMSO and coumarin causing altered rates of mortality and malformation cannot be completely discounted.

Because of the relative insolubility of 13-cis retinoic acid, stocks were prepared by measuring 1 mg into 1 L FETAX solution, stirring and filtering with 0.45 μm Millipore filter paper. The stock concentration was then determined by spectrophotometry with a wavelength setting of 354 nm and extinction coefficient of 39,800 (Merck, 1983).

At least one range and three definitive concentration-response tests were conducted for each compound. Tests run separately by different technicians were analyzed together statistically. The pH of all compounds tested was between 7.0 and 8.0. Embryos were cultured at 24^{o} C $\pm$ 1 throughout the test.

Solutions were renewed every 24 hr for a total exposure time of 96 hr and dead embryos were removed daily. At the termination of the tests, surviving embryos were anesthetized with 3-aminobenzoic acid ethyl ester (methanesulfonate salt) and fixed with 3.0% (w/v) formalin. The number of dead, number and type of malformations, and developmental stages were recorded and determined using a dissecting microscope (Nieuwkoop and Faber, 1975).

Probit analysis (Tallarida and Murray, 1980) determined the 96-hr LC50 (median lethal concentration), 96-hr EC50 (concentration inducing malformations in 50% of the surviving embryos) and 95% confidence intervals for each test compound. In order to compare and assess levels of teratogenic potential, a Teratogenic Index [TI=LC50/EC50(malformation)] was also determined (Courchesne and Bantle, 1985; Dawson and Bantle, 1987a; Dawson et al., 1989; Fort et al., 1988; Bantle and Dawson, 1988; and Dawson et al., 1988b).

Head-tail length (growth) data were collected at the end of each definitive test using an IBM-compatible computer equipped with digitizing software (Jandel

Scientific, Corte Madera, CA). The Minimum Concentration to Inhibit Growth (MCIG) was calculated using the t-Test for grouped observations ($p<0.05$).

Results

Final results from the definitive tests with FETAX are presented in Table 1. Representative concentration-response and growth-inhibition curves for the five compounds are illustrated in Figures 1 and 2, respectively.

In this investigation the FETAX solution control mortality and malformation rates were 44 of 1300 (3.4%) and 72 of the 1256 survivors (5.7%), respectively. Control data for dimethyl sulfoxide, the solvent used in the testing of coumarin, was 1 of 130 (0.8%) for mortality and 5 of 129 survivors (3.8%) for malformation. Acceptable rates of control mortality and malformation are generally < 10%.

The most common malformation induced by ascorbic acid was failure of the gut to coil. At concentrations > 10 mg/ml loose gut coiling was common along with slight musculoskeletal kinking. At concentrations > 13 mg/ml facial, eye and brain malformations were noted. Growth was stunted and severe malformations of the gut, musculoskeletal system, face, eye and heart occurred at concentrations > 19 mg/ml.

TABLE 1.

DEVELOPMENTAL TOXICITY OF FIVE COMPOUNDS TESTED WITH FETAX

Compound	Test #	LC50[a]	EC50[b]	TI[c]	MCIG[d]	MCIG[e]
Ascorbic	1	19.2	11.6	1.7	10.0	52
acid		(17.8-20.7)	(10.2-13.2)			
	2	20.3	12.8	1.6	10.0	49
		(18.7-21.9)	(12.4-13.3)			
	3	19.6	12.0	1.6	10.0	51
		(18.9-20.3)	(10.4-13.8)			
Sodium	1	0.017	0.006	3.0	0.014	82
selenate		(0.016-0.017)	(0.002-0.013)			
	2	0.019	0.007	2.8	0.006	32
		(0.017-0.019)	(0.006-0.008)			
	3	0.027	0.009	3.1	0.008	30
		(0.026-0.029)	(0.006-0.012)			
Coumarin	1	0.15	0.038	4.0	0.01	7
		(0.14-0.15)	(0.025-0.059)			
	2	0.14	0.038	3.5	0.05	36
		(0.13-0.14)	NA			
	3	0.10	0.045	2.2	0.04	40
		NA	(0.037-0.055)			

TABLE 1. (Continued)

Compound	Test #	LC50[a]	EC50[b]	TI[c]	MCIG[d]	MCIG[e]
Serotonin	1	2.74	0.35	7.8	0.25	9
		(2.55-2.93)	(0.19-0.66)			
	2	3.27	0.39	8.4	0.6	18
		(3.18-3.36)	(0.21-0.72)			
	3	3.21	0.48	6.7	1.0	3
		NA	(0.43-0.54)			
13-Cis retinoic	1	37.3×10^{-9}	1.98×10^{-9}	18.8	71×10^{-9}	19
acid		($24.5\text{-}56.5 \times 10^{-9}$)	($1.50\text{-}2.61 \times 10^{-9}$)			
	2	18.3×10^{-9}	1.99×10^{-9}	9.2	20×10^{-9}	NA
		($15.5\text{-}21.6 \times 10^{-9}$)	($1.27\text{-}3.10 \times 10^{-9}$)			
	3	35.7×10^{-9}	3.54×10^{-9}	10.1	10×10^{-9}	28
		($34.1\text{-}37.4 \times 10^{-9}$)	($3.13\text{-}4.00 \times 10^{-9}$)			

[a] LC50, mortality, mg/ml with (95% C.I.)

[b] ED50, malformation, mg/ml with (95% C.I.)

[c] Teratogenic Index (LC50/EC50)

[d] Minimum Concentration to Inhibit Growth as mg/l.

[e] Minimum Concentration to Inhibit Growth as a percent of LC50.

NA Not available.

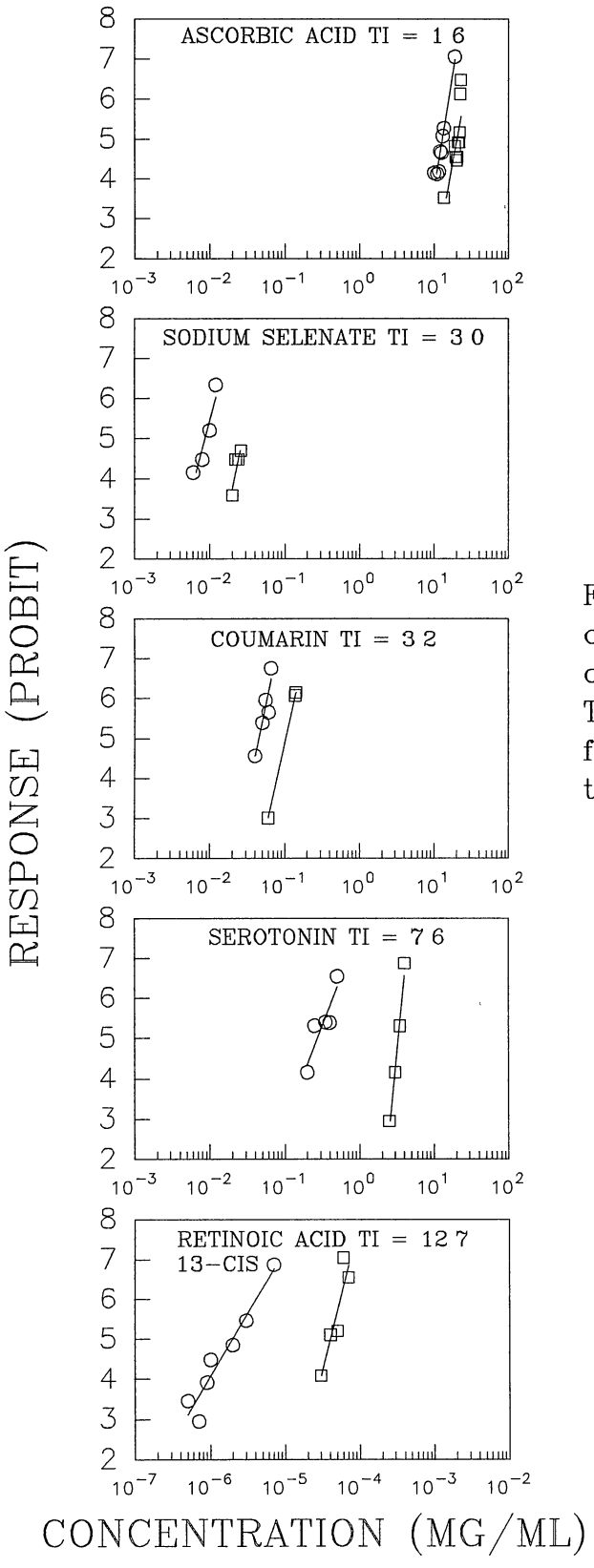

Figure 1 Representative concentration–response curves and respective Teratogenic Index values for the five compounds tested with FETAX

○ Malformation
□ Mortality

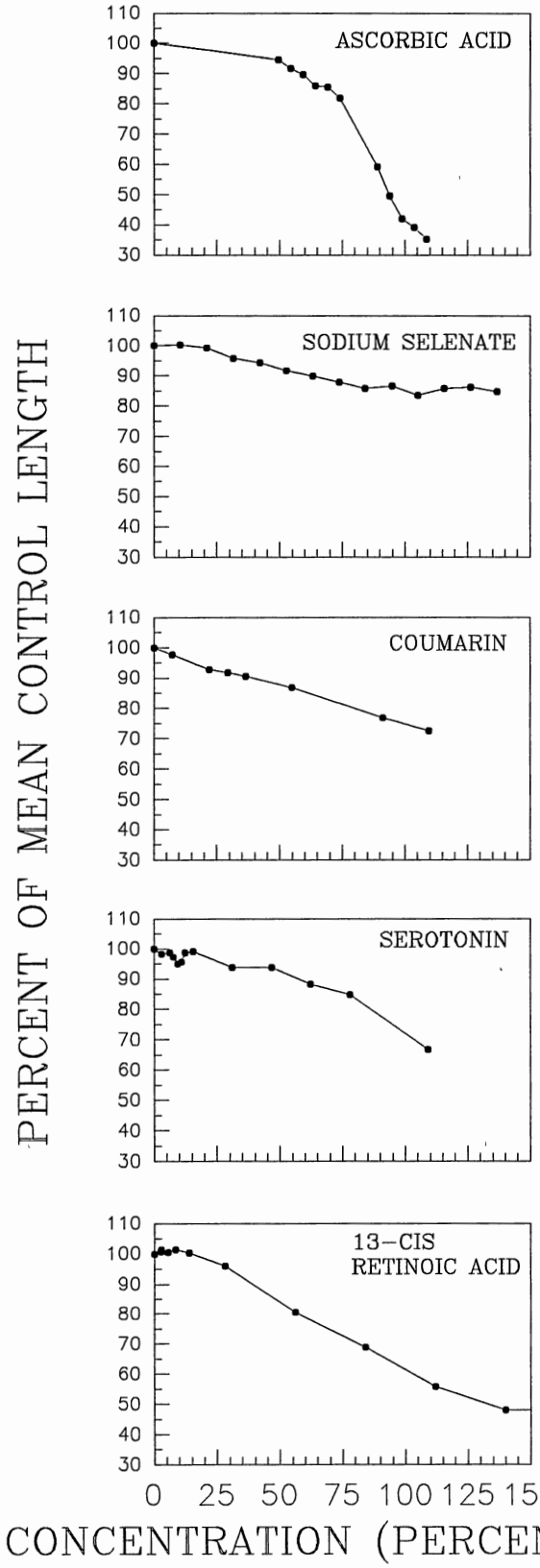

Figure 2. Representative embryo growth curves for the five compounds

Concentrations are expressed as percent of the respective compound LC50.

Growth is expressed as percent of mean FETAX solution control length

Sodium selenate at concentrations > 0.002 mg/ml gut, heart and face. Blistering was evident at concentrations > 0.012 mg/ml.

Coumarin induced musculoskeletal kinking, loose gut coiling and craniofacial malformations at concentrations > 0.01 mg/ml. Concentrations > 0.04 mg/ml induced craniofacial malformations consisting of a reduced head size and downward tilting of the head. Edema, eye and gut malformations occurred at concentrations > 0.07 mg/ml. The malformations mentioned above became more severe at concentrations > 0.13 mg/ml.

Serotonin caused minor malformations and stunting at most of the concentrations tested. Embryos gradually became smaller, shorter in length and less developed with blunter nose and looser gut coil. At concentrations > 1.0 mg/ml, microencephaly and blistering of the dorsal fin were noticed. Embryos were severely stunted at concentrations > 3.0 mg/ml.

13-cis retinoic acid caused loose gut coiling and musculoskeletal kinking at concentrations > 0.5×10^{-6} mg/ml. Concentrations > 2.0×10^{-6} mg/ml resulted in eye and brain malformations. Cyclopia, eye pigment ruptures, edema, spinal kinking and craniofacial abnormalities were induced in concentrations > 10×10^{-6} mg/ml.

Discussion

FETAX determines teratogenic potential by comparing TI values, embryo growth, and the type and severity of induced malformations. In general, TI values < 1.5 indicate low teratogenic potential and higher values indicate an increase in the potential hazard (Courchesne and Bantle, 1985; Dawson and Bantle, 1987a; Dawson et al., 1989; Fort et al., 1988; Bantle and Dawson, 1988; and Dawson et al., 1988b. With higher TI values, the mortality and malformation dose-response curves become separated and the potential for the production of deformed embryos in the absence of lethality increases (Dawson and Bantle, 1987).

In this investigation ascorbic acid exhibited an overlapping of the curves as presented in Figure 1. It is, therefore, considered embryolethal at high concentrations and is not a potential teratogen. Sodium selenate and coumarin represent compounds with increasing separation of the curves and potential teratogenic hazard. Serotonin and 13-cis retinoic acid exhibit even wider separation of the curves, and are examples of compounds with strong teratogenic hazard. Because all chemicals are potential teratogens if administered in appropriate doses at sensitive stages of development (Karnofsky, 1965), it is important to consider the types and severity of terata and the concentrations at which they occur. Compounds with TI values < 1.5 may pose a hazard to developing organisms, possibly as embryotoxins.

Developmental toxicity may also be assessed by considering the MCIG (expressed as % compound LC50) (Courchesne and Bantle, 1985; Dawson and Bantle, 1987a; Dawson et al., 1989). Rates of growth inhibition (i.e. slope) and overall reduction in embryo growth vary with the severity of the teratogen. Dawson et al. (1989) suggest that compounds with significant teratogenic potential generally inhibit growth at concentrations < 30% of the respective LC50 values. Ascorbic acid begins to inhibit growth at > 50% of the LC50. Sodium selenate and coumarin cause inhibition between 28 and 48% of the LC50. Serotonin and 13-cis retinoic acid cause inhibition between 10% and 16% of each particular LC50. In addition, serotonin and 13-cis retinoic acid with the highest TI values show a sharper decrease in slope compared to the other compounds. This characteristic is presented in Figure 2 by the growth-inhibition curves.

The five compounds presented here have been selected as part of the validation process of FETAX because of the availability of mammalian literature for each (Shepard, 1986; Smith et al., 1983). Although FETAX results cannot be directly extrapolated to mammalian developmental toxicity tests, comparisons are beneficial in order for FETAX to be useful as a screening assay.

Ascorbic acid which tested negative in FETAX, has been tested with mice, rats and rabbits in studies following FDA Segment II guidelines. No effects were observed in rats up

to 500 or 1000 mg/kg/dy (Carpi and Scarinic, 1974; Frohberg et al., 1983). No effect was observed in mice up to 1000 mg/kg (Frohberg et al., 1983) nor in rabbits up to 500 mg/kg/dy (Carpi and Scarinic, 1974. Frohberg et al.(1983) administered up to 1000 mg by mouth to pregnant mice and rats on days 6-15 with no adverse effects found. In fact, ascorbic acid (ascorbate) has been found to protect against the embryolethality of N-acetoxy-2-acetylaminofluorene and 2-nitrosofluorene (NF), and decreased the number of flexure abnormalities caused by NF in a rat whole embryo culture system (Faustman-Watts et al., 1986).

FETAX results for sodium selenate also agree with the majority of mammalian literature reports. As a potential teratogen it tested positive in FETAX. Selenium induced malformations have resulted from livestock grazing on seleniferous ranges (Rosenfeld and Beath, 1939; Beath et al., 1939). Beath et al. (1939) reported malformations in lambs consisting of multiple cysts in eyes, microphthalmia and deformities of the extremities. Similar effects were reported with horses (Smith et al, 1936). However, early laboratory studies with rats and cats fed a continuous diet of selenium did not result in any malformations in the progeny (Westfall, et al., 1938).

As a result of the interest in the Kesterson Reservoir and Kesterson National Wildlife Refuge, Merced County, California, in which selenium contamination was a factor, several developmental toxicity studies have emerged.

Sodium selenite and selenomethionine were tested in the laboratory with mallards. Malformations found included hydrocephaly, microphthalmia, lower bill and foot defects, edema and stunted growth (Hoffman and Heinz, 1988). High rates of embryonic mortality and abnormalities were also reported in wild aquatic bird populations at Kesterson (Ohlendorf, 1979; Ohlendorf et al, 1986). An *in situ* study was conducted using 10 species of mammals collected from Kesterson Reservoir and a low rate of abnormalities was found (Clark, 1987). Nobunaga et al. (1979) conducted a study with mice in which the malformation rate of sodium selenite was not significantly different from controls. Species differences in developmental toxicity caused by selenium are apparent, however FETAX results agree with the species tested other than rats and mice and further studies are needed.

Coumarin is a compound which also has conflicting reports in the mammalian literature but is generally considered to be a developmental toxin, especially for humans. Coumarin-induced abnormalities are known as the fetal warfarin syndrome (Hall et al., 1980) and the most consistent malformations in humans have been described by Shaul and Hall (1977) as nasal hypoplasia, stippling of the bones, ophthalmologic abnormalities, intrauterine growth retardation and developmental delay. When administered to mice, coumarin elicited a low incidence of gross fetal malformations including cleft lip and cleft palate

(Knonick, 1974). A study on rabbits and mice found that coumarin adversely affected normal implantation and placentation, but no mention was made of any malformations (McCallion et al., 1971). In a similar study by Hirsch et al. (1970), rabbits exposed to coumarin gave birth to stillborn fetuses with hemorrhages. One discrepancy between nonhuman and human data may be due to the fact that in man and the baboon, coumarin is metabolized to 7-hydroxycoumarin (Shilling et al., 1974; Gangolli et al., 1974) but this metabolic pathway is relatively minor in the rat. FETAX tested coumarin as a positive and in this case was a better indicator of teratogenic hazard than some laboratory non-primate tests.

Serotonin tested positive in FETAX and agreed with mice, rat, and human data which state that serotonin poses a teratogenic hazard. Defects reported in laboratory mice include kidney, abdomen, eye, limb, tail, skull, brain and CNS abnormalities (Poulson et al., 1963; Thompson and Gautieri, 1969). The teratogenic effects of serotonin in the laboratory rat include anophthalmia, hydrocephaly, exencephaly, omphalocele and vacuolization of myocardial cells (Reddy et al., 1963). Reddy et al. (1963) also reported evidence of the effects of serotonin in human pregnancy.

The teratogenicity of 13-cis retinoic acid (Vitamin A) has been observed in all species tested including rat, mouse, rabbit, monkey and also Xenopus laevis (Geelen,

1979; and Kamm, 1982). Nervous system and craniofacial defects are the most common terata reported. Human birth defects as a result of the use of 13-cis retinoic acid are documented and described as a syndrome of central nervous system, aural and cardioaortic defects (Rosa et al., 1986). J.A.G. Geelen (1979) has published a survey on malformations reported in the literature for Vitamin A and its congeners. A strong positive result in FETAX for 13-cis retinoic acid, confirms the advice that this treatment for cystic acne should be avoided in pregnant women.

The results of this study indicate that all five compounds agree with the majority of mammalian literature available regarding teratogenicity. Ascorbic acid, cited as a negative teratogen in mammalian literature (Carpi and Scarinic, 1974; Frohberg et al, 1973; and Faustman-Watts et al, 1986), tested negative in FETAX. Sodium selenate and coumarin, variable positives in mammalian literature, tested positive in FETAX (Rosenfeld and Beath, 1939; Beath et al., 1939; Smith et al., 1936; Westfall et al, 1938; Hoffman and Heinz, 1988; Ohlendorf, 1979; Clark, 1987; Nobunaga et al, 1979; Hall et al, 1980; Shaul and Hall, 1977; Kronick et al, 1974; McCallion et al., 1971; Hirsch et al, 1970; Hirsch et al 1970; Shilling et al, 1969; and Gangolli et al., 1974). Serotonin and 13-cis retinoic acid, listed as positives in mammalian literature, tested positive in FETAX (Poulson et al., 1963; Thompson and Gautieri, 1969; Reddy et al., 1963; Geelen, 1979; Kamm,

1982; and Rosa et al, 1986). Overall, FETAX currently has a predictive accuracy of 89% including compounds tested with metabolic activation.

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CHAPTER IV

Species Sensitivity Differences in Developmental Toxicity Observed with Xenopus laevis and Pimephales promelas Embryos

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Abstract

The developmental toxicity and teratogenicity of sodium acetate (SA), caffeine (CAF), and 5-fluorouracil (FLU) were determined by measuring growth, malformation, and mortality with Xenopus laevis (frog) and Pimephales promelas (fish) embryos. A modified protocol of the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) was followed which allowed similar temperatures, exposure length, and embryological events to occur during the test. Chemical uptake by each species was determined using ^{14}C labeled material and liquid scintillation counting of whole embryo residue. Results suggest that frog and fish embryos exhibit similar malformations when exposed to SA, CAF, and FLU. Frogs had a higher concentration of ^{14}C SA per body weight than fish and were more sensitive to SA than fish at all endpoints tested. Fish had a higher concentration of ^{14}C CAF per body weight than frogs, and fish were more sensitive to CAF at growth and malformation endpoints. Fish had a higher concentration of ^{14}C FLU per body weight than frogs, and fish were more sensitive to FLU at growth endpoints. Test compound uptake correlated with the most sensitive indicator (growth).

Key words: Development; Xenopus laevis; Pimephales promelas; Sodium Acetate; Caffeine; 5-Fluorouracil

Introduction

Today there are over 600 teratogenic agents in the environment known to cause developmental abnormalities in experimental animals (Shepard, 1980). The Frog Embryo Teratogenesis Assay--Xenopus (FETAX) was developed by Dumont et al. (1983) to provide a standard screening test for developmental toxicants and teratogens. FETAX has been used to evaluate surface waters and sediment samples, groundwater, pure chemicals, solvents, and compounds requiring metabolic activation (Dawson et al., 1985; Dawson et al., 1988; Fort and Bantle, 1990; Bantle et al., 1989).

Amphibian and fish tests are useful for evaluating the developmental toxicity of chemicals and environmental mixtures (Birge et al.,1983; Birge et al., 1985; Dawson et al., 1988). A developmental toxicant should have similar effects on amphibians and fish if the organisms are exposed during the time homologous vertebrate structures are formed (Cameron et al., 1985). Fish and amphibian embryos exhibit similar in malformations when exposed to various agents but differ in their responses to a particular concentration of material (Dawson et al.,1985).

The fathead minnow, Pimephales promelas, is a member of Cyprinidae--the largest family of freshwater fish in North America. Fathead minnows are used as standard bioassay fish to assess the toxicity of complex environmental mixtures and pure compounds (Brungs, 1969; Devlin et al., 1985; Holcombe et al., 1982; McKim, 1977; Pickering and

Gast, 1972). Fathead minnows have been tested for survival, growth, and reproductive responses, and teratogenic assays (Benoit et al.,1982; Birge et al., 1985; McKim et al., 1977).

Birge et al. (1979;1983) as well as Dawson et al. (1985) used differing exposure periods (time to hatching plus four days) to compare sensitivities between different species of frogs and fish. Dawson et al. (1985) exposed Xenopus laevis (4 dy) and Pimephales promelas (6 dy) to effluents from Tar Creek and the Neosho River, Oklahoma. While the test samples containing heavy metals and zinc were teratogenic to both species, fish were found to be more sensitive than frogs.

Several studies have attempted to explain toxicological differences between aquatic organisms using rate of uptake, bioconcentration, and toxicant distribution and elimination as the basis for comparison (Werner and Kimerle, 1982; Muir et al., 1985; Ingebrigsten, 1988; and Mayes, 1985).

In this study Xenopus laevis (frog) and Pimephales promelas (fish) embryos were exposed to sodium acetate (SA), caffeine (CAF), and 5-fluorouracil (FLU). A modification of standard FETAX protocol (ASTM, 1991) was followed and by 120 hours the frog and fish embryos were hatched, free-swimming, and had undergone major organogenesis. The developmental stages for both species are similar through this period of time (Table 2).

Table 2.

SCHEDULE OF SOME FEATURES OF XENOPUS AND PIMEPHALES DEVELOPMENT AT 22-24°C IN MODIFIED FETAX SOLUTION

Stage No.			Approximate time (hrs) homologous structure appears in:	
Frog[a]	Fish[b]	Characteristics	Xenopus	Pimephales
0	1	Single cell ovum	0	0
6	8	Advanced 32-cell	3	2.5
9	11	Fine cell blastula	7	5.5
10	12	Initial gastrula	8.5	6
19	17	Neurula, 4-6 somites	20.5	16.5
26	20	Otic vesicle protruding	29.5	20
29/30	21	Distinct tailbud	35	21
32	25	Retinal pigmentation	40	40
33/34	23	Heartbeat	44.5	30
36	25	S-shaped heart	50	40
40	27	Mouth formation	66	60
40	29	Liver circulation	66	85
42	NA	Operculum development	80	95
36	NA	Beginning of hatching	50	105

[a] Nieuwkoop, P.D. and J. Faber, 1975. Normal tables of *Xenopus laevis* (Daudin). 2nd ed. North Holland, Amsterdam, 1975.

[b] Devlin, E.W., J.D. Brammer, and R.L. Puyear, 1985. Effect of toluene on fathead minnow (*Pimephales promelas* Rafinesque) development. Arch. Environ. Contam. Toxicol. 14, 595-603.

Materials and Methods

Test Organisms

Xenopus culture, breeding procedures, and egg sorting were as described previously (Dawson and Bantle, 1987). *Pimephales* embryos were obtained from the Water Quality Research Laboratory, Oklahoma State University, Oklahoma. Frog embryos at small-cell blastula stage (Nieuwkoop, 1975) and fish embryos at high blastula stage (Devlin, 1982) were chosen for testing. Fish embryos were separated according to Gast (1973).

Chemicals

Sodium acetate CAS# 6131-90-4 (SA), caffeine CAS# 58-08-2 (CAF), and 5-fluorouracil CAS# 51-21-8 (FLU) were chosen as test compounds based on their range of teratogenicity, cost, and available mammalian data. Test compounds were obtained for initial testing from Sigma (St. Louis, MO). Radiolabeled [^{14}C] SA was obtained from ICN Biomedicals, Inc. and had a radiochemical purity > 98% (specific activity: 7.1 mCi/mmol). Radiolabeled CAF, [1-methyl-^{14}C] was obtained from NEN Research Products and had a radiochemical purity of 97% (specific activity: 55.7 mCi/mmol). 5-fluorouracil-2-^{14}C was obtained from Sigma Chemical Company and had a radiochemical purity > 98% (specific activity: 22 mCi/mmol).

Concentration-Response Studies

Dilutions of test materials were made with modified FETAX solution (MFS) which allowed normal development of both frog and fish embryos (Dawson, 1988). This reconstituted water medium contained 400 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 14 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$, and 75 mg $MgSO_4$ per liter of deionized distilled water.

A range test and at least two definitive tests were conducted for each compound. Forty frog embryos and 30 - 40 fish embryos were exposed per dilution in separate dishes. Tests consisted of four control dishes and two dishes per dilution (8 ml total solution in each dish).

Static-renewal tests were conducted for 5 days (120 hrs) at 24± 2°C for both species. Test material was replaced every 24 hr during the test. Test organisms were incubated with a photoperiod (16 hr light, 8 hr darkness) to allow for maximum fish hatching. During the tests, pH was measured daily and dead embryos were counted and removed.

At dy 5 surviving embryos were anesthetized with 3-aminobenzoic acid ethyl ester (methanesulfonate salt) and fixed with 3.0% (w/v) formalin. Malformations were determined with a dissecting microscope. Head-to-tail lengths were measured using an IBM-compatible computer equipped with Sigma Scan digitizing software (Jandel Scientific, Corte Madera, CA).

Analysis of Radioactivity

Frog and fish embryos were exposed to radiolabeled ^{14}C SA, CAF, and FLU in order to measure uptake. Each labeled compound was tested in a separate dish containing 10 frog or 10 fish embryos and 4 ml total solution. Four control dishes (10 embryos each) were used for the tests.

A preliminary test with ^{14}C SA determined that an addition of 11 x 10^6k disintegrations per minute (dpm) to the dish gave sufficient radioactivity at the end of the test. Therefore, for the final definitive radiolabeled assay, 11 x 10^6 counts of each chemical was added to the appropriate dishes. Each dish contained 100 μl of SA (concentration of 0.05 mCi/ml); 50 μl of CAF (0.05 mCi/0.5 ml) and 25 μl of FLU (0.2 mCi/1 ml).

The test was conducted statically. Radiolabeled chemicals were added on day 0 to dishes containing frog embryos at small cell blastula stage and fish embryos at high blastula stage. Dead embryos were removed daily. At day 5 the test was ended.

Whole embryos were sampled at the end of the test. The number of frog and fish embryos analyzed (45 out of 50: 90% and 42 out of 50: 84%, respectively) depended on mortality during the test and animals damaged or lost during processing.

The embryos were carefully rinsed to remove adsorbed chemicals and were wet weighed before analysis. Samples

were solubilized by placing each animal into a small tube, adding TS-1 tissue solubilizer, and allowing the samples to digest for 2 dy. Neutralizer cocktail was added to the digestion medium and the contents of the tube were mixed. The samples were then analyzed using a Beckman LS-3100 Series Liquid Scintillation Spectrometer. A quench curve determined that little or no quench was occurring.

Radioactivity as counts per minute (cpm) was counted for each sample consisting of an individual fish or frog and was converted to concentration ^{14}C-labeled compound (mg) per bodyweight (mg).

Statistical Analysis

The EC50 (median concentration inducing malformation in 50% of surviving embryos) and the LC50 at 120 hr were determined using Litchfield-Wilcoxin probit analysis (Tallarida and Murray, 1980). The Minimum Concentration to Inhibit Growth (MCIG) was determined by t-test for grouped observations ($p = 0.05$).

Potency ratios were obtained for compound uptake, EC50s, and LC50s by t-test for grouped observations. Frog and fish responses (i.e. how much greater one responded over the other) could then be compared.

Results and Discussion

Teratogenic Potential

Results of FETAX are used to determine the teratogenic hazard of test materials based on embryo growth, Teratogenic Index values [TI=LC50/EC50 (malformation)], and the type and severity of induced malformations. In general, TI values < 1.5 indicate low teratogenic hazard. At higher TI values, the mortality and malformation concentration-response curves are separated and the potential for the survival of deformed embryos increases (Dawson and Bantle, 1987a).

This study tested one nonteratogen (SA), one moderate teratogen (CAF) and one strong teratogen (FLU). Representative concentration-response curves for SA, CAF, and FLU are presented in Figure 3. Results for the concentration-response studies and tests with radiolabeled compounds are found in Table 3.

Growth Inhibition

Growth was the most sensitive indicator of stress in a study of the effects of phenolics on the early life stages of the fathead minnow (Holcombe et al, 1982). Growth was also more sensitive than survival in early life stage tests by Norberg and Mount (1985) with fathead minnows.

Representative growth curves for SA, CAF, and FLU are presented in Figure 4. The teratogenicity of SA, CAF, and

Table 3.

CONCENTRATION RESPONSE AND RADIOASSAY RESULTS FOR XENOPUS AND PIMEPHALES EMBRYOS EXPOSED TO THREE COMPOUNDS

Compound	MCIG[a]	EC50[b]	LC50[c]	TI[d]	Uptake[e]
Sodium Acetate CAS# 6131-90-4					
Frog	2.5	3.29 (3.10-3.49)	4.24 (4.02-4.46)	1.29	7.96×10^{-4}
Fish	7.5	9.13 (8.56-9.73)	13.33(12.43-14.31)	1.03	4.35×10^{-4}
Caffeine CAS# 58-08-2					
Frog	0.08	0.13 (0.12-0.13)	0.19 (0.18-0.21)	1.55	3.57×10^{-6}
Fish	0.02	0.07 (0.04-0.11)	0.72 (0.50-0.11)	11.08	1.15×10^{-5}
5-Fluorouracil CAS# 51-21-8					
Frog	0.20	0.08 (0.06-0.10)	0.53 (0.42-0.62)	6.49	2.37×10^{-6}
Fish	0.20	0.40 (0.17-0.91)	2.42 (1.28-4.56)	6.11	5.97×10^{-6}

[a] Mean Minimum Concentration to Inhibit Growth, mg/ml.
[b] Mean 120 hr EC50 (malformation) with (95% confidence interval), mg/ml.
[c] Mean 120 hr LC50 with (95% confidence interval), mg/ml.
[d] Mean Teratogenic Index
[e] Mean Concentration of Radiolabeled Compound per Mean Weight Embryo, mg/mg.

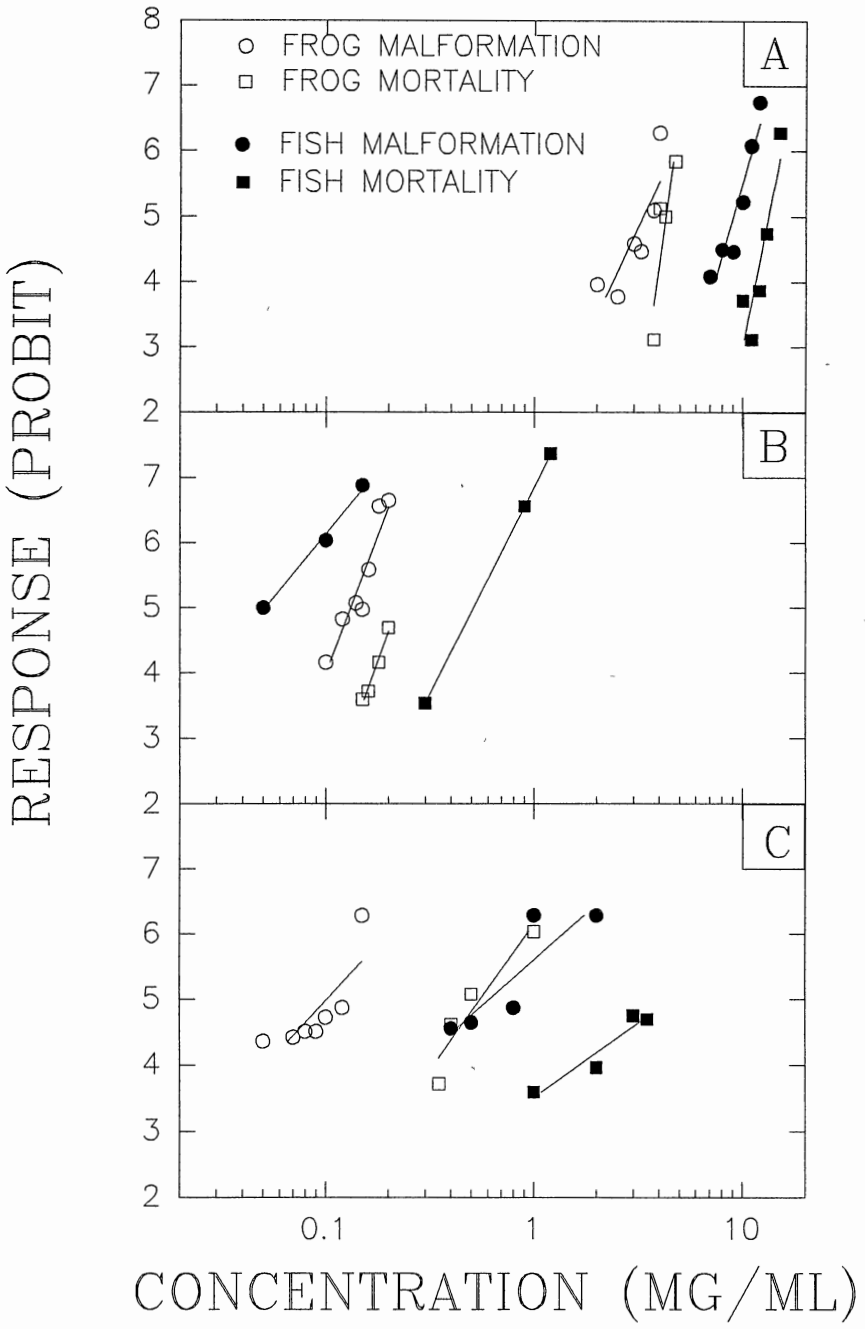

FIGURE 3. Representative concentration–response curves for sodium acetate (A), caffeine (B), and 5–fluorouracil (C) tested with Xenopus and Pimephales embryos.

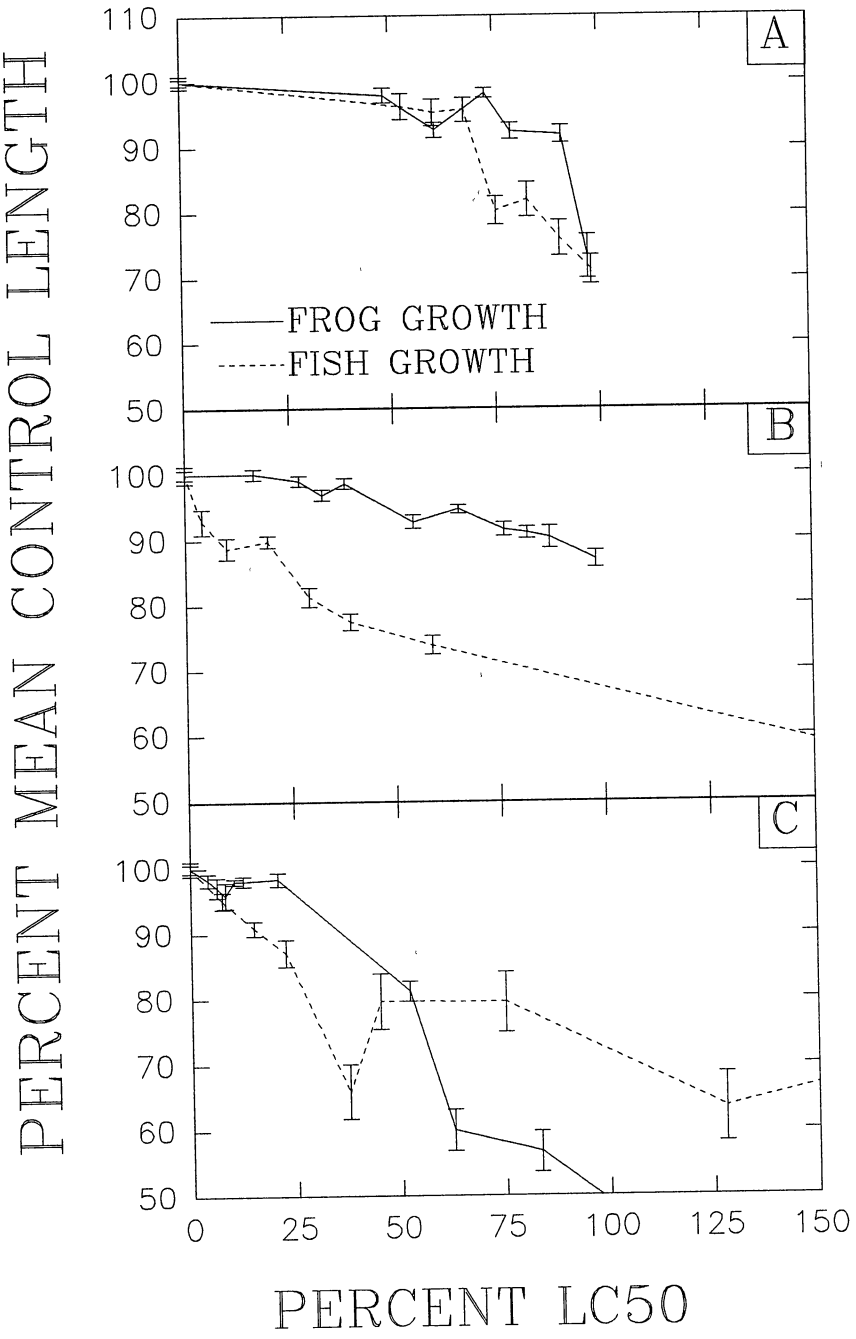

FIGURE 4. Representative growth curves for sodium acetate (A), caffeine (B), and 5-fluorouracil (C) tested with Xenopus and Pimephales embryos

FLU was assessed by considering the Minimum Concentration to Inhibit Growth (MCIG; also expressed as % compound LC50). Rates of growth inhibition (i.e. slope) and overall reduction in embryo growth vary with the severity of the teratogen. Dawson et al.(1989) suggest that compounds with significant teratogenic potential generally inhibit growth at concentrations < 30% of the respective LC50 values. Growth data was used to make comparisons between species responses and was an important indicator of effects which occurred at low levels of exposure.

Controls

Frog control mortality and malformation rates were 50 out of 560 (8.9%) and 16 out of 510 survivors (3.1%), respectively. Fish control mortality and malformation rates were 18 out of 460 (3.9%) and 14 out of 442 survivors (3.2%), respectively. Acceptable rates of control mortality and malformation in FETAX are less than 10% and 7%, respectively.

Effects of Sodium Acetate on Development and Growth

Sodium acetate was tested in mammals for antifertility effects by Dutta and Fernando (1972). Although SA reduced the number of surviving neonates, no abnormalities were reported in the offspring. SA was also tested on rats and no teratogenic effects were observed (Miller, 1971).

Sodium acetate (SA) failed to demonstrate teratogenicity to either frogs or fish, although similar mild malformations were observed for both. Frogs were more sensitive to SA than fish at all endpoints tested (growth, EC50, and LC50) possibly due to an increased uptake. Accumulation of ^{14} SA in frogs was 1.4 x greater than that accumulated by fish.

The most common malformations induced by SA in frogs were failure of the gut to coil, optic and facial malformations, and edema at concentrations > 2.0 mg/ml. At concentrations > 3.5 mg/ml spinal kinking and stunting was common. At concentrations > 4.5 mg/ml severe kinking, optic and facial malformations, and edema occurred.

The most common malformations induced by SA in fish were spinal kinking and stunting at concentrations > 7.0 mg/ml. Heart edema and facial malformations were also common at these concentrations. At concentrations > 13.0 mg/ml, severe spinal kinking, optic and facial malformations and edema occurred.

Effects of Caffeine on Development and Growth

Smith et al. (1983) report caffeine (CAF) as a weak teratogen in mammals. Al-Hachim (1989) found evidence for the teratogenicity of CAF in humans, mice, rats, and rabbits. In previous 4 dy tests with FETAX, CAF exhibited moderate teratogenic potential in Xenopus with a TI of 2.01

(Dawson and Bantle, 1987a; 1987b). Teratogenic data was not available for Pimephales.

Caffeine (CAF) was slightly teratogenic to frogs but was strongly teratogenic to fish. Both organisms demonstrated similar types of malformations, however fish malformations were more severe. Fish were more sensitive at growth and malformation endpoints than frogs possibly due to a difference in uptake. Accumulation of ^{14}C CAF in fish was 2.31 times greater than that accumulated by frogs.

The most common malformations induced by CAF in frog embryos was spinal kinking at concentrations > 0.03 mg/ml. At concentrations > 0.14 mg/ml spinal kinking, facial malformations, and improper gut coiling were common. At concentrations > 0.16 mg/ml moderately severe spinal kinking, stunting, and edema were observed.

The most common malformations induced by CAF in fish were spinal kinking and stunting at concentrations > 0.02 mg/ml along with occasional facial and eye malformations. At concentrations > 0.10 mg/ml moderate spinal kinking, heart defects, and edema were noted. At concentrations > 0.20 mg/ml severe curling of the tail and growth stunting occurred.

Effects of 5-Fluorouracil on Development and Growth.

Smith et al. (1983) reported 5-fluorouracil (FLU) as a strong teratogen in humans, mice, rats, and in chicks. In previous 4-day tests with FETAX, FLU exhibited strong

teratogenic potential in Xenopus with a TI value of 11.82 (Dawson and Bantle, 1987a; 1987b). Teratogenic data was not found for Pimephales.

5-Fluorouracil (FLU) was strongly teratogenic to both frogs and fish, and severely malformed and stunted embryos were observed with both. Fish were more sensitive to CAF than frogs at growth endpoints and had more severe malformations at low concentrations, possibly due to an increased uptake. Accumulation of ^{14}C FLU in fish was 2.4 times greater than that accumulated by frogs.

Malformations in frogs exposed to FLU consisted of slight to moderate abnormalities of the gut, face, eye, brain, heart and spine at 0.01 mg/ml. At concentrations > 0.15 mg/ml similar malformations occurred with greater severity in addition to stunting, edema and blistering. At concentrations > 0.50 mg/ml embryos were so severely stunted that vitality was difficult to determine.

Malformations in fish exposed to FLU consisted of moderate to severe spinal kinking, optic, and facial abnormalities at 0.05 mg/ml. At concentrations > 0.10 mg/ml the severity of brain, optic, spinal kinking, heart defects, and edema increased. At concentrations > 0.50 mg/ml vitality was difficult to determine and embryos had large yolk sacs, severe multiple malformations, and stunted growth.

Summary and Conclusion

In order to conduct a species comparison assay and to determine sensitivity differences, as many test factors as possible must be kept constant. Sensitivity to a toxicant, and thus the test results, can be affected by species, strain, previous exposure, age, size, health, and animal handling procedures (Adelman and Smith, 1976). In addition, exposure time variations can influence bioassay results (McCarty, 1986). A testing protocol that allows for similar developmental stages to be exposed over the same time period is beneficial for understanding species differences. However, as organogenesis rates and order of development differs among organ systems and species, differences in development are still a factor in species comparison tests.

Species sensitivity differences in fish have been attributed to uptake which is influenced by environmental and physiological factors such as ventilation volume, metabolic rate, temperature, and fish behavior (Spacie and Hamelink, 1982). Bioaccumulation differences between a fish and snail species was attributed to differences in uptake, temperature, and lipid content (Spehar, 1983). In a study with trout, catfish, and bluegills exposed to benomyl and carbendazim, differential toxicity to catfish could not be explained by residue dynamic studies. It was likely that the selective toxicity to catfish was

associated with events stemming from the pharmacodynamic phase of toxicant action (Palawski and Knowles, 1986).

Uptake studies with amphibians are scarce in the literature. However, one study dealt with the uptake of ^{14}C DDT [1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane] by wood frog embryos (Licht, 1985). Embryos at different stages and temperatures were exposed to radiolabeled DDT and it was found that the jelly capsules restricted the uptake of DDT.

The results of this study indicate that species differences between Xenopus and Pimephales embryos exposed to developmental toxicants may be explained by differences in uptake. Further testing is necessary to understand why frogs were able to accumulate a higher concentration of sodium acetate and fish were able to acumulate a higher concentration of caffeine and 5-fluorouracil.

The TI values generated for frogs in this 5 dy study for CAF and FLU were slightly lower than the TI values found by Dawson et al. in a 4 dy study (1987a;1987b). This was probably due to increased mortality caused by an additional day of exposure. As the malformation rate did not increase as much over one day as the mortality, the TI was reduced. Thus, the teratogenic hazard of a toxicant may be altered by differing exposure periods.

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CHAPTER V

SUMMARY AND CONCLUSIONS

The developmental toxicity of five compounds was evaluated with the Frog Embryo Teratogenesis Assay: Xenopus (FETAX) and the results were compared to mammalian literature. Small cell Xenopus laevis blastulae were exposed to ascorbic acid, sodium selenate, coumarin, serotonin and 13-cis retinoic acid for 96 hr. Three separate static-renewal assays were conducted for each compound. Teratogenic potential of the test materials was determined based on Teratogenic Index values [TI=LC50/EC50 (malformation)], types and severity of induced malformations and embryo growth. Ascorbic acid had little or no teratogenic potential. Sodium selenate and coumarin tested as having moderately positive teratogenic potential. Serotonin scored as having moderately strong teratogenic potential and 13-cis retinoic acid scored as having strong teratogenic potential. Results were consistent with mammalian data and support the use of FETAX for the screening of developmental toxicants.

The results of this study indicate that all five compounds agree with the majority of mammalian literature available regarding teratogenicity. Ascorbic acid, cited

as a negative teratogen in mammalian literature (Carpi and Scarinic, 1974; Frohberg et al, 1973; and Faustman-Watts et al, 1986), tested negative in FETAX. Sodium selenate and coumarin, variable positives in mammalian literature, tested positive in FETAX (Rosenfeld and Beath, 1939; Beath et al., 1939; Smith et al., 1936; Westfall et al, 1938; Hoffman and Heinz, 1988; Ohlendorf, 1979; Clark, 1987; Nobunaga et al, 1979; Hall et al, 1980; Shaul and Hall, 1977; Kronick et al, 1974; McCallion et al., 1971; Hirsch et al 1970; Shilling et al, 1969; and Gangolli et al., 1974). Serotonin and 13-cis retinoic acid, listed as positives in mammalian literature, tested positive in FETAX (Poulson et al., 1963; Thompson and Gautieri, 1969; Reddy et al., 1963; Geelen, 1979; Kamm, 1982; and Rosa et al, 1986). Overall, FETAX currently has a predictive accuracy of 89% including compounds tested with metabolic activation.

The developmental toxicity and teratogenicity of sodium acetate (SA), caffeine (CAF), and 5-fluorouracil (FLU) were determined by measuring growth, malformation, and mortality with Xenopus laevis (frog) and Pimephales promelas (fish) embryos. A modified protocol of the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) was followed which allowed similar temperatures, exposure length, and embryological events to occur during the test. Chemical uptake by each species was determined using ^{14}C labeled material and liquid scintillation counting of whole embryo

residue. Results suggest that frog and fish embryos exhibit similar malformations when exposed to SA, CAF, and FLU. Frogs had a higher concentration of ^{14}C SA per body weight than fish and were more sensitive to SA than fish at all endpoints tested. Fish had a higher concentration of ^{14}C CAF per body weight than frogs, and fish were more sensitive to CAF at growth and malformation endpoints. Fish had a higher concentration of ^{14}C FLU per body weight than frogs, and fish were more sensitive to FLU at growth endpoints. Test compound uptake correlated with the most sensitive indicator (growth).

In order to conduct a species comparison assay and to determine sensitivity differences, as many test factors as possible must be kept constant. Resistance to a toxicant, and thus the test results, can be affected by species, strain, previous exposure, age, size, health, and animal handling procedures (Adelman and Smith, 1976). In addition, exposure time variations can influence bioassay results (McCarty, 1986). Radiolabeling procedures are useful tools for evaluating the uptake of chemicals to determine differences between species. Further testing with radiolabeled compounds and a testing protocol which allows for the same developmental stages to be exposed over the same time period would be beneficial to further understanding species differences.

The TI values generated for frogs in this 5 dy study for CAF and FLU were slightly lower than the TI values found by Dawson et al. in a 4 dy study (1987a;1987b). This was probably due to increased mortality caused by an additional day of exposure. As the malformation rate did not increase as much over one day as the mortality, the TI was reduced. Thus, the teratogenic hazard of a toxicant may be altered by differing exposure periods.

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VITA 2

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Master of Science

Thesis: FETAX VALIDATION AND SPECIES DIFFERENCES IN DEVELOPMENTAL TOXICITY OBSERVED WITH XENOPUS LAEVIS AND PIMEPHALES PROMALES EMBRYOS

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