VALIDATION AND APPLICATIONS OF THE FROG EMBRYO TERATOGENESIS ASSAY-*XENOPUS* (FETAX)

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VALIDATION AND APPLICATIONS OF THE FROG EMBRYO

TERATOGENESIS ASSAY-XENOPUS (FETAX)

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PREFACE

The Frog Embryo Teratogenesis Assay-Xenopus (FETAX) is an in vitro bioassay designed to determine the potential teratogenic risk a compound or mixture poses to developing organisms. The system utilizes embryos of the South African clawed frog, Xenopus laevis, in 96 hour exposures to a solution of toxicant and evaluates malformation, mortality, and growth endpoints in order to determine the teratogenic risk of the compound. For this test system to be routinely used for assessing the developmental toxicity of chemical agents it must be standardized and validated. This work was conducted to increase acceptance of FETAX by refining and validating the assay and applying it to a variety of situations. It is hoped that the studies presented here will encourage scientists in both biomedical and environmental toxicology to use FETAX as a screening test for developmental toxicants.

The three studies encompassing this research involved developing a defined medium for use in the assay, validating FETAX by comparing the toxicity and teratogenicity of several compounds in Xenopus embryos with that reported in mammalian tests, and evaluating metal contaminated sediments for developmental toxicity using both frog and fathead minnow embryos. A report on the results of each of the three studies has been accepted for publication. Chapter II has been published in the Journal of Applied Toxicology (Volume 7, pages

237-244, 1987); Chapter III was published in Teratology (Volume 35, pages 221-227, 1987); Chapter IV was scheduled for publication in the December, 1987 edition of Environmental Toxicology and Chemistry. Each of these chapters is presented in the format of the respective journal.

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CHAPTER I

INTRODUCTION

Background

Teratology is the study of abnormal structural development. During animal development, precisely regulated processes give rise to a complete multicellular organism. Many situations or events, such as exposure to chemical agents, may disrupt these normal processes leading to malformation. The effects of these chemicals may occur at concentrations far below that which induces general cytotoxicity, increasing the chance an exposed organism will be alive and malformed. The testing of chemical agents, especially drugs, for teratogenic hazard has been a focal point of teratological research ever since the 1960's, when limb-reduction malformations in newborn children were correlated to the use of thalidodmide by pregnant women (Wilson, 1977). New compounds that are developed each year need to be specifically tested for potential teratogenicity to prevent similar occurences.

Presently, teratogenesis testing involves the use of small mammals such as rats, mice, hamsters, or rabbits but primates may be used as well. These procedures are time-consuming and expensive and have become overtaxed by the increasing number of chemicals that need to be tested (Kimmel et al., 1982). To help reduce these problems many alternative test systems have been developed that may prove useful in prioritizing compounds for further mammalian testing (Best and Morita,

1982; Bournias-Vardiabasis and Teplitz, 1982; Fantel, 1982; Greenburg, 1982; Jelinek, 1982; Johnson et al., 1982; Kochhar, 1982; Sadler et al., 1982; Schuler et al., 1982; and Dumont et al., 1983). One or more of these assays may eventually be used to directly predict the embryotoxic hazards an agent poses to humans (Kimmel et al., 1982).

One system is the Frog Embryo Teratogenesis Assay-Xenopus (FETAX), developed by J.N. Dumont and T.W. Schultz at the Oak Ridge National Laboratory (Dumont et al., 1983). This assay utilizes embryos of the South African clawed frog, Xenopus laevis, to determine the relative teratogenic risk of a test compound or environmental contaminant. Several studies have indicated that FETAX is useful as an assay for detecting compounds or mixtures that may cause abnormal structural development (Browne and Dumont, 1979; Dumont and Schultz, 1980; Davis et al., 1981; Courchesne and Bantle, 1985; Sabourin et al., 1985; and Schultz et al., 1985).

The overall objective of this dissertation research was to couple the development and validation of FETAX as a standard teratogenesis test with additional applications for aquatic toxicology research. To do this three areas of investigation were emphasized: refinement, validation, and applications for use.

Refinement

Reducing sources of variation in FETAX may ultimately determine the value of this system as a standard test method. By developing specific procedures to reduce variation and by standardizing the test protocol data comparison and interpretation can be facilitated. One way to reduce variation was to develop a defined medium to be used by all FETAX researchers. This would provide a greater degree of quality

control necessary in standardized testing protocols.

Several reconstituted water formulae were tested and the most promising was selected for further tests with validation compounds. This allowed a determination to be made on the use of the new solution as the standard medium for FETAX researchers. It was necessary to develop this solution early in the research program so that later research would benefit from the increased quality control.

Validation

Validation studies were needed to demonstrate that FETAX result were similar to the results of established mammalian tests (Kimmel et al., 1982; Smith et al., 1983). As many as 100 compounds may need to be tested to fully validate the assay. Validation of FETAX using one set of test chemicals has been partially completed (T.D. Sabourin, personal communication). In this research, further testing was conducted within time and cost restraints to more fully validate FETAX. Test compounds were chosen from several lists (Smith et al, 1983; Seidenberg et al., 1986; and Shepard, 1986) and included known human teratogens. Completed validation should demonstrate that FETAX is a system of choice for rapidly determining potential teratogenic hazards.

The testing of validation compounds represents only a small portion of chemicals presently in use and does not consider the effects of compound interactions. Therefore, an additional method of validation was conducted based on two teratogenic interactions studies performed using mammalian systems (Ritter et al., 1982; Nakatsuka et al., 1983). In these studies a pattern of teratogenic potentiation (a more than additive effect) was apparent when methylxanthines were coadministered with inhibitors of DNA, protein, and nucleic acid synthesis.

Demonstration of a similar pattern of potentiation in Xenopus embryos would further validate the assay. Compound interactions can be tested more rapidly and economically using FETAX. Therefore, successful completion of this study would help increase acceptance of FETAX as a model system for studying the toxic and teratogenic effects of multiple compounds on developing organisms.

Applications

An important factor for eventual acceptance and use of FETAX will be the variety of practical situations to which it can be applied. Previous research has indicated that FETAX can be adapted to test water samples from contaminated sites in the environment (Dumont et al., 1983; Dawson et al., 1985). Aqueous extracts of metal-contaminated sediment were tested using FETAX to further demonstrate the value and utility of the assay for environmental testing. The extraction procedure used was designed to enable researchers to determine the potential developmental toxicity that might be observed if metals were leached from aquatic sediments (McCormick, 1985). This study included testing of fathead minnow embryos as well to help demonstrate that the responses of Xenopus embryos were similar to those of other aquatic vertebrates. This would increase acceptance of Xenopus as a test organism for aquatic toxicology research and increase the use of FETAX. It was hoped that the study would clearly demonstrate that a malformation endpoint can provide valuable information to aquatic toxicologists, by assessing toxicant hazards during embryonic development. Early-life stages are a sensitive point in the life-cycle of aquatic organisms (Holcombe et al., 1982; Birge et al., 1985). A specific malformation endpoint is not presently used in most testing of

environmental samples.

By not limiting this research to test compound validation, more was accomplished toward the primary goal of increasing the acceptance of FETAX as a standard test procedure. In focusing on these areas of refinement, validation, and applications of FETAX, the assay may become more widely used for determining developmental hazards in biomedical and environmental research.

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CHAPTER II

DEVELOPMENT OF A RECONSTITUTED WATER MEDIUM AND PRELIMINARY VALIDATION OF THE FROG EMBRYO TERATOGENESIS ASSAY-*XENOPUS* (FETAX)

Abstract

A reconstituted water medium was developed for use in the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). FETAX solution was then tested on three compounds with known mammalian teratogenicity (ethanol, caffeine, and 5-fluorouracil) as was a non-teratogen (saccharin). The results obtained were then compared with results from tests on these compounds in two other media that had previously been used in the assay. Saccharin was not teratogenic. Ethanol and caffeine were weak and moderate teratogens, respectively. 5-fluorouracil was a strong teratogen. The results compare favorably with those obtained in mammalian studies. The amount of growth inhibition in embryos in the 96 h tests was positively correlated with the degree of teratogenicity of the compound. Final validation of FETAX will allow it to be used to screen and rank compounds for further testing and as a tool for studying the basic mechanisms of teratogenesis.

Introduction

Embryos of the South African clawed frog, Xenopus laevis, have been used to determine the toxic and teratogenic effects of; fungicides and herbicides,[1] chemicals,[2] heavy metals,[3] and complex effluents.[4] Similar studies by other researchers have made use of Xenopus and other amphibians. The Frog Embryo Teratogenesis Assay-Xenopus (FETAX) was developed by Dumont et al.[5] and is presently being validated as a screening test to determine the teratogenic risk of chemical agents and polluted waters.

Recent work in this laboratory has shown that the system has great potential for detecting the teratogenicity of individual compounds[6] and complex mixtures.[7] Studies such as these are vital to the acceptance of FETAX as an acceptable method of teratogenic risk assessment. Successful validation of FETAX should allow the screening test to be used alone or in conjunction with other in vitro assay systems to rank compounds for further testing in mammals. Nationwide use of FETAX would allow for the establishment of a large data base, which may ultimately allow it to be used directly to predict the teratogenic risk of a compound or mixture to a variety of organisms, including humans.

To gain acceptance, FETAX must be properly devised so that both intra- and interlaboratory variation is minimal. It has been common in aquatic toxicity bioassays for the water used as a diluent to vary greatly between labs, thereby making results difficult to interpret.[8] Due to this variation, the use of dechlorinated tap water in FETAX would be acceptable only for continuous-flow experiments.[8,9] Therefore, we set out to develop a reconstituted water formula that

would allow for proper Xenopus embryo development and at the same time reduce the variation associated with an undefined medium.

This report describes the development of a reconstituted water formula for use in FETAX, which we have named FETAX solution. This medium allows for excellent development of Xenopus embryos and consistently resulted in lower percentages of malformation and mortality and increased rates of development and growth of control embryos over those we had tested in dechlorinated tap water or modified Amphibian Ringers. FETAX solution was then used as the diluent water for tests on four compounds previously tested in the other media. The results of these comparison tests on a non-teratogen (saccharin), two weak teratogens (ethanol and caffeine), and a strong teratogen (5-fluorouracil),[10] are presented here as well. The results not only support the use of FETAX solution but serve as a preliminary report on the validation procedure outlined by Smith et al.[10] for in vitro teratogenesis assay validation.

Materials and Methods

The Xenopus adults were obtained from Xenopus I (Ann Arbor, MI) and maintained in glass aquaria and/or fiberglass raceways in dechlorinated tap water. This water was filtered through activated carbon and aerated for 48 h prior to use. It was periodically tested to ensure that the pH, dissolved oxygen content, hardness, and content of heavy metals and total organic carbon were at acceptable levels.[6] Adult frogs were fed beef liver and lung supplemented with liquid vitamins (Polyvisol).

Several reconstituted water formulae were tested to determine if normal Xenopus embryo growth and development took place. From initial tests one formula (FETAX solution) was selected for further testing and for comparison with results from tests using dechlorinated tap water or modified Amphibian Ringers. FETAX solution is composed of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$, and 75 mg $MgSO_4$ per 1 of deionized, distilled water. The pH was 7.9 after mixing and gentle aeration. No additional buffer was required to maintain the pH at 7.6 to 7.9. Modified Amphibian Ringers contained 1300 mg of NaCl and 30 mg each of KCl, $CaCl_2$, and $MgSO_4$ per 1 of deionized, distilled water. One-half ml of 300 mM Hepes was required to maintain a pH of 7.2 to 7.4 after adjustment.

Breeding tanks and all glassware were washed in dilute HCl, rinsed, washed in dilute NaOH, and then rinsed thoroughly in deionized water. The tanks were filled with the water medium to be used in the test and aerated for a short time before introducing the animals.

To induce mating, the male and female received 500 and 1000 IU, respectively, of human chorionic gonadotropin (Sigma, St. Louis, MO.)

via injection into the dorsal lymph sac. Amplexus normally ensued within 2 to 6 h and the deposition of eggs took place from 9 to 12 h after injection.

Following breeding the adults and fecal material were removed from the tank and the embryos collected in 60 mm plastic Petri dishes. The jelly coating surrounding the embryos was removed by gentle swirling for 3 to 4 m in a 2% w/v cysteine solution, prepared in the appropriate medium. The pH of the cysteine solution was adjusted to 8.1 with NaOH.

Following the removal of the jelly coat, abnormally cleaving embryos and necrotic eggs were removed from the collection of embryos. A second selection ensured that only normally developing embryos (at blastula) were used in the tests. For each test concentration two sets of 20 embryos each were placed in plastic Petri dishes containing a total of 8 ml of solution. At least four sets of 20 embryos were used as controls for each test. The control solutions contained 8 ml of the specific medium. The dishes were covered to minimize evaporation.

Four compounds were tested in FETAX solution for comparison with results obtained in the other media. These compounds: saccharin, ethanol, caffeine, and 5-fluorouracil, were tested at least twice each in dechlorinated tap water and at least once in modified Amphibian Ringers, except saccharin which was not tested in the latter.

All test compounds used in this study were obtained from Sigma. The embryos were maintained in the test dishes at 23-24C for 96 h. At 24, 48, and 72 h the solutions were changed. Appropriate dilutions were made from freshly prepared stock solutions. At the time of daily test solution changes, dead embryos were removed and the number dead recorded. Determination of gross structural malformations and mortality were made using a dissecting microscope. All embryos were

scored by the same individual. Death at 24 (stages 26,27)[11] and 48 h (stages 37-39) was determined by skin pigmentation, structural integrity, and irritability of the embryos, while at 72 (stage 42) and 96 h (stage 46) the absence of a heartbeat in the transparent embryos was also used as an indicator of death. In addition the number of surviving malformed embryos was determined for each dish and the stage of development of the embryos at each concentration noted.

Structural anomalies typically scored as malformations included head and eye irregularities, lack of or improper gut coiling, skeletal and heart defects, pericardial, fin, and head edema, and blistering.

At 96 h, dead embryos were removed from the dishes and the surviving embryos were fixed in formalin (0.5 to 0.75% w/v). The numbers of dead and malformed embryos were then noted and recorded. Malformed embryos that died prior to 96 h were not included in the number malformed. Embryos surviving to fixation were then individually measured (head-tail length) using a Radio Shack digitizer and model 16 microcomputer.

Dose-response bioassays, evaluated according to Litchfield-Wilcoxon,[12] were used to determine the EC50 (malformation) and LC50 values for the test compounds. The 96 h EC50 (malformation) is the concentration at which 50% of the embryos were malformed at 96 h. Similarly, the 96 h LC50 is the concentration that produces 50% lethality in the test at 96 h. Division of the 96 h LC50 by the 96 h EC50 (malformation) for a test resulted in a Teratogenicity Index (TI) value that was useful in estimating the teratogenic risk associated with a compound.[5] A compound with a TI of <1.3 was tentatively called a non-teratogen. Compounds with TI values of 1.3 to 2.0, 2.0 to 3.0, and >3.0 were designated as weak, moderate, and strong teratogens,

respectively. The final determination of the teratogenicity of a compound included the types and severity of malformations and the percent embryo mortality at the lowest concentration where 100% of the surviving embryos were malformed. The t-test for grouped observations[12] was used to analyze for a significant reduction in growth, at $p<0.05$, in order to obtain the minimum concentration to inhibit growth (MCIG) for each test.

Results

The results of initial tests on several reconstituted formulae indicated that further testing of one formula (now called FETAX solution) was desirable. For brevity only data for FETAX solution is presented here, with comparison to control and experimental results from tests with the previously used media. After preparation and gentle aeration FETAX solution had the following water quality parameters. The dissolved oxygen content was 7.0 to 7.2 mg/l, the specific conductivity varied from 1600 to 1620 S, and the hardness was 112 to 120, expressed as mg/l of $CaCO_3$.

Table I compares results for control embryos used in FETAX experiments over a two-year period. Statistical analysis using the t-test for grouped observations showed a significant ($p<0.05$) increase in mean length of control embryos in FETAX solution over those in the other media. Untreated embryos in FETAX solution typically developed to stage 46 at 96 h (the time of fixation) while these embryos in the other media normally developed only to stage 45 at 96 h. Although the results were not statistically significant, the average control mortality and malformation percentages were lower in FETAX solution.

Table II shows the results for the media tests on saccharin. In all tests the TI was approximately 1.0. The 96 h EC50 (malformation) and LC50 in both media were from 18 to 21 mg/ml concentration. Figure 1A represents the dose-response curve for saccharin in FETAX solution. The malformations included slight to moderate edema in the pericardial region and the dorsal fin, slight tail kinking, and loose or improper gut coiling. These malformations were the same in both media and were minor in severity. The lowest concentration at which 100% of the survivors were malformed always occurred where at least 70% of the test

embryos were dead. The minimum concentration to inhibit growth (MCIG) of the embryos varied from 12.0 to 17.0 mg/ml. At 20.0 mg/ml growth was inhibited by 9 to 15% in dechlorinated tap water and by 13% in FETAX solution, when compared to mean control lengths (Figure 2A).

Results from tests on ethanol in all three media are shown in Table II. The TI values for all tests were between 1.3 and 1.9. The 96 h EC50 (malformation) range was from 1.0 to 1.2% ethanol (v/v). The range for the 96 h LC50 was 1.4 to 2.0%. The dose-response curve for ethanol, in FETAX solution, appears in Figure 1B. The malformations were edema, tail kinking, and loose and improper gut coiling. These malformations were slight to moderate at the 96 h EC50 (malformation) concentrations and more severe above the 96 h LC50 values. All embryos were malformed at concentrations where more than 50% of the embryos were dead. The MCIG range was from 0.6 to 1.0% ethanol. At 1.4% concentration the growth of embryos was inhibited by 10 and 13% in dechlorinated tap water, by 10 and 15% in modified Amphibian Ringers, and by 15% in FETAX solution (Figure 2B).

Caffeine was also tested in all three media and the results are shown in Table II. Teratogenicity Index values varied from 1.8 to 2.4 in the tests. The EC50 (malformation) and LC50 values at 96 h ranged from 0.11-0.15 and 0.25-0.30 mg/ml, respectively. The dose-response curve for caffeine is shown in Figure 1C. The malformations included edema in the head, pericardial, and fin regions, blistering, tail kinking, and improper gut coiling. These malformations were severe at all concentrations above 0.175 mg/ml. On all occasions malformations in 100% of the survivors first occurred at 0.20 to 0.22 mg/ml caffeine. These concentrations were always below the 96 h LC50 value for that test. The MCIG ranged from 0.04 to 0.10 mg/ml. At 0.2 mg/ml the

growth inhibition when compared to controls was 13 and 22% in dechlorinated tap water, 22% in modified Amphibian Ringers, and 13% in FETAX solution (Figure 2C).

Tests on 5-fluorouracil resulted in TI values from about 10 to 12 (Table II). The 96 h EC50 (malformation) and LC50 concentrations were from 0.12-0.14 and 1.22-1.62 mg/ml, respectively. Figure 1D shows the dose-response curve for this compound. Severe kinking, absence of or improper gut coiling, and severe head and eye abnormalities (micropthalmia, microencephaly, and anencephaly) were the typical malformations noted. The malformations were severe at concentrations near the 96 h EC50 (malformation) values. All surviving embryos were malformed at 0.2 mg/ml. This was much lower than the 96 h LC50 values. The MCIG was approximately 0.1 mg/ml. The results of the first test in dechlorinated tap water are included in Table 2 for comparative purposes. In this test the lowest concentration tested was 0.2 mg/ml, all these embryos were malformed. At 0.2 mg/ml growth was inhibited by 10 and 17% in dechlorinated tap water, by 21% in modified Amphibian Ringers, and by 10% in FETAX solution. At 0.7 mg/ml growth inhibition was always greater than 40% (Figure 2D).

Discussion

Although the percentages of control malformation and mortality in FETAX solution were lower than in the other media, the difference was not statistically significant. It is apparent, however, that FETAX solution is superior to the other media for other reasons. Advantages of FETAX solution over dechlorinated tap water include the significant increase in growth of the controls, the more rapid rate of development, and that it is a defined medium. Dechlorinated tap water may contain substances that could interact with the test compound, positively or negatively. However, we have seen no evidence of this in the present test scheme.

The advantages of FETAX solution over modified Amphibian Ringers are the better growth and development rates in the controls, that no additional buffer is needed, and the lower percentage of edema in controls. This edema accounted for the higher malformation percentage and standard error of the mean in controls tested in the modified Ringers solution (Table I). This was the main reason we did not like this solution as a defined medium for the assay. The growth inhibition plots (Figure 2) show that there was some slightvariation in growth in the different media. The mean head-tail length was slightly smaller in the modified Ringers but no consistent pattern was detected between dechlorinated tap water and FETAX solution.

In this study the criteria used for determining the teratogenicity of a compound incorporated information on the severity and extent of the malformations, the Teratogenicity Index (TI), and the percent embryo mortality at the lowest concentrations where all surviving embryos were malformed. The TI was proposed by Dumont et al.[5] as a potential measure of teratogenic risk assessment. The use of a ratio

such as this in *in vitro* teratogenesis test systems has been discussed.[10] The TI is similar to the therapuetic index in pharmacology that is used as a relative measure of the margin of safety for the use of a drug in comparison to other drugs.[13] The Teratogenicity Index is not as useful as other ratios which employ both adult and developmental testing endpoints.[10,14]

The TI values for the compounds tested represent the degree of separation between the malformation and mortality curves (Figure 1A-D). This is directly useful for determining the teratogenic risk of a compound. For example, there was a large separation of the malformation and mortality curves for 5-fluorouracil (Figure 1D) indicatingteratogenic effects were observed at far lower concentrations than embryo lethality. At the lowest concentrations where most or all of the surviving embryos were malformed there was little if any mortality. The risk of being alive and malformed at these concentrations was very high. By comparison, the malformation and mortality curves for saccharin (Figure 1A) covered the same concentrations and crossed-over. This indicated the chance of being alive and malformed was equal to or less than the chance of being dead.

The degree of teratogenic risk indicated by the TI can not be used alone to determine if a compound is a teratogen or not. Courchesne and Bantle[6] tested actinomycin D on *Xenopus* embryos and observed a cross-over of the malformation and mortality curves similar to that noted for saccharin in this study. A calculated TI for actinomycin D would have been <1, indicating a lower risk teratogen. However, the malformations induced in embryos exposed to this compound were so severe that it was considered teratogenic.[6] Complete testing of the compounds suggested by Smith et al.[10] should establish the validity

of the criteria used in FETAX for determining the teratogenicity of a compound. Additional methods of analyzing the data to determine compound teratogenicity are being evaluated to improve on the present scheme.

All tests of a compound produced similar types and severity of malformations and similar TI values in each medium. For saccharin the TI always fell in the range of values <1.3, that for non-teratogens. The malformations were minor in nature and 100% of the tadpoles were malformed only at concentrations where 70% or more of the test embryos were dead. The cross-over of the malformation and mortality curves for this compound, shown in Figure 1A, indicated it was toxic in this range. In addition the concentration required to produce harmful effects was very high (15 mg/ml). Our conclusion was that saccharin was not a teratogen to the embryos under our conditions of testing. This concurs with its designation by Smith et al.[10] who suggested inclusion of saccharin as a negative teratogen for *in vitro* teratogenesis assay validation.

The tests on ethanol produced TI values in the range from 1.3 to 2.0, the range for a weak teratogen. The dose-response curve for ethanol (Figure 1B) showed a slight separation of the malformation and mortality curves. This was used as an indication the compound had some teratogenic risk. The increased severity of malformations at higher concentrations and the occurence of malformations in 100% of the embryos just above the 96 h LC50 value, in all tests, added support to the TI designation that ethanol was a weak teratogen. Ethanol was listed as a weak teratogen by Smith et al.[10]

We have concluded that caffeine has moderate teratogenicity in *Xenopus*. The TI fell, variously, in the ranges from 1.3-2.0 and

2.0-3.0. In three of the four tests the TI was in the latter range, indicating that caffeine was a moderate teratogen. There was clearly a larger separation of the malformation and mortality curves for caffeine (Figure 1C) (along their entire lengths) than for ethanol (Figure 1B). Using the additional information on the severity of the malformations, especially kinking and blistering, at concentrations just above the 96 h EC50 (malformation) but below the 96 h LC50 concentrations and the occurrence of malformations in all surviving tadpoles below the 96 h LC50 for each replicate, our conclusion that caffeine was a moderate teratogen, in Xenopus, is supported. This conclusion does not specifically agree with its designation by Smith et al.[10] as a weak teratogen, in mammals. However, both conclusions indicate the potential teratogenicity of caffeine.

The large separation of the malformation and mortality curves (Figure 1D), the severity of malformations, the degree of separation between the lowest point where all tadpoles were malformed (0.2 mg/ml) and the 96 h LC50 values (1.22-1.62 mg/ml) in conjunction with the TI values from 10 to 12 (Table 2) indicated that 5-fluorouracil was a strong teratogen. This conclusion is in agreement with Smith et al.[10]

The malformations observed in Xenopus embryos due to exposure to the four compounds tested were similar to those seen in mammals. A disadvantage ofthe 96 h FETAX test is the inability to detect limb and digital defects. However, the skeletal kinking observed in the embryos may bear some relationship to skeletal limb defects seen in mammalian tests. The term skeletal kinking is used to differentiate from kinking due to muscular contraction occasionally observed in Xenopus tadpoles. Tests with saccharin in rats showed no malformations above

control levels.[15] In this study minor malformations, especially edema and gut miscoiling, were observed. These abnormalities were probably a result of the toxic effects exerted on the embryos at the very high concentrations used. Ethanol produced skeletal, cardiovascular, head, and eye defects in mice.[16] Early exposure to ethanol in Xenopus embryos resulted in craniofacial abnormalities typical of those seen in Fetal Alcohol Syndrome,[17] while skeletal kinking and edema were the predominant malformations observed in this study. The malformations in Xenopus embryos due to caffeine exposure were severe skeletal kinking, edema, and gut miscoiling. Similarly, prolonged caffeine administration in rats resulted in a high incidence of generalized edema in addition to visceral and skeletal abnormalities[18] and limb defects have been noted.[19] Exposure of Xenopus embryos to 5-fluorouracil resulted in severe kinking, gut miscoiling, and reduced size of the head, eye, and brain. The malformations observed in mice, due to exposure to this compound, were head, tail, and limb defects.[20] Exposure of hamsters to 5-fluorouracil induced tail, limb, palate, gut, eye, and brain malformations. Fetal growth was also impaired.[21]

Growth inhibition appeared to be a very sensitive endpoint that may be used to aid in determining the degree of teratogenicity of a compound. Figure 2 is a comparison plot of the growth inhibition for each test compound, in each medium tested. The MCIG for saccharin (in FETAX solution) was 17.0 mg/ml or 92.5% of its 96 h LC50. The MCIG for ethanol (1.0%), caffeine (0.1 mg/ml), and 5-fluorouracil (0.12 mg/ml) (in FETAX solution) were 69.4%, 38.9%, and 7.4% of the respective 96 h LC50 values. As the teratogenicity of the compound increased the rate of growth inhibition and the total reduction in length increased.

Although data from many more compounds is needed, this method of growth comparison may prove useful in the final determination of the degree of teratogenicity associated with a compound.

Smith et al.[10] have suggested using compounds from the National Toxicology Program (NTP) repository. Because of limited supplies of certain NTP compounds and the limited objectives of this study we used test compounds purchased from Sigma.

The variation seen in results for the 4-5 replicate tests on each compound can, for the most part, be attributed to variation; due to the water medium used, in the actual stock and expected exposure concentrations from test to test (and day to day), and in the genetics of offspring from separate breedings (especially for the MCIG). Reducing variations such as these for both intra- and interlaboratory comparisons is important to the utility of any such test system. We have addressed the water medium variation in this study. The next step is to test FETAX solution, and the assay itself, for interlaboratory reproducibility.

FETAX has broad applicability as an *in vitro* teratogenesis screening assay. It can be used for testing pure compounds[6,22] and complex mixtures[5,7] to determine potential harm, not only to aquatic organisms but mammals (eventually including humans) as well. For now FETAX is immediately useful as a rapid assay to screen and rank samples for further mammalian testing. Following complete validation, FETAX should enable standardization of information on the teratogenicity of a variety of agents and the mechanisms involved in the process. These can be done in a rapid and cost-effective manner.

Our conclusion for this study is that FETAX solution appears to be an excellent medium for use in teratogenic assays. While results of

experiments on the four test compounds can not be interpreted as a validation of the assay, it does provide incentive and encouragement for continued validation efforts of FETAX.

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Table I. Comparison of FETAX Test Media on Control Embryo Development and Growth.[a]

Medium	Control Embryos[b] (n)	Mortality[c] (%)	Survivors Malformed[d] (%)	Mean Length[e] (mm)
Tap Water[f]	1480	3.8±0.7	3.3±0.6	8.84±0.08
Amphibian Ringers	560	4.6±1.4	7.1±2.0	8.92±0.15
FETAX solution	2000	2.3±0.9	2.7±0.5	9.52±0.11[g]

[a] Grouped observations were compared by t-test.

[b] Total number of control embryos tested in the medium from Jan. 84 to Jan. 86.

[c] The mean control mortality percentage ± standard error of the mean.

[d] The mean percentage of surviving malformed control embryos ± standard error of the mean.

[e] The average mean lengths of the control embryo replicates ± standard error of the mean.

[f] Dechlorinated.

[g] Value significantly different from the others at $p<0.05$.

Table II. Results of media tests on four *in vitro* teratogenesis assay validation compounds.

Compound Medium-Test #	96h EC50[a] (malformation) (95% Confidence Limits)	96h LC50[a] (95% Confidence Limits)	TI[b]	MCIG[c]
A. Saccharin (mg/ml)				
Dechlorinated Tap Water - #1	20.71 (N.A.)	21.09 (18.9-23.5)	1.02	12.0
Dechlorinated Tap Water - #2	18.12 (17.4-18.9)	18.32 (17.8-18.8)	1.01	N.A.
Dechlorinated Tap Water - #3	18.05 (17.8-18.3)	17.94 (17.6-18.3)	0.99	17.0
FETAX Solution - #1	19.34 (18.7-20.0)	18.37 (18.0-18.8)	0.95	17.0
B. Ethanol (% concentration)				
Dechlorinated Tap Water - #1	1.09 (1.00-1.16)	1.45 (1.20-1.70)	1.33	1.0

Table II cont.

Dechlorinated	1.20	1.75	1.46	0.6
Tap Water - #2	(1.06-1.36)	(1.51-2.02)		
Amphibian	1.06	1.99	1.88	0.6
Ringers - #1	(0.94-1.19)	(1.91-2.09)		
Amphibian	1.04	1.78	1.71	0.6
Ringers - #2	(0.98-1.10)	(1.74-1.81)		
FETAX	1.01	1.44	1.43	1.0
Solution - #1	(0.95-1.09)	(1.20-1.71)		

C. Caffeine (mg/ml)

Dechlorinated	0.146	0.297	2.03	0.075
Tap Water - #1	(0.07-0.30)	(0.26-0.32)		
Dechlorinated	0.152	0.276	1.83	0.040
Tap Water - #2	(0.14-0.16)	(0.25-0.30)		
Amphibian	0.107	0.252	2.36	0.080
Ringers - #1	(0.10-0.12)	(0.22-0.29)		
FETAX	0.128	0.257	2.01	0.100
Solution - #1	(0.12-0.14)	(0.21-0.31)		

Table II cont.

D. 5-fluorouracil (mg/ml)

Dechlorinated Tap Water - #1	N.A.	1.47 (1.29-1.66)	N.A.	N.A.
Dechlorinated Tap Water - #2	0.120 (0.11-0.13)	1.22 (1.07-1.39)	10.17	0.0875
Amphibian Ringers - #1	0.124 (0.12-0.13)	1.26 (1.17-1.36)	10.16	0.090
FETAX Solution - #1	0.137 (0.12-0.16)	1.62 (1.56-1.68)	11.82	0.120

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[a] Statistical analysis using the Litchfield-Wilcoxon dose-response test.

[b] Teratogenicity Index = 96h LC50/96h EC50 (malformation).

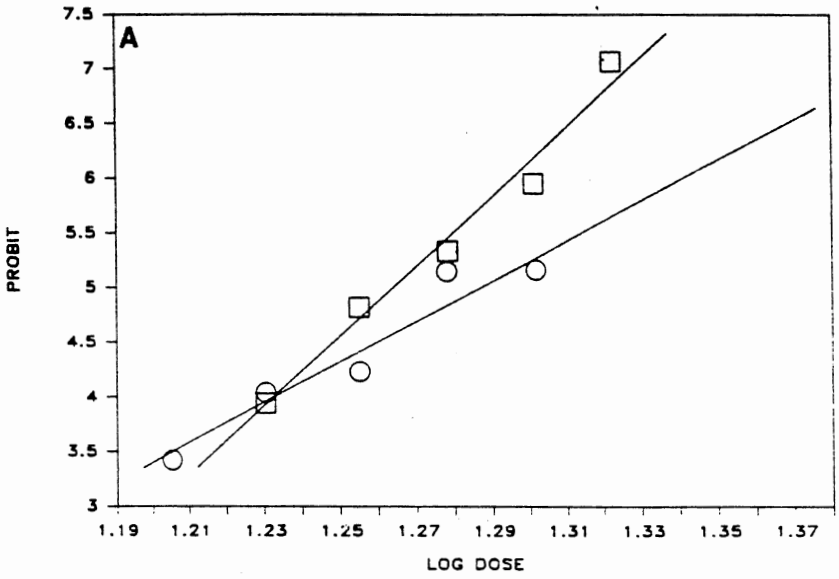
[c] MCIG - Minimum Concentration to Inhibit Growth; analyzed by the t-test for grouped observations ($p<0.05$).

N.A. not available.

Figure Legends

Figure 1. Dose-response curves for Xenopus embryo malformation (◯) and mortality (□) after 96 hours of exposure to A. saccharin (mg/ml), B. ethanol (% concentration), C. caffeine (mg/ml), and D. 5-fluorouracil (mg/ml), in FETAX solution.

Figure 2. Representative growth curves for Xenopus embryos after 96 hours of exposure to: A. saccharin, B. ethanol, C. caffeine, D. 5-fluorouracil; prepared in dechlorinated tap water (◯), modified Amphibian Ringers (▲), or FETAX solution (□).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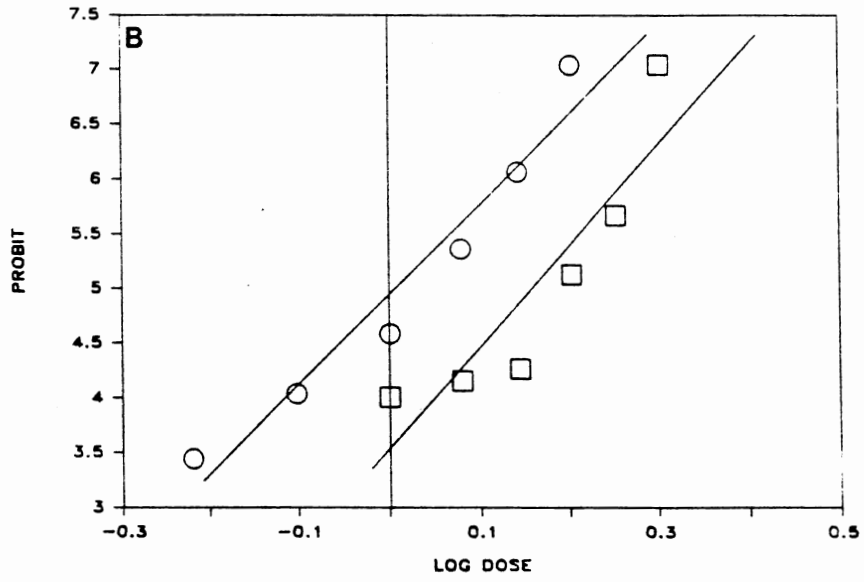
B
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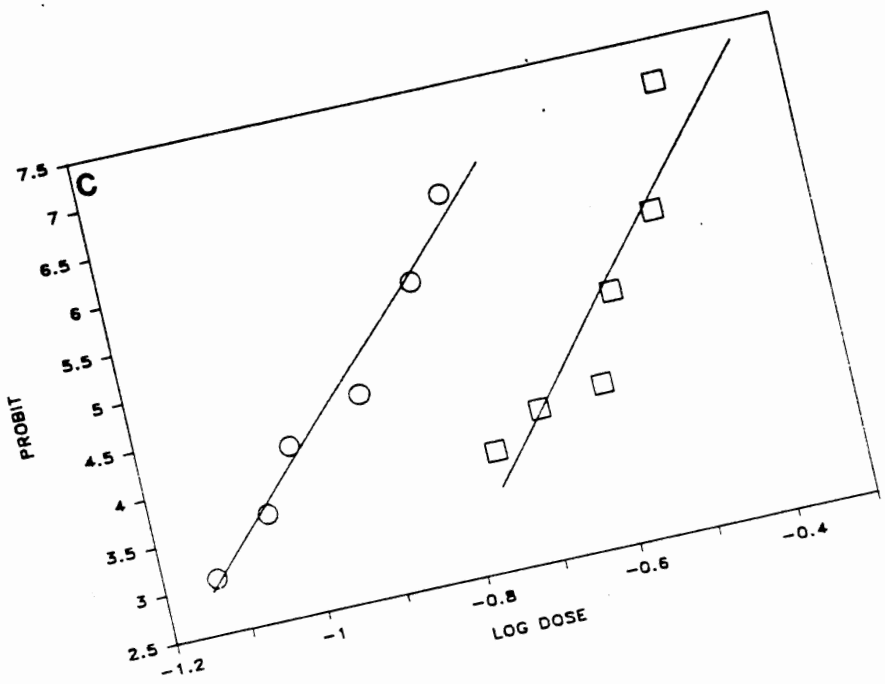
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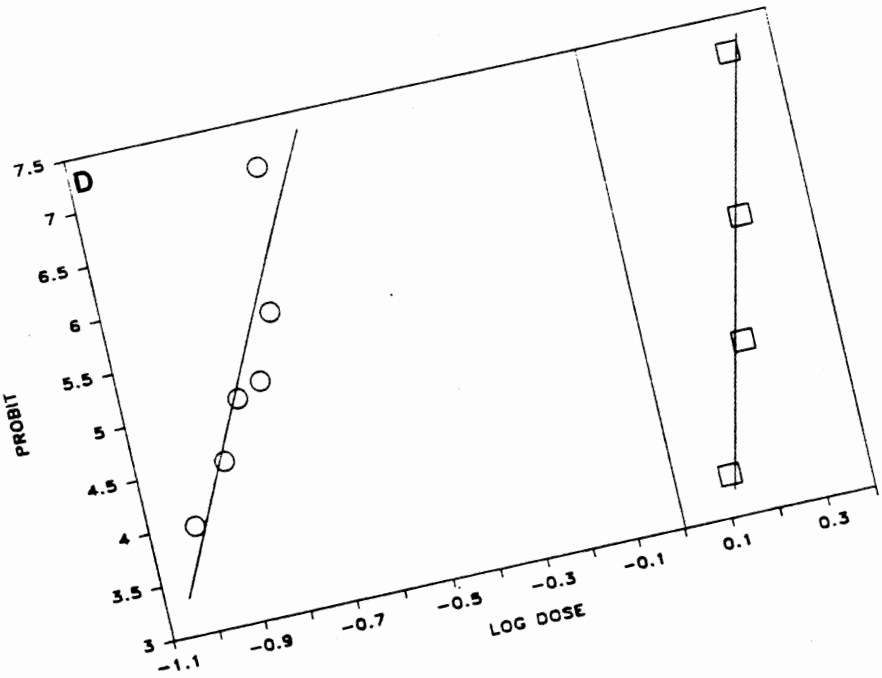
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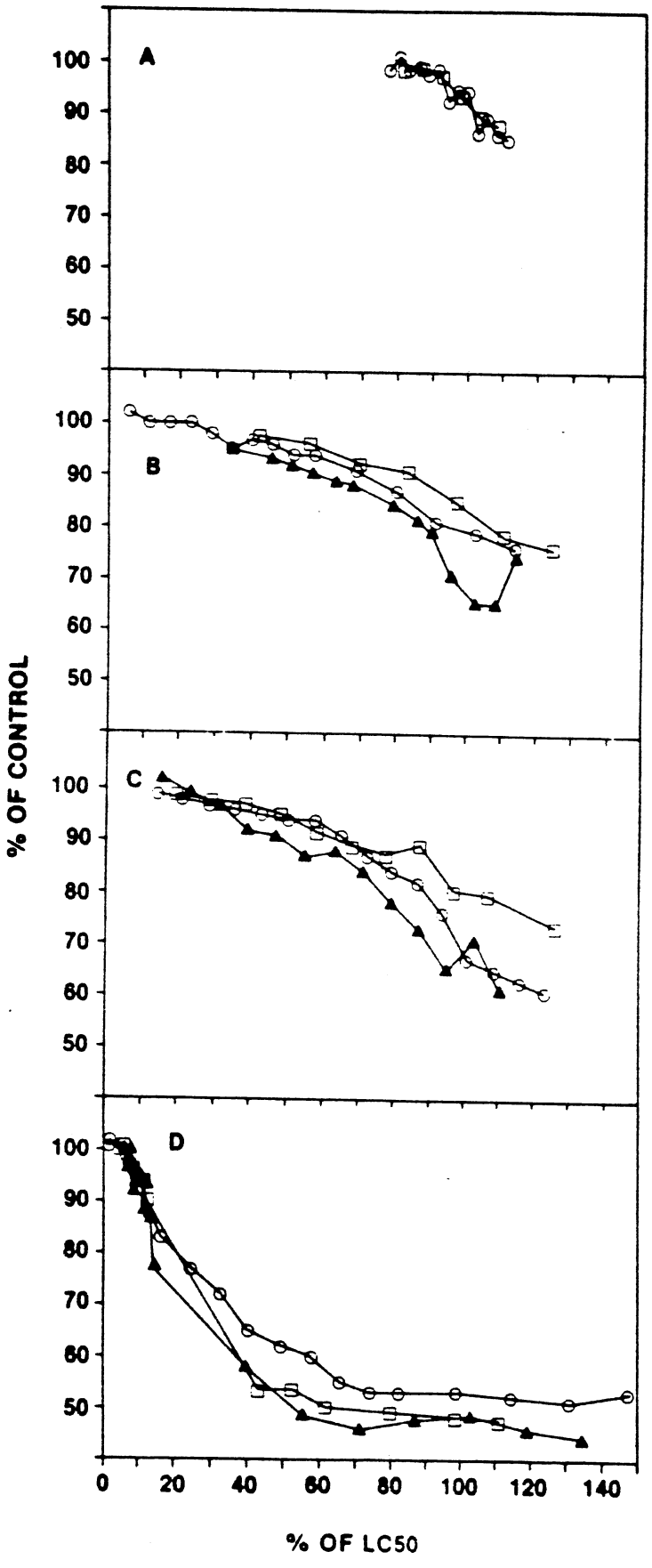
A
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50
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60
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100
120
140
% OF CONTROL
% OF LC50

CHAPTER III

COADMINISTRATION OF METHYLXANTHINES AND INHIBITOR COMPOUNDS POTENTIATES TERATOGENICITY IN *XENOPUS* EMBRYOS

Abstract

Inhibitors of DNA synthesis (hydroxyurea and cytosine arabinoside), protein synthesis (cycloheximide and emetine), and nucleic acid synthesis (5-fluorouracil) were administered with each of three methylxanthines (caffeine, theophylline, and theobromine) to determine if teratogenic effects could be potentiated in *Xenopus laevis* embryos. The animals were exposed for 96 hours to methylxanthine and inhibitor concentrations that alone, produced low percentages of malformations.

Coadministration of caffeine or theophylline with each inhibitor greatly increased the incidence of malformed embryos. Similar potentiation was induced when theobromine and the protein synthesis inhibitors were tested. A lesser potentiative response was produced when theobromine and the nucleic acid synthesis inhibitor were administered together. Teratogenic potentiation did not occur when theobromine was administered in conjunction with the DNA synthesis inhibitors. Growth reduction in the treatments proved to be the most sensitive indicator of the potentiative effects.

This study had two significant findings: the teratogenicity of the

protein synthesis inhibitors was greatly increased upon coadministration with each methylxanthine, even though they are typically not very teratogenic by themselves, and coadministration of the DNA synthesis inhibitors with theobromine did not result in teratogenic potentiation. Additionally, this study serves as one method of validating the Frog Embryo Teratogenesis Assay-Xenopus (FETAX), since the results obtained concur with results from similar mammalian studies.

Introduction

Amphibian embryos have been employed by a number of investigators to evaluate the teratogenic and toxic potential of a variety of chemicals and agents (Dial, '76; Greenhouse, '76; Birge et al., '85). A frog embryo teratology screen ("FETAX") has been developed (Dumont et al., '83) and tested in several laboratories (Courchesne and Bantle, '85; Dawson et al., '85; Sabourin et al., '85). As a part of the validation procedure for this assay, we investigated the phenomenon of teratogenic potentiation between two agents in Xenopus embryos. Three methylxanthines (caffeine, theophylline, and theobromine) were selected and their interactions with inhibitors of DNA (hydroxyurea, cytosine arabinoside), protein (cycloheximide, emetine), and nucleic acid synthesis (5-fluorouracil) studied. Earlier studies in in vivo mammalian systems (Ritter et al., '82; Nakatsuka et al., '83) had indicated potentiation with two of these xanthines (caffeine and theophylline but not theobromine).

Materials and Methods

The Xenopus adults were obtained from Xenopus I (Ann Arbor, MI) and maintained in glass aquaria and/or fiberglass raceways in dechlorinated tap water. This water was filtered through activated carbon and aerated for 48 hours prior to use. The water was periodically tested to ensure that the pH, dissolved oxygen content, hardness, and content of heavy metals and total organic carbon were at acceptable levels (Courchesne and Bantle, '85). Adult frogs were fed beef liver and lung supplemented with baby vitamins (Polyvisol).

Breeding tanks (and all glassware used in this study) were washed in dilute HCl, rinsed, washed in dilute NaOH, and then rinsed thoroughly in deionized water. The tanks were filled with FETAX Solution (Dawson and Bantle, 1987) and aerated for a short time before introducing the animals. FETAX Solution is composed of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$, and 75 mg $MgSO_4$ per liter of deionized distilled water.

To induce mating, the male and female received 500 and 1000 IU, respectively, of human chorionic gonadotropin (Sigma, St. Louis, MO.) via injection into the dorsal lymph sac. Amplexus normally ensued within two to six hours and the deposition of eggs took place from nine to twelve hours after injection.

Following breeding the adults and fecal material were removed from the tank and the embryos collected in 60 mm plastic Petri dishes. The jelly coating surrounding the embryos was removed by gentle swirling for three to four minutes in a 2% w/v cysteine solution, prepared in FETAX Solution. The pH of the cysteine solution was adjusted to 8.1 with NaOH. After removal of the jelly coat, abnormally cleaving

embryos and necrotic eggs were removed from the collection of embryos. A second selection ensured that only normally developing embryos (at blastula) were used in the tests.

Preliminary tests were conducted to determine the best concentration of caffeine and each inhibitor to use in the experiments. The concentrations of theophylline and theobromine used were equimolar to caffeine. For each separate clutch of embryos four sets of 20 embryos each were placed in plastic Petri dishes containing 8 ml of FETAX Solution, as controls. Controls for each methylxanthine and inhibitor compound were set up in a similar manner, as were the methylxanthine and inhibitor mixtures. All dishes contained a total of 8 ml of the appropriate solution(s), which had been diluted from stocks prepared in FETAX Solution. The dishes were covered to minimize evaporation. Two separate experiments were performed on each inhibitor compound.

All test compounds used in this study were obtained from Sigma. The embryos were maintained in the test dishes at 23-24^{o}C for 96 hours. At 24, 48, and 72 hours the solutions were changed. Appropriate dilutions were made from freshly prepared stock solutions. At the time of daily solution changes dead embryos were removed and the number dead recorded. Death at 24 (stages 26,27) (Nieuwkoop and Faber, '75) and 48 hours (stages 37-39) was determined by skin pigmentation, structural integrity, and irritability of the embryos, while at 72 (stage 42) and 96 hours (stage 46) the absence of a heartbeat (visible) was also used as an indicator of death. In addition the number of surviving malformed embryos was determined for each dish and the stage of development of the embryos noted. Structural anomalies typically scored as malformations included head and eye irregularities, absence

of or improper gut coiling, skeletal and heart defects, pericardial, fin, and head edema, and blistering.

At 96 hours, dead embryos were removed from the dishes and the surviving embryos were fixed in formalin (0.5 to 0.75%). The numbers of dead and malformed embryos were then determined. Malformed embryos that died prior to fixation were not included in the number malformed. Embryos surviving to fixation were then individually measured (head-tail length) using a Radio Shack digitizer and model 16 microcomputer.

Analysis of variance and the Student-Newman-Keuls range test were used to analyze for statistical significance at $p<0.05$ and $p<0.001$ (Steel and Torrie, '80). The analysis was conducted by comparing mean malformation and mortality percentages and mean growth lengths between treatments, for the tests with each inhibitor.

Results

Eighty embryos were treated as experimental controls for each clutch of embryos. Similar methylxanthine control treatments were established for each breeding. Mortality and malformation percentages for the experimental and methylxanthine controls may overlap to some degree between inhibitor compounds, depending on the number of inhibitors tested on each clutch. Specific experimental and methylxanthine control results for the tests with each inhibitor are presented in Tables III-VII. In the study a total of 560 embryos were treated as controls. Five of these died (0.9%) and 13 of the survivors were malformed (2.3%). The malformations were limited to pericardial and dorsal fin edema with the exception that two also had skeletal kinking. Caffeine at 0.08 mg/ml exposure concentration and theophylline and theobromine at 0.075 mg/ml (equimolar to caffeine) were used as the methylxanthines in all of the experiments. Each methylxanthine alone was tested on a total of 560 embryos as well. The mortality percentages for the methylxanthine control treatments, in the order presented above, were 1.6%, 2.3%, and 3.6%. The malformation percentages for the surviving embryos were 8.0%, 7.3%, and 4.1%, respectively. Throughout the study the malformations observed in each of the methylxanthine control treatments were limited to edema in the pericardium and dorsal fin and, occasionally, slight skeletal kinking.

The results of the coadministration of each methylxanthine and hydroxyurea on Xenopus embryo development, survival, and growth after 96 hours of exposure are presented in Table III. The average replicate malformation percentage in the embryos treated with 0.3 mg/ml of hydroxyurea, a DNA synthesis inhibitor, was 20.6%. None of the embryos

in this treatment died. The malformations observed were slight to moderate skeletal kinking and occasional fin edema and improper gut coiling. When caffeine or theophylline was coadministered with hydroxyurea, at the appropriate concentrations, a significant increase in both mortality ($p<0.05$) and malformation ($p<0.001$) percentages was observed over that seen for the hydroxyurea treatment alone. Caffeine and hydroxyurea together produced 6.9% death in the embryos and 89.0% of the survivors were malformed. Theophylline in combination with hydroxyurea resulted in death in 8.1% of the embryos while 50.6% of the survivors were malformed. No additional types of malformations were noted, but the severity of the edema and kinking was judged to be greater, overall. Theobromine coadministered with hydroxyurea resulted in death in 3.1% of the embryos and 25.2% of the survivors were malformed. There was no statistical difference in these percentages from those obtained for hydroxyurea alone. No enhancement of the skeletal kinking was observed when theobromine was coadministered with hydroxyurea but the degree of edema was variable. Throughout the study, the mean head-tail length (growth) of the embryos appeared to be the most sensitive indicator of treatment effects.

The results of tests on the interaction of the DNA synthesis inhibitor cytosine arabinoside with each methylxanthine are shown in Table IV. There was no significant difference in mortality in any of the treatments. Cytosine arabinoside alone, at 0.6 mg/ml, produced malformations in 23.6% of the survivors. The malformations were limited to edema and skeletal kinking, ventrally, at the tip of the tail. As with hydroxyurea, coadministration of caffeine or theophylline with this inhibitor produced a statistically significant

($p<0.001$) increase in the average replicate malformation percentage. In this case the malformation percentages for these two coadministration treatments were approximately the same, with 77.6% of the surviving embryos in the caffeine and cytosine arabinoside treatment malformed and 76.0% of the survivors in the theophylline and inhibitor treatment malformed. In both of these treatments the typical malformations observed were edema and a bowing of the tail with the ventral tail flexure at the tip. The embryos were judged to be more severely malformed due to the additional bowing in the tail. A second theobromine concentration was included in the tests with this inhibitor, in order to determine whether a higher concentration of theobromine would result in potentiation of the teratogenic effects. This concentration (0.12 mg/ml) produced 5.6% death and 11.1% of the surviving embryos were malformed. These values were slightly higher than occurred in the lower theobromine concentration (Table IV) but they were not statistically different. At both concentrations of theobromine in combination with cytosine arabinoside the percentages of the malformations (30.4% and 34.1% respectively) were approximately the sum of the malformation percentages for the respective theobromine and cytosine arabinoside treatments alone. These percentages were not statistically different from the malformation percentage for cytosine arabinoside alone at $p<0.001$. However, the malformation percentage for the higher theobromine concentration with the inhibitor was statistically different from that for cytosine arabinoside alone at $p<0.05$. The malformations observed in the coadministration treatments of theobromine and cytosine arabinoside were typical of those seen in cytosine arabinoside alone.

The results of the interaction of the nucleic acid synthesis inhibitor 5-fluorouracil (5-FU) and each methylxanthine are shown in Table V. The mortality percentages for the treatments were not statistically different. The percentage of malformed survivors in 5-FU alone, at 0.08 mg/ml, was 24.1%. Skeletal kinking and improper gut coiling were typically observed, while pericardial and fin edema were occasionally noted. Coadministration of caffeine or theophylline with 5-FU produced malformations in 85.7% and 87.8% of the survivors, respectively. In addition to more severe skeletal kinking, improper eye development was noted. Theobromine in combination with 5-FU also produced a statistically significant ($p<0.001$) increase in the percentage of malformed survivors (42.5%) compared to that obtained for 5-FU alone. The skeletal kinking was judged to be only slightly more severe and only a few of the malformed embryos had the eye defect.

Cycloheximide, a protein synthesis inhibitor, was also tested with each methylxanthine and these results are presented in Table VI. There was a statistical difference in mortality percentage between some of the treatments. Cycloheximide at a concentration of 0.04 ug/ml, resulted in malformations in 18.4% of the survivors. Slight kinking, edema, and necrosis of a portion of the dorsal fin were noted in these embryos. Coadministration of each methylxanthine with this inhibitor resulted in a statistically significant increase ($p<0.001$) in the average replicate malformation percentage. The malformation percentages were 80.7%, 69.6%, and 70.6% for cycloheximide administered with caffeine, theophylline, or theobromine, respectively. The kinking and edema were only slightly more severe but blistering in the dorsal

fin was observed in the area where the necrosis was noted in malformed embryos in cycloheximide alone.

A second protein synthesis inhibitor, emetine, was also coadministered with the methylxanthines. The results are shown in Table VII. Emetine alone, at 0.7 ug/ml, produced malformations in 28.9% of the survivors. The malformations included slight lateral kinking of the tail, predominantly to the left, and occasional blistering along the edge of the eyes. When emetine was coadministered with caffeine, theophylline, or theobromine the percentage of malformed survivors, 81.4%, 87.5%, and 81.7% respectively, was increased significantly ($p<0.001$). In all three cases the tail kinking was of the same relative order of severity and was similar to that observed in emetine alone. Nearly all of the malformed survivors in the coadministration treatments had the blistering around the eyes, of varying severity. As with cycloheximide, the malformation percentages between the coadministration treatments were the same statistically.

Discussion

The results of this study showed that coadministration of caffeine or theophylline with each of the inhibitor compounds resulted in potentiation of the teratogenic effect. Coadministration of theobromine and the protein synthesis inhibitors (cycloheximide, emetine) or 5-fluorouracil (5-FU) also resulted in teratogenic potentiation. In contrast only an additive effect was observed between theobromine and the inhibitors of DNA synthesis (hydroxyurea, cytosine arabinoside). We failed to note potentiation of cytosine arabinoside teratogenicity even when the dose of theobromine was raised from 0.075 to 0.12 mg/ml. A lack of potentiation between theobromine and mitomycin C (a DNA synthesis inhibitor) has been reported by Nakatsuka et al. ('83) in mice. Mitomycin C teratogenicity was potentiated by both caffeine and theophylline (Nakatsuka et al., '83). Ritter et al. ('82) demonstrated that coadministration of caffeine with hydroxyurea, cycloheximide, and emetine resulted in potentiation of embryo toxicity in rats.

In all treatments where potentiation took place the types of malformations in the combined treatments were typical of those noted with the inhibitors alone. The increased number of eye malformations observed when a methylxanthine was administered with emetine or 5-FU indicated that the teratogenicity of the inhibitors was potentiated by the action of the methylxanthines since none of the methylxanthines alone typically produced eye malformations. Dose-response tests on emetine and 5-FU alone have demonstrated a high incidence of eye malformations (unpublished observations).

The results of statistical comparisons of growth data from the tests produced the most sensitive indicator of the treatment effects (Tables III-VII). In general, growth was inhibited most in the treatments where teratogenic potentiation occurred. Emetine was an exception in the tests since embryos exposed to this inhibitor alone had a mean head-tail length equal to that of the controls (Table VII). Methylxanthine and emetine treatment only slightly reduced head-tail length.

The goal of this study was to determine whether the teratogenic effects of DNA, protein, and nucleic acid synthesis inhibitors could be potentiated in Xenopus embryos upon coadministration with methylxanthines. By demonstrating that such potentiation did occur, as it had in mammals, the study helps to validate the use of FETAX as an in vitro teratogenesis assay. This study has also demonstrated that FETAX could be used to study the structure-activity relationships of a family of compounds with respect to teratogenicity and that it has utility as a basic research tool for studying teratogenic processes. Our emphasis was on validation, therefore we did not attempt experiments that would elucidate the molecular nature of these effects. Future studies can be performed that may help to determine the mechanisms involved in this type of potentiation and to determine why theobromine does not potentiate the teratogenicity of DNA synthesis inhibitors.

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Table III. Effects of hydroxyurea and methylxanthines alone and in combination on Xenopus embryo development and growth at 96 hours[1].

Treatment	No. of embryos[2]	mean length[3] (mm)	No. dead (%)	No. of survivors malformed (%)[4]
Controls	160	9.46 ± 0.11^{a}	0 (0)^{g}	1 (0.6)^{i}
Hydroxyurea (Hyd)	160	9.25 ± 0.13^{b}	0 (0)^{g}	33 (20.6)^{j}
Caffeine (Caf)	160	9.09 ± 0.11^{c}	0 (0)^{g}	13 (8.1)^{i}
Caf & Hyd	160	$8.30 \pm 0.09^{d*}$	11 (6.9)^{h}	132 (89.0)^{k}
Theophylline (Tp)	160	9.05 ± 0.10^{c}	0 (0)^{g}	12 (7.5)^{i}
Tp & Hyd	160	$8.64 \pm 0.14^{e*}$	13 (8.1)^{h}	74 (50.6)^{l}
Theobromine (Tb)	160	9.30 ± 0.09^{b}	4 (2.5)^{g,h}	7 (4.5)^{i}
Tb & Hyd	160	$8.86 \pm 0.15^{f*}$	5 (3.1)^{g,h}	39 (25.2)^{j}

Concentrations: Hyd = 0.3 mg/ml; Caf = 0.08 mg/ml; Tp & Tb = 0.075 mg/ml.

[1]Analysis of variance and Student-Newman-Keuls range test.

[2]Two separate experiments, each with four replicates of twenty embryos.

[3]Mean head-tail length of surviving embryos $\pm$ standard error of the mean.

[4]Average of the replicate percentages of surviving malformed embryos.

^{a,b,c,d,e,f}are significantly different from each other at $p<0.05$.

^{g,h}are significantly different from each other at $p<0.05$.

^{i,j,k,l}are significantly different from each other at $p<0.001$.

*indicates significance from all other treatments at $p<0.001$.

Table IV. Effects of cytosine arabinoside and methylxanthines alone and in combination on Xenopus embryo development and growth at 96 hours[1].

Treatment	No. of embryos[2]	mean length[3] (mm)	No. dead (%)	No. of survivors malformed (%)[4]
Controls	160	9.36 ± 0.08^{a}	3 (1.9)	5 (3.1)^{g}
Cytosine arabinoside (Ara-C)	160	$9.12 \pm 0.08^{b,c}$	3 (1.9)	37 (23.6)^{h}
Caffeine (Caf)	160	8.93 ± 0.06^{d}	3 (1.9)	12 (7.7)^{g}
Caf & Ara-C	160	8.47 ± 0.09^{e}	4 (2.5)	121 (77.6)^{i}
Theophylline (Tp)	160	$9.01 \pm 0.08^{c,d}$	3 (1.9)	11 (6.9)^{g}
Tp & Ara-C	160	8.41 ± 0.09^{e}	5 (3.1)	118 (76.0)^{i}
Theobromine (Tb)	160	9.21 ± 0.07^{b}	7 (4.4)	6 (3.8)^{g}
Tb & Ara-C	160	8.88 ± 0.14^{d}	6 (3.8)	47 (30.4)^{h}
Theobromine 0.12 (Tbx)	160	$9.02 \pm 0.08^{c,d}$	9 (5.6)	17 (11.1)^{g}
Tbx & Ara-C	160	8.69 ± 0.10^{f}	2 (1.3)	56 (34.1)^{h*}

Concentrations: Ara-C = 0.6 mg/ml; Caf = 0.08 mg/ml; Tp & Tb = 0.075 mg/ml.

[1] Analysis of variance and Student-Newman-Keuls range test.

[2] Two separate experiments, each with four replicates of twenty embryos.

[3] Mean head-tail length of surviving embryos $\pm$ standard error of the mean.

[4] Average of the replicate percentages of surviving malformed embryos.

^{a,b,c,d,e,f} are significantly different from each other at $p<0.001$.

^{g,h,i} are significantly different from each other at $p<0.001$.

* indicates significance from Ara-C alone treatment at $p<0.05$.

Table V. Effects of 5-fluorouracil and methylxanthines alone and in combination on Xenopus embryo development and growth at 96 hours[1].

Treatment	No. of embryos[2]	mean length[3] (mm)	No. dead (%)	No. of survivors malformed (%)[4]
Controls	160	$9.38 \pm 0.06^{a*}$	1 (0.6)	4 (2.5)^{h}
5-fluorouracil (5-Fu)	160	9.02 ± 0.06^{b}	3 (1.9)	38 (24.1)^{i}
Caffeine (Caf)	160	$8.98 \pm 0.03^{b,c}$	3 (1.9)	12 (7.7)^{h}
Caf & 5-Fu	160	$8.18 \pm 0.10^{d*}$	6 (3.8)	132 (85.7)^{j}
Theophylline (Tp)	160	$9.13 \pm 0.02^{e*}$	7 (4.4)	12 (7.8)^{h}
Tp & 5-Fu	160	$8.63 \pm 0.07^{f*}$	3 (1.9)	138 (87.8)^{j}
Theobromine (Tb)	160	$9.29 \pm 0.05^{g*}$	5 (3.1)	7 (4.4)^{h}
Tb & 5-Fu	160	8.93 ± 0.07^{c}	0 (0)	68 (42.5)^{k}

Concentrations: 5-Fu & Caf = 0.08 mg/ml; Tp & Tb = 0.075 mg/ml.

[1]Analysis of variance and Student-Newman-Keuls range test.

[2]Two separate experiments, each with four replicates of twenty embryos.

[3]Mean head-tail length of surviving embryos ± standard error of the mean.

[4]Average of the replicate percentages of surviving malformed embryos.

^{a,b,c,d,e,f,g}are significantly different from each other at $p<0.05$.

^{h,i,j,k}are significantly different from each other at $p<0.001$.

*indicates significance from all other treatments at $p<0.001$.

Table VI. Effects of cycloheximide and methylxanthines alone and in combination on Xenopus embryo development and growth at 96 hours[1].

Treatment	No. of embryos[2]	mean length[3] (mm)	No. dead (%)	No. of survivors malformed (%)[4]
Controls	160	9.38 ± 0.06^{a}	1 $(0.6)^{i}$	4 $(2.5)^{k}$
Cycloheximide (Cyh)	160	$8.77 \pm 0.09^{b*}$	3 $(1.9)^{i}$	29 $(18.4)^{l}$
Caffeine (Caf)	160	$8.98 \pm 0.03^{c*}$	3 $(1.9)^{i}$	12 $(7.7)^{k,l}$
Caf & Cyh	160	$8.35 \pm 0.05^{d*}$	12 $(7.5)^{i,j}$	119 $(80.7)^{m+}$
Theophylline (Tp)	160	$9.13 \pm 0.02^{e*}$	7 $(4.4)^{i,j}$	12 $(7.8)^{k,l}$
Tp & Cyh	160	8.62 ± 0.06^{f}	7 $(4.4)^{i,j}$	106 $(69.6)^{m+}$
Theobromine (Tb)	160	9.29 ± 0.05^{g}	5 $(3.1)^{i,j}$	7 $(4.4)^{k}$
Tb & Cyh	160	8.54 ± 0.06^{h}	16 $(10.0)^{j}$	102 $(70.6)^{m+}$

Concentrations: Cyh = 0.04 µg/ml; Caf = 0.08 mg/ml; Tp & Tb = 0.075 mg/ml.

[1]Analysis of variance and Student-Newman-Keuls range test.

[2]Two separate experiments, each with four replicates of twenty embryos.

[3]Mean head-tail length of surviving embryos ± standard error of the mean.

[4]Average of the replicate percentages of surviving malformed embryos.

^{a,b,c,d,e,f,g,h}are significantly different from each other at $p<0.05$.

^{i,j}are significantly different from each other at $p<0.05$.

^{k,l,m}are significantly different from each other at $p<0.05$.

*indicates significance from all other treatments at $p<0.001$.

+indicates significance from all treatments lacking this designation at $p<0.001$.

Table VII. Effects of emetine and methylxanthines alone and in combination on Xenopus embryo development and growth at 96 hours[1].

Treatment	No. of embryos[2]	mean length[3] (mm)	No. dead (%)	No. of survivors malformed (%)[4]
Controls	160	9.93±0.17[a]	3 (1.9)	6 (3.8)[f]
Emetine (Eme)	160	9.97±0.21[a]	4 (2.5)	45 (28.9)[g]
Caffeine (Caf)	160	9.47±0.20[b,c]	4 (2.5)	16 (10.3)[f]
Caf & Eme	160	9.34±0.17[c]	4 (2.5)	127 (81.4)[h]
Theophylline (Tp)	160	9.60±0.19[b,d]	4 (2.5)	13 (8.3)[f]
Tp & Eme	160	9.33±0.21[c]	8 (5.0)	133 (87.5)[h]
Theobromine (Tb)	160	9.82±0.18[a,e]	8 (5.0)	8 (5.1)[f]
Tb & Eme	160	9.69±0.18[d,e]	12 (7.5)	121 (81.7)[h]

Concentrations: Eme = 0.7 µg/ml; Caf = 0.08 mg/ml; Tp & Tb = 0.075 mg/ml.

[1]Analysis of variance and Student-Newman-Keuls range test.

[2]Two separate experiments, each with four replicates of twenty embryos.

[3]Mean head-tail length of surviving embryos ± standard error of the mean.

[4]Average of the replicate percentages of surviving malformed embryos.

[a,b,c,d,e]are significantly different from each other at $p<0.05$.

[f,g,h]are significantly different from each other at $p<0.001$.

CHAPTER IV

EVALUATION OF THE DEVELOPMENTAL TOXICITY OF METAL-CONTAMINATED SEDIMENTS USING SHORT-TERM FATHEAD MINNOW AND FROG EMBRYO-LARVAL ASSAYS

Abstract

The effects of metal-contaminated sediment extracts and a reference toxicant (zinc sulfate) were determined by examining the developmental morphology, growth, and mortality of exposed fathead minnow (Pimephales promelas) and frog (Xenopus laevis) embryos. Sediments from two contaminated stream sites were extracted with reconstituted culture water at various pH's for 24 h. Developmental toxicity tests were performed using the FETAX protocol. The results suggested Zn was the major developmental toxicant in the sediment extracts. The measured Zn concentration in the sediment extracts which caused 50% malformation (EC50) of the fish embryos was 0.5 to 1.4 mg/L (normalized to 100 mg/L hardness). EC50 values for the reference toxicant tests were 0.6 and 0.8 mg/L Zn. The frog embryo EC50 for the extracts ranged from 2.2 to 3.6 mg/L Zn and was 3.6 mg/L Zn in the reference toxicant test. In 67% of the tests malformation was a more sensitive endpoint than growth inhibition. Mortality was the least sensitive endpoint, i.e., the LC50 in the reference toxicant tests were 3.6 mg/L Zn for the fathead minnow and 34.5 mg/L for the frog. The

extraction procedure may be useful for determining potential toxicity in the event metals are leached from aquatic sediments due to dredging or acidification.

Introduction

A major obstacle in evaluating the effects of aqueous contaminants has been the gap between the use of short-term lethal effects assays and assays that measure long-term chronic responses. Attempts to assess long-term effects without doing complete life cycle studies have focused on assays that measure effects on especially sensitive life processes such as reproduction and development. Therefore, examining gross terata during morphological development may help determine sublethal effects of contaminants [1].

Survival, growth, and reproduction responses of fathead minnows have been widely used for assaying contaminant effects [2-4] while inclusion of teratogenic effects has been a relatively recent development [1,5-6]. Frog embryos have been used for more than a decade to detect abnormal development caused by exposure to aqueous contaminants [7-9]. FETAX (Frog Embryo Teratogenesis Assay - Xenopus) was developed to rapidly and inexpensively determine the deleterious effects of sublethal levels of contaminants upon animal development [9-12]. The assay includes malformation as a distinct measure of effect, separate from survival and growth endpoints.

FETAX was used to detect heavy metal teratogens in water samples from Tar Creek, a stream in northeastern Oklahoma that had been contaminated by acidified mine-water surface seeps [12]. Heavy metals, especially Zn, accumulated in the sediments of Tar Creek and the Neosho River as a result of the contamination. McCormick developed a sediment extraction procedure with reconstituted water to permit bioassay evaluation of the release of harmful levels of metals from these

aquatic sediments prior to actual leaching that might occur during events such as hydraulic dredging or acidification [13].

In this study, metal-contaminated sediments were extracted in reconstituted water and the effects of the extracts and a reference toxicant (zinc sulfate) on morphological development, growth, and survival of fathead minnow and frog embryos were examined in order to evaluate the potential toxicity of the contaminated aquatic sediments.

Materials and Methods

Composite sediment samples from two sites, Tar Creek (S5, T28N, R23E) and Neosho River (S26, T27N, R23E) in Ottawa County, Oklahoma, were prepared for extraction [13] in modified FETAX solution (MFS). This reconstituted water medium contained 400 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$, and 75 mg $MgSO_4$ per liter of deionized distilled water. The pH of MFS was 7.9 with an average hardness and alkalinity of 106 and 72 mg/L $CaCO_3$, respectively. Preliminary tests indicated both fathead minnow and frog embryos could develop normally in this solution.

Extraction was modified from the method of McCormick [13] with extractions performed at pH 4, 5, 6, or 7. For each extraction, 50 g of sediment was added to 1 liter of MFS and the pH was then adjusted to the desired level with acetic acid (pH 4, 5, and 6) or NaOH (pH 7). Extractions were performed separately on the sediment sample aliquots from each site, using a new aliquot of sediment at each pH. The extraction procedure was repeated a second time at pH 4, 5, or 6, using new sediment aliquots for each site and pH. Extractions at pH 7 were not repeated. The extractions were performed in polypropylene bottles for 24 h, using a rotating tumbler. At 1, 6, 16, and 24 h of extraction the pH was readjusted, if necessary. During extraction, the pH varied by no more than $\pm$ 0.8, 0.6, 0.3, and 0.2 S.U. from the desired extraction pH at 1, 6, 16, and 24 h, respectively. An equipment malfunction necessitated stopping the first pH 5 and the pH 7 extractions after 20 h. The suspended sediment material was allowed to settle for 24 h after extraction. The solutions were then centrifuged

at 1475 x g for 10 min and the supernatants filtered (Whatman #1 filter paper). All extraction filtrates were readjusted to pH 7 with NaOH prior to use in the developmental toxicity tests. Readjustment to pH 7 was necessary to avoid toxic effects due to low pH. At each pH, a sample of MFS alone was carried through the extraction procedure, to serve as a control. An aliquot of each pH readjusted extract and control solution was analyzed for nonfilterable metals concentration by atomic absorption [14].

A portion of one Neosho River sediment extract (pH 4) was passed twice over Chelex-100 (Bio-Rad) to remove divalent metal ions, to determine if metals were primarily responsible for the effects observed [12,15]. An extracted MFS sample was also chelated as a control.

Animal culture and breeding procedures were as described for fathead minnows (Pimephales promelas) [16] and frogs (Xenopus laevis) [12]. Normally developing embryos at the gastrula (fathead minnow) or blastula (frog) stages were placed in various dilutions of the pH readjusted (7.0) extracts. These dilutions were based on the measured Zn concentration of each sample. The tests were conducted in covered plastic Petri dishes [12]. At each dilution, 10 to 20 fathead minnow embryos were tested in each of two dishes containing 10 ml of solution while 15 to 20 frog embryos were tested in each of two dishes containing 8 ml of solution [12]. Controls were tested in both MFS and extracted MFS.

Static renewal tests were conducted for 6 d with fathead minnow embryos (to allow hatching to take place) and 4 d with frog embryos [9]. During the tests, pH, conductivity, and dissolved oxygen parameters were measured daily on each sample while hardness and

alkalinity were determined at the start and end of each test and on various other days. All tests were conducted at 22 to 24°C. Dead organisms were counted and removed at daily renewal. At the end of each test surviving larvae were fixed in formalin. Gross terata (eg. head, eye, gut, skeletal, and cardiovascular abnormalities and edema) were determined using a dissecting microscope. Malformed individuals that died prior to test termination were not included in the number malformed [9]. Head-tail length of the organisms was used as an index of growth [12].

Since previous work had implicated Zn as the suspect deleterious contaminant, zinc sulfate ($ZnSO_4 \cdot 7H_2O$) was chosen as the reference toxicant to permit comparison of the observed effects of the sediment extracts with a known toxicant. Initial tests using frog embryos determined the response ranges to zinc sulfate. Full reference toxicant tests were conducted twice for the fish embryos and once for the frogs. As with the extract tests, the reference toxicant tests were conducted at pH 7. The test procedures were as described above with the stock Zn concentration determined by atomic absorption analysis.

To facilitate comparison, the Zn concentration of each test dilution was normalized to 100 mg/L $CaCO_3$ hardness [17,18]. The 50% malformation and lethal concentrations [EC50(malformation) and LC50] were determined as mg/L Zn, using the TOXDAT Multi-method program [16]. In addition, the 50% effects concentrations for mortality and malformation in the reference toxicant tests were calculated using the Litchfield-Wilcoxon test [19]. The t-Test for grouped observations was used to determine the minimum concentration to inhibit growth (MCIG) in

each test (p=0.05) [12]. All EC50, LC50, and MCIG values were determined after the Zn concentration of each dilution of the sediment extracts and reference toxicant had been normalized to 100 mg/L hardness. Therefore, all test results are presented in terms of normalized Zn concentration. Teratogenicity Index (TI) values were calculated for the reference toxicant tests [9].

Results

The measured Zn concentration in the undiluted sediment extracts ranged from 1.0 mg/L at pH 7 to 53.6 mg/L at pH 4 (Table VIII). Iron was the next most abundant metal detected in the extracts with a range from 2.1 to 13.0 mg/L. Trace levels of nickel, chromium, cadmium, arsenic, and selenium were detected in some extracts. No detectable quantities of lead or copper were found in any sample. Mean alkalinity and hardness values for the undiluted extract samples ranged from 62 to 1900 mg/L and 110 to 560 mg/L $CaCO_3$, respectively. The dissolved oxygen content of the exposure solutions ranged from 6 to 8 mg/L while the range for pH was 6.8 to 7.6 during the tests.

The EC50(malformation) for fathead minnow embryo/larvae exposed to the sediment extracts ranged from 0.5 to 1.1 mg/L Zn (normalized concentrations), except for one pH 7 extract with an EC50 of 1.4 mg/L Zn (Table IX). In two reference toxicant tests the EC50 values were 0.6 and 0.8 mg/L Zn. The minimum concentration to inhibit growth (MCIG) for the extracts ranged from 0.4 to 1.6 mg/L Zn and were 0.6 and 0.9 mg/L Zn in the reference toxicant tests. The 6 d LC50s for the pH 4 and 5 extracts ranged from 1.5 to 2.2 mg/L Zn, however, 50% mortality was not obtained in the pH 6 and 7 extracts. The LC50 for the second reference toxicant test was 3.6 mg/L Zn, whereas the concentration range for the first test was not high enough to obtain an LC50. Control malformation was usually zero and never >10%. Mortality in the controls was always 5% or less. The concentration-response curves for malformation of fathead minnow embryos exposed to the sediment extracts and the reference toxicant (zinc sulfate) essentially overlapped. The concentration-response curves for the pH 5 extracts and the

reference toxicant appear in Figure 3. Curves for the other extracts were similar and are, therefore, not shown.

The EC50 for frog embryos exposed to the sediment extracts ranged from 2.2 to 3.6 mg/L Zn and was 3.6 mg/L Zn for the reference toxicant test (Table IX). The MCIG was between 2.0 and 4.2 mg/L Zn for the extracts and was 4.2 mg/L Zn for the reference toxicant test. There was little or no mortality in the extracts after 4 d of exposure. The 4 d LC50 for the reference toxicant test was 34.5 mg/L Zn. Control malformation and mortality was usually <5% and always <10%. The concentration-response curves for malformation of frog embryos exposed to the sediment extracts and the reference toxicant also essentially overlapped. The response curves for frog embryos exposed to the pH 5 extracts and the reference toxicant appear in Figure 4.

The malformation and mortality curves for the reference toxicant tests were in separate concentration ranges for both species (Fig. 5). The Teratogenicity Index values [TI = LC50/EC50 (malformation)] were 5.1 for fathead minnows and 9.6 for frogs.

The types and severity of malformations observed in fathead minnow larvae were similar between the sediment extract and reference toxicant tests. The malformations were more severe with increasing Zn concentration. Below 0.5 mg/L Zn, abnormalities were mild kinking or edema which were also observed in some controls. Above 1 mg/L, gross malformations were observed: edema (gut, pericardial, and eye), kinking, fin abnormalities and blistering. At higher Zn concentrations the mouth was incompletely formed and hemorrhaging and cardiac abnormalities were noted. Some organisms did not hatch. All grossly malformed individuals were incapable of swimming normally, if at all.

Some premature hatching took place on days 3 and 4 at 1 to 4 mg/L Zn. This did not appear to affect survival. Above 4 mg/L, membrane rupturing was often observed on days 1 and 2. These organisms appeared to be abnormal and soon died.

The types and severity of malformations observed in frog larvae were also similar between the sediment extract and reference toxicant tests and the malformations were more severe with increasing Zn concentration. A noticeable increase in pericardial edema and abnormal gut coiling occurred in frog embryos exposed to 2 to 5 mg/L Zn. Above 5 mg/L, skeletal kinking and edema in the dorsal fin were also observed. Above 8 mg/L Zn, fin blistering and edema around the eyes were noted. There was also incomplete gut coiling, the mouth was improperly formed, and the size of the head, eye, and brain reduced. Above 20 mg/L Zn, in the reference toxicant test, all tadpoles lacked proper pigmentation. Heart coiling was absent in some embryos. Facial and fin necrosis were noted in live tadpoles. Severely malformed organisms displayed a shaking movement, otherwise swimming behavior was absent. Animals that died appeared to be paralyzed before death.

Thirty frog embryos that developed normally for 72 h in MFS were exposed to one extract from 72 to 96 h. All of these embryos had severe edema in the heart and gut regions at 96 h and gut coiling was halted.

Fathead minnow and frog embryos showed no malformation or mortality above control levels after exposure to the chelated portion of an extract (Neosho River #1 pH 4). More than 99% of the zinc was removed by chelation, leaving an effective Zn concentration of 0.24 mg/L (normalized concentration) in the sample. When fathead minnow embryos

were exposed to the unchelated portion of this sample, all were dead at 48 h while all frog embryos were severely malformed by the end of the test.

Discussion

The Teratogenicity Index (TI) values for the reference toxicant tests indicated Zn was teratogenic to the developing aquatic vertebrates [19]. The results suggested Zn was the major causative agent for the teratogenic effects observed in organisms exposed to the sediment extracts. Other metals present in the extracts may have interacted to affect developmental toxicity. Chelation, specific for divalent metal ions, removed >99% of the heavy metals and reduced malformation to control levels, therefore, organics were unlikely to have caused the malformations in the extract samples.

The fathead minnow embryo 6 d LC50 for the pH 4 and 5 extracts were somewhat lower than that for the reference toxicant test. This indicated that another factor in addition to Zn toxicity was involved. The additional toxicity may have been due to one or more of the other metals in these extracts, possibly nickel. In contrast, 50% mortality was not observed in three pH 6 extracts with measured Zn concentrations above the reference toxicant LC50 for fathead minnow embryos (3.6 mg/L Zn). This lack of expected mortality can be explained by the effective Zn concentration of less than 3 mg/L in these samples after normalization for hardness. Similarly, the lack of frog embryo mortality in the pH 4 extracts can be explained by the effective Zn concentration of less than 16 mg/L in the samples after normalization. In the frog embryo reference toxicant test there was no mortality at 16 mg/L Zn.

The types of malformations in both test species were similar and increased in severity as Zn increased. In 67% of the tests in which effects were observed, the EC50(malformation) was lower than the

minimum concentration to inhibit growth (MCIG), demonstrating the sensitivity of the malformation endpoint. Gross terata were easily detected using a dissecting microscope. Some malformations were obvious to the unaided eye.

Thirty normally developing frog larvae exposed at 72 h to an extract sample with 12.5 mg/L Zn were all severely malformed 24 h later. This demonstrated that even a brief exposure to Zn during organogenesis was detrimental to the developing aquatic vertebrates.

The sensitivity of the results in this study compare favorably with previous work. For example, in a 10 month chronic study using adult fathead minnows, Brungs demonstrated that reproduction was inhibited at as low as 0.1 mg/L Zn (concentration normalized to 100 mg/L hardness for comparison) [2]. In the present study, the no observed effects concentrations (NOEC) for malformation of fathead minnow embryos was 0.12 mg/L Zn in the reference toxicant tests. The lowest observed effects concentration (LOEC) for malformation was 0.43 mg/L Zn. Therefore, the LOEC in our short-term study approached the LOEC for the 10 month life cycle study.

Brungs exposed eggs spawned in control water to Zn, although there was no mention of malformations, reported fry mortality after 20 d exposure was 2 to 12% at 0.37 mg/L and 51 to 70% at 0.73 mg/L Zn (concentrations normalized to 100 mg/L hardness)[2]. In the present study, 20% of the fathead minnow embryos were malformed after 6 d exposure to 0.58 mg/L Zn while 48% were malformed at 0.76 mg/L Zn. Fry survivability would be impaired if abnormal development inhibited feeding and/or swimming abilities.

The Teratogenicity Index (TI) is a relative measure of the separation of the malformation and mortality curves [9]. The TI values obtained for Zn in this study (5.1 and 9.6 for fish and frog embryos, respectively) were relatively high [Dawson and Bantle, in press]. This indicated that the lowest Zn concentration needed to cause mortality of the embryos was approximately 5 to 10 times greater than the lowest concentration needed to produce malformations. In contrast to Zn, the TI for Cd in frog embryos was 1.3 [10], indicative of a small separation of the malformation and mortality curves. In published Zn and Cd life cycle studies using fathead minnows, a similar pattern was apparent for the separation of the reproductive inhibition and mortality curves. For Zn, the lowest concentration necessary to cause mortality in adults was more than 10 times greater than the lowest concentration needed to inhibit reproduction [2]. For Cd, the lowest concentration necessary to cause mortality was less than 2 times greater than that needed to inhibit reproduction [3]. Further tests evaluating gross terata, reproductive inhibition, and mortality are needed to determine if there is a consistent relationship between these parameters. If so, the malformation endpoint and TI of FETAX used in this investigation may prove useful for rapidly determining sublethal effects levels of aqueous contaminants.

The mechanism of Zn teratogenicity is presently unknown although the inhibition of DNA synthesis by excess Zn is the most likely explanation. Zinc is a cofactor for many enzymes such as DNA polymerase and thymidine kinase [20]. Due to this requirement, a slight increase in Zn concentration can stimulate DNA synthesis while a large deficiency or excess leads to an inhibition of DNA synthesis

[21]. DNA synthesis inhibitors have proven to be teratogenic in a variety of animals regardless of their mode of action [22-23]. Zinc deficiency is clearly teratogenic in mammals while Zn excess seems to be less teratogenic [24-25]. This may be due to the action of maternal liver and placental metallothioneins which prevent excess Zn from disrupting embryonic DNA synthesis. Oviparous aquatic organisms would not receive such protection and would likely be more sensitive to large excesses of Zn, causing abnormal development. In this study, the relatively large TI values and severity of the malformations in the Zn reference toxicant tests were consistent with previous FETAX studies in which known DNA synthesis inhibitors were tested [22]. Further tests are needed to more fully evaluate DNA synthesis inhibition as the mechanism for Zn teratogenicity.

The potential toxicity of the sediments tested in this study related primarily to the effective Zn concentration in the extracts. Therefore, the acid-extraction technique for metal-contaminated aquatic sediments [13] appears to be useful for evaluating potential toxicity in the event metals are released from aquatic sediments due to dredging or acidification.

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Table VIII. Metals content,[a] alkalinity, and hardness of the undiluted sample extracts.

Sediment Extracts	Zn	Fe	Ni	Cr	Cd	As	Se	Alk.[b]	Hard.[b]
pH 7 extracts									
Tar Creek #1	1.7	7.06	<0.13	0.010	<0.005	0.035	0.011	83	110
Neosho River #1	1.0	7.62	0.13	0.012	<0.005	0.025	0.010	62	151
pH 6 extracts									
Tar Creek #1	4.4	7.05	<0.13	0.007	<0.005	0.014	0.011	95	167
Tar Creek #2	4.6	5.24	<0.13	0.008	<0.005	<0.005	0.016	49	175
Neosho River #1	4.1	4.44	<0.13	<0.005	<0.005	<0.005	0.010	111	215
Neosho River #2	2.4	4.96	<0.13	0.008	<0.005	<0.005	0.016	95	205
pH 5 extracts									
Tar Creek #1	16.0	4.10	0.19	<0.005	<0.005	0.005	0.009	153	373
Tar Creek #2	16.2	2.07	0.20	<0.005	<0.005	<0.005	0.016	206	287
Neosho River #1	14.6	3.16	0.18	0.006	<0.005	0.005	0.013	239	354
Neosho River #2	12.0	4.95	0.16	<0.005	<0.005	<0.005	0.013	229	319

Table VIII. cont.

pH 4 extracts

Tar Creek #1	53.6	10.49	0.66	<0.005	0.025	0.010	0.029	1280	453
Tar Creek #2	53.2	12.96	0.70	<0.005	0.029	0.012	0.049	1460	515
Neosho River #1	48.3	5.02	0.58	<0.005	0.060	0.017	0.036	1700	510
Neosho River #2	45.7	6.36	0.59	<0.005	0.036	0.017	0.042	1900	560
Neosho River chelated[c]	0.4	<0.05	<0.13	<0.005	<0.005	0.020	0.040	1490	182

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[a] Values are mg/L as determined by atomic absorption analysis.

[b] Alk. - alkalinity, Hard. - hardness: Mean value of undiluted extracts, expressed as mg/L $CaCO_3$.

[c] Sample was passed over Chelex-100, a bound ion-exchange resin, to remove heavy metal ions.

Table IX. Malformation, growth, and mortality responses of fish and frog embryos in the extract and zinc tests.

Samples	FATHEAD MINNOW			FROG		
	EC50[a](95%CI)	MCIG[b]	LC50 (95%CI)	EC50[a](95%CI)	MCIG[b]	LC50 (95%CI)
	(mg/L Zn - normalized to 100 mg/L hardness)					
pH 7 extracts						
Tar Creek #1	1.4 (±1.4)	1.6	---[c]	---	---	---
Neosho River #1	---	---	---	---	---	---
pH 6 extracts						
Tar Creek #1	0.8 (±0.3)	1.1	---	---	2.9	---
Tar Creek #2	0.7 (±0.3)	1.1	---	2.8 (±0.2)	2.9	---
Neosho River #1	0.9 (±0.4)	0.9	---	---	2.0	---
Neosho River #2	1.1 (±1.1)	0.8	---	---	---	---
pH 5 extracts						
Tar Creek #1	0.7 (±0.2)	1.2	1.6 (±0.6)	2.7 (±0.5)	2.8	---
Tar Creek #2	0.8 (±0.4)	1.4	2.2 (±0.8)	3.3 (±0.5)	2.4	---
Neosho River #1	0.6 (±0.2)	1.1	1.3 (±0.7)	2.5 (±0.5)	3.2	---
Neosho River #2	0.5 (±0.4)	1.0	1.6 (±0.7)	2.5 (±0.3)	2.3	---

Table IX. cont.

pH 4 extracts						
Tar Creek #1	0.8	0.5	1.7 (±1.0)	3.2 (±1.0)	3.9	---
Tar Creek #2	0.6 (±0.6)	1.4	1.5 (±1.5)	3.5 (±1.0)	3.8	---
Neosho River #1	0.5 (±0.5)	0.4	2.1 (±1.2)	2.9 (±1.2)	3.4	---
Neosho River #2	0.8 (±0.4)	1.2	2.1 (±1.0)	3.6 (±0.7)	4.2	---
Zinc						
Zinc Sulfate #1	0.6 (±0.6)	0.9	---	3.6 (±0.5)	4.2	34.5 (±1.2)
Zinc Sulfate #2	0.8 (±0.3)	0.6	3.6 (±0.9)			

[a] EC50 for malformation only, values are for 6 d tests for fathead minnow and 4 d tests for frog embryos.

[b] Minimum concentration to inhibit growth (p=0.05).

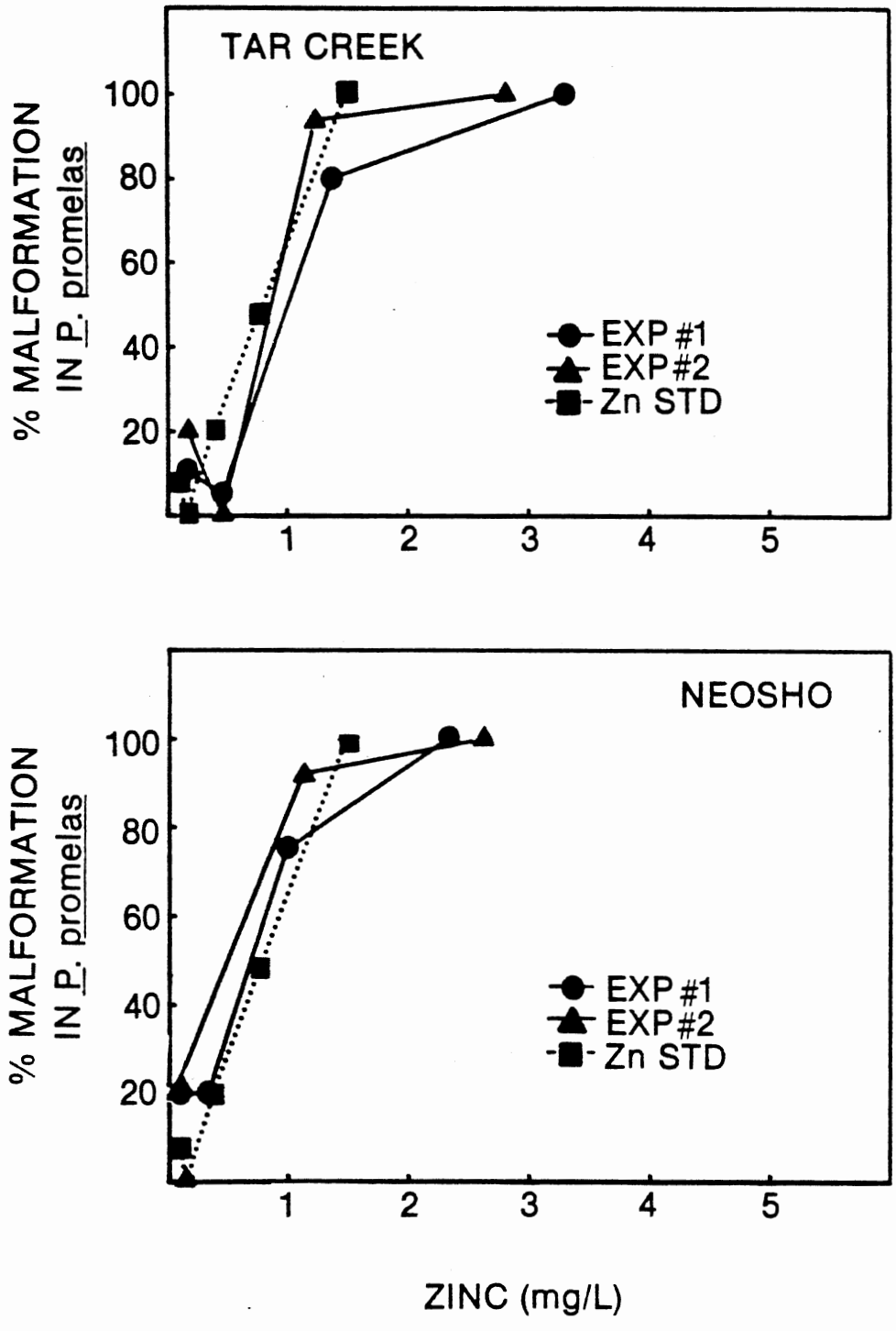
[c] --- indicates 50% effect not obtained or growth not inhibited.

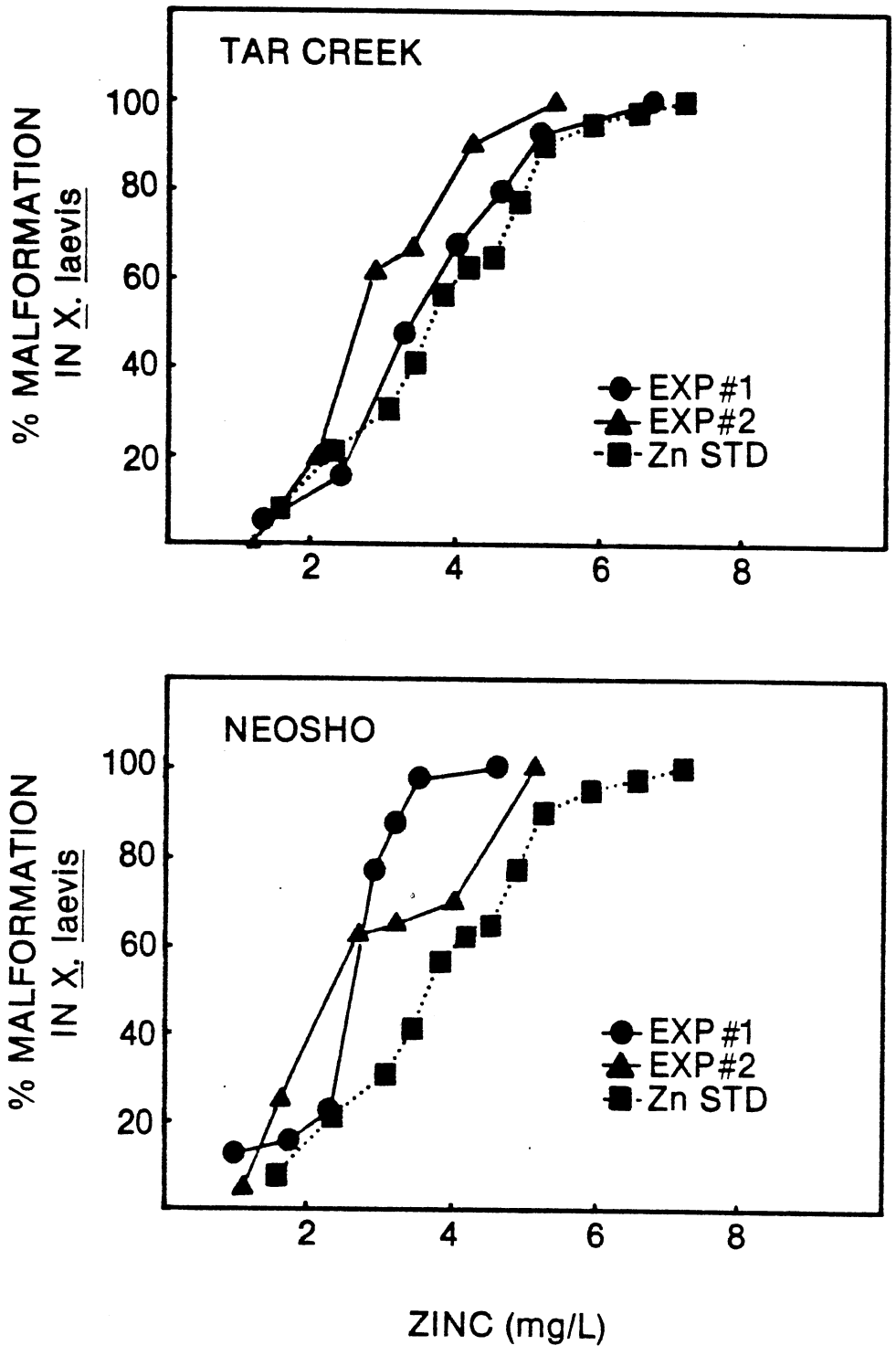
Figure Legends

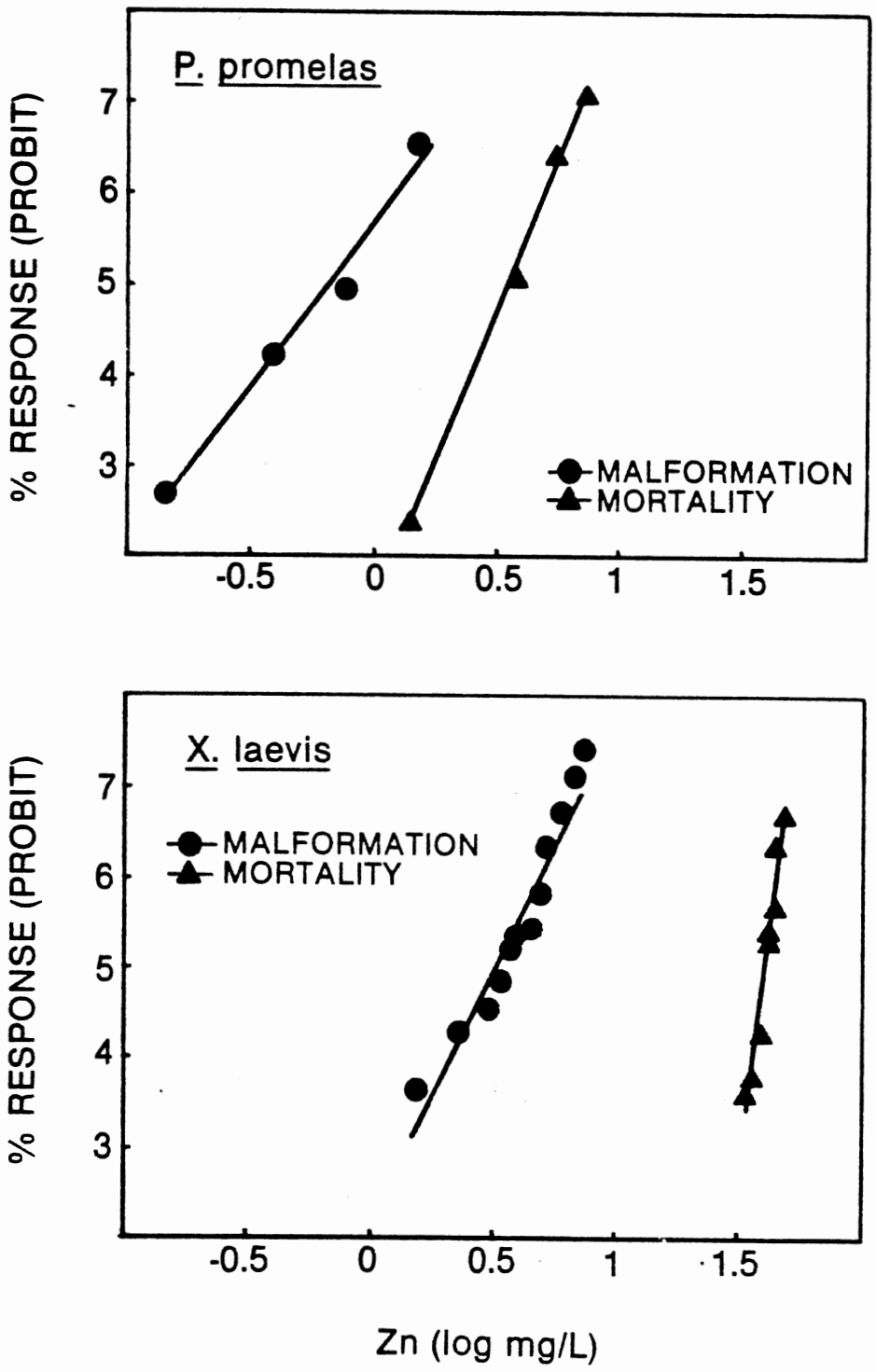
Figure 3. Zinc concentration-response curves for malformation of fathead minnow embryos exposed for 6 d to Tar Creek and Neosho River sediments extracted at pH 5 (Exp #1 = first extract exposure, Exp #2 = second extract exposure) and the zinc sulfate reference toxicant. Tests were conducted at pH 7.

Figure 4. Zinc concentration-response curves for malformation of frog embryos exposed for 4 d to Tar Creek and Neosho River sediments extracted at pH 5 (Exp #1 = first extract exposure, Exp #2 = second extract exposure) and the zinc sulfate reference toxicant. Tests were conducted at pH 7.

Figure 5. Concentration-response curves for malformation and mortality of fathead minnow and frog embryos exposed for 6 and 4 d, respectively, to the zinc sulfate reference toxicant. Tests were conducted at pH 7.


TAR CREEK
% MALFORMATION IN P. promelas
100
80
60
40
20
1
2
3
4
5
EXP #1
EXP #2
Zn STD
NEOSHO
% MALFORMATION IN P. promelas
100
80
60
40
20
1
2
3
4
5
EXP #1
EXP #2
Zn STD
ZINC (mg/L)


TAR CREEK
% MALFORMATION IN X. laevis
100
80
60
40
20
2
4
6
8
EXP #1
EXP #2
Zn STD
NEOSHO
% MALFORMATION IN X. laevis
100
80
60
40
20
2
4
6
8
EXP #1
EXP #2
Zn STD
ZINC (mg/L)


P. promelas
% RESPONSE (PROBIT)
7
6
5
4
3
-0.5
0
0.5
1
1.5
MALFORMATION
MORTALITY
X. laevis
% RESPONSE (PROBIT)
7
6
5
4
3
-0.5
0
0.5
1
1.5
MALFORMATION
MORTALITY
Zn (log mg/L)

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