DEVELOPMENTAL TOXICITY TESTING WITH THE FROG EMBRYO TERATOGENESIS ASSAY: XENOPUS (FETAX) AND AN EXOGENOUS METABOLIC ACTIVATION SYSTEM: EVALUATION AND APPLICATIONS

By

DOUGLAS JAMES FORT

Bachelor of Science
Southwestern College
Winfield, Kansas
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Master of Science
Oklahoma State University
Stillwater, Oklahoma
1988

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Thesis Approved:

John C. Bantle
Thesis Adviser

Cyril R Clarke

James T Blankenship

H. L. Burks

Norman N Durham
Dean of the Graduate College

PREFACE

The Frog Embryo Teratogenesis Assay: Xenopus is an in vitro bioassay designed to determine the potential teratogenic hazard a compound or complex mixture poses to developing organisms. Metabolism is a significant obstacle for most in vitro developmental toxicity assays and should not be overlooked in a proper screening test. If FETAX is to be routinely used for assessing potential hazard it must be combine with a properly validated exogenous metabolic activation system. Thus, this project was conducted to increase the overall acceptance of FETAX and an exogenous bioactivation system by evaluating its predictability, applying it to a variety of situations, and investigating potential short-comings of the system. By so doing, it is hoped that this research will spur other scientists in both biomedical and environmental toxicology to use FETAX routinely as a screen for potential developmental toxicants.

This dissertation is divided into four chapters. Chapter I provides background and introductory information. Chapters II through IV describe evaluation and application studies of the exogenous metabolic activation system. Chapter II describes the evaluation of the Aroclor 1254-induced metabolic activation system with compounds thought

to be either proteratogenic, bioinactivated by cytochrome P-450, or virtually unaffected by mixed functional oxidase metabolism in mammalian test systems. This work has been published in the Journal of Applied Toxicology (Volume 9, pages 377-388, 1989). Chapter III provides a discussion of the developmental toxicity of the tuberculostatic drug isoniazid. This section provides an explanation of why isoniazid produces false-positive test results with in vitro developmental toxicity screening systems when tested without a suitable bioactivation system. A discussion of the counter-productivity of Aroclor 1254-induced metabolic activation systems is also provided. Chapter III has been submitted to Teratogenesis, Carcinogenesis, and Mutagenesis and is currently being reviewed. Chapter IV describes an investigation of the toxicological mechanisms of action of the known mammalian teratogen, diphenylhydantoin, using FETAX, an in vitro metabolic activation system, and various metabolic inhibitors. Chapter IV has been accepted for publication by Fundamental and Applied Toxicology.

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CHAPTER I

INTRODUCTION

Background

Increasing demands for developmental toxicity screening necessitate the development, validation and use of alternative screening systems (1-10) to the traditional mammalian test systems. In vitro teratogenesis screening systems may provide a rapid, cost-effective method of indentifying compounds and complex environmental mixtures which may be potential teratogenic hazards (11). Alternative models may also alleviate burdens associated with increasing concern for animal welfare. A fundamental problem shared by many in vitro developmental screening systems is the inability to metabolize xenobiotics. This presents a major problem in the detection of proteratogenic compounds, as well as compounds inactivated by the mixed-functional oxidase system (MFO).

The Frog Embryo Teratogenesis Assay: Xenopus (FETAX) is a 96-hour static-renewal bioassay designed to screen potential developmental toxicants in the workplace or the environment (12). Since Xenopus embryos lack many metabolic systems including the MFO system prior to 96-hours of

development an exogenous metabolic activation system was developed using Aroclor 1254-induced rat liver microsomes (13).

Objectives

The specific objective of this dissertation research was to combine evaluation of the exogenous metabolic activation system with creative applications of this system using FETAX. To accomplish this objective two areas of investigation were emphasized: evaluation and applications. The evaluation process was further subdivided into validation studies and estimation of potential short-comings of the Aroclor 1254-induced metabolic activation system.

Metabolic Incompetence

A recognized obstacle in most *in vitro* assay systems is the inability to biotransform compounds into active teratogenic species. Metabolism must not be overlooked in a proper screening test (11). Furthermore, compounds should be tested both with and without a metabolic activating system, exogenous when necessary.

Obviously, some metabolic activation systems are incompatible with the viability of the test. Of those tests supplying a source of metabolism, the Ames mutagenicity test (14) and a cultured whole rat embryo screen (15) employ rat liver S-9 supernatant. *Drosophila* have been successfully cultured with both *Drosophila* and rat liver S-27 and S-9

supernatant (16). In addition, cultured mouse erythrocytes have been used to mediate the the metabolic activation of cyclophosphamide in the Saccharomyces cerevisiae mutagenicity test (17).

Noshiro and Omura (18) demonstrated that Xenopus embryos exhibit little xenobiotic metabolism although the adult has limited capabilities. Therefore, through 96 h of development Xenopus embryos are virtually useless for detecting proteratogenic compounds without an exogenous metabolic activation system.

Experimental Design

Detection of Teratogenic Agents Using Xenopus Embryos

The original study on a bioassay employing Xenopus to detect environmental teratogens was performed by Greenhouse (19). Greenhouse used a short-term (48-hour) protocol exposing embryos to N-phenyl-a-napthylamine and various hydrazines to demonstrate the deleterious effects on developing embryos. Dumont et al. (12) demonstrated the hazardous effects of coal-conversion, shale and oil products on Xenopus embryos using a 96 h exposure protocol.

In order to measure the teratogenic potential of a substance, Xenopus embryos in the late blastulae stage were exposed to different concentrations of a toxicant. Mortality, malformation, and growth were measured at the end

of the 4 day test. As a measure of predicting the teratogenic hazard of a given substance, a teratogenic index (TI) was calculated by dividing the 96 h LC50 (median lethal concentration) by the EC50 (malformation) (median teratogenic concentration) (12). Compounds with TI values greater than 1.5 are considered to have strong teratogenic potential. Subsequently, several laboratories (10,12,20) have used FETAX for the screening of potential developmental toxicants.

Design of Metabolic Activation System

Five days prior to microsome preparation, an adult Sprague-Dawley (SD) CD strain male rat (200-280 g) was injected with 500 mg/Kg Aroclor 1254 in corn oil (21). Liver was perfused with 50 ml of 1.15% KCl containing 0.02 M Tris-HCl buffer, pH 7.5. Homogenization was performed in 1.15% KCl containing 0.02 M Tris-HCl buffer and 0.5% bovine serum albumin (BSA). The homogenate was centrifuged successively at 600 and 9000 x g avg. The crude S-9 supernatant was further purified by two additional ultracentrifugation steps at 102,000 x g avg. The microsomal pellet was then resuspended in 0.05 M Tris-HCl buffer, pH 7.5, put into 1 ml aliquots, and immediately frozen in liquid nitrogen (22). Protein was determined by the method of Bradford (23) (BioRad, Richmond, CA). P-450 activity was inferred by the measurement of formaldehyde

generated from the N-demethylation of aminopyrine by the method of Nash (24). Assay conditions were described by Lucier et al. (25). Several aliquots of microsomes were chemically reduced with dithionite and pretreated with carbon monoxide (CO) to selectively inactivate P-450 activity (26).

Animal Care and Breeding

Xenopus adult care, breeding, and embryo collection was performed according to Courchesne and Bantle (20).

Assay Protocol

For each separate clutch of embryos, four sets of 20 are placed in 60 mm covered plastic Petri dishes containing FETAX solution, a reconstituted water media (27), and 100 U/ml penicillin-100 ug/ml streptomycin in 8 ml of total solution. These were designated FETAX controls. Treatments were performed in duplicate with 20 embryos per dish. Each test dish received microsomal protein, NADPH generating system, 100 U/ml penicillin-100 ug/ml streptomycin, and various concentrations of toxicant. The NADPH generating system consisted of 3.5 mM glucose 6-phosphate, 0.31 U/ml glucose 6-phosphate dehydrogenase, 0.1 mM NADP, and 0.007 mM NADPH. Metabolic activation system, activated cyclophosphamide [positive control], and CO-gassed microsomes and toxicant [negative control] were prepared in a similar manner. All dishes contained 8 ml of the

appropriate solutions, which were previously diluted from stocks prepared in FETAX solution. All solutions were removed every 24 h of the four d test and fresh solutions added in a static-renewal fashion.

Data Collection and Analysis

Dead embryos were removed every 24 h and the number recorded. Death at 24 and 48 h was ascertained by embryo skin pigmentation, structural integrity, and irritability. At 72 and 96 h the lack of a heart beat was an unambiguous sign of death in the transparent embryo. Surviving larvae were fixed in 0.6% formalin, pH 7.0. The number of live-malformed embryos and the stage of development according to Nieuwkoop and Faber (28) were determined. Dose-response bioassays evaluated according to Litchfield-Wilcoxon (29) were used to determine the 96 h LC50 and EC50 (malformation). The 96 h LC50 was divided by the EC50 (malformation) yielding a teratogenic index (TI) which has proven useful in assessing teratogenic hazard (13,20,27,30). Head-tail length was measured using a Radio Shack digitizer and model 16 microcomputer.

Approach

Evaluation

Validation. Validation is the process of selecting a number of compounds which have well characterized mammalian

teratogenic potential in vivo, testing them in the model system, and comparing the results (31). Thus, evaluation of the system was needed to demonstrate that the metabolic activation system was adequately predicting the potential teratogenic hazard of chemicals tested in FETAX. As many as 50 compounds of varying teratogenic potency may need to be tested to fully validate the assay (11). In this portion of the study, testing was completed within time and cost restraints to evaluate the predictability of FETAX and the Aroclor-induced exogenous metabolic activation system. Three compounds suspected to be proteratogenic in mammalian test systems, one compound unaffected by mixed-functional oxidase metabolism, and one compound inactivated by cytochrome P-450 were selected from several published lists. Ideally, evaluation of this metabolic activation system should determine whether or not it is the system of choice for FETAX.

Short-Comings. Aroclor 1254 induces P-450 isozymes characteristic of the two prototype inducers 3-methylcholanthrene (3-MC) and phenobarbital (PB) (32). Thus, Aroclor 1254-induced microsomes are quite effective in metabolizing a diverse array of xenobiotics. Interestingly, several investigators (33,34) have found decreased levels of other putative P-450 isozymes, including the pyrozole-inducible P-450j. The Aroclor 1254-induced system should be highly effective for investigating the developmental toxicity of compounds metabolized by 3-MC or PB inducible

isozymes. However, compounds metabolized by isozymes repressed by Aroclor 1254 would have a greater chance of being falsely scored, thus causing Aroclor 1254-induction to be counter-productive. In this study, attempts were made to determine the magnitude of this problem using the tuberculostatic drug isoniazid and metabolic activation system induced by different chemical agents. Isoniazid was found previously to be metabolized by P-450j (35). The specific aim of this portion of the research was to determine whether or not a potential problem exists and develop modifications if needed. Ultimately, this would improve the predictive value of the metabolic activation system and should aid in overall acceptance of FETAX by future investigators.

Applications. In order for FETAX to gain acceptance as an in vitro developmental toxicity screening assay a variety of practical applications and overall versatility must be demonstrated. Since Xenopus embryos lack MFO activity prior to 96-h of development, testing with and without an exogenous metabolic activation system provides a means of assessing the role MFO metabolism plays in the potential developmental toxicity of a compound. Evaluating the potential teratogenicity of both parent compound and metabolites with the inclusion of a bioactivation system allows for a more accurate account of what may be occurring in the mammal in vivo. By selectively inhibiting different fractions of cytochrome P-450 (P-450) isozymes, as well as, epoxide hydrolase with known inhibitors this study helped to

demonstrate that FETAX could be used to investigate toxicological mechanisms of action. Occassionally, mammalian test systems fail to provide an adequate explanation of toxicological mechanisms of action of teratogenesis, as is the case with diphenylhydantoin. Although FETAX can not pharmacologically represent the fetal-maternal system, it was hoped that the study would demonstrate the utility of FETAX for studying toxicological mechanisms *in vitro*.

Without the ability to metabolize xenobiotics, it is doubtful that *in vitro* tests such as FETAX would be suitable for screeing human developmental toxicants. Hopefully, the results of previously mentioned studies will suggest that FETAX is able to overcome the obstacle of metabolic incompetence. By extending this research beyond test compound validation, a greater contribution was made toward the primary goal of increasing the acceptance of FETAX as a screen of developmental toxicants.

Literature Review

In Vivo Teratogenicity Testing

Multigeneration Studies. In multigeneration tests, animals are continuously exposed to a test agent in the food or water for a period of three generations (see 36 for review). This protocol was primarily developed to assess the developmental toxicity of chemicals that are likely to

bioaccumulate with long-term exposure, such as pesticides. Exposure generally begins shortly after weaning. Upon reaching reproductive maturity, animals are mated to produce the F_1 generation. F_1 offspring are then selected to produce the F_2 generation, and the F_2 individuals to produce the F_3 offspring which are, subsequently killed and examined at weaning. The same treatment is administered to each successive generation. Typically, three treatment groups and one control group are utilized, with a minimum of 20 pregnant females per group per generation. Use of rodent species as test organisms for multigeneration tests (F_3) allows completion of the study within twenty months. Fertility, litter size, sex ratio, neonatal viability, and fetal growth endpoints are commonly used to assess the effects of the compound on reproductive performance.

Short-Term Techniques. Methods of evaluating the effects of short-term exposure may be divided into three-segment single generation studies (see 37-39 for review). Phase I tests examine effects on fertility and general reproductive performance. Phase II studies, the most commonly employed protocol, evaluates developmental toxicity. Phase III tests provide a method of evaluating peri- and postnatal effects.

Phase I tests consist of treatment of male and female rodents for approximately 70 and 14 days, respectively. Female exposure occurs during mating, pregnancy and lactation. Half of the dams are killed at mid-pregnancy and

the uterine contents examined for pre- and postimplantation embryo death. The remaining females are allowed to deliver and nurse their brood. The offspring are subsequently killed and autopsied for the presence of terata.

Segment II studies involve treatment of a pregnant females during critical periods of organogenesis. Females are killed and fetuses removed one day prior to parturition. Early embryo or late fetal death, abnormal development, and fetal body weight endpoints are then used to assess potential developmental toxicity.

Pregnant females are exposed to potential developmental toxicants during the final third of gestation and through the weaning process in Phase III tests (39). The ultimate objective of these studies are to provide information concerning the effects on late fetal development, labor and delivery, lactation, and neonatal viability/growth.

Today there is growing concern over the adequacy and practicality of these methods for evaluating teratogenicity in vivo, since they were developed in response to the thalidomide controversy nearly twenty years ago. Several modifications have been proposed for both the multigeneration and short-term segment tests, as well as, provisions for the inclusion of cost- and time-effective in vitro teratogenesis screening assays (40).

In Vitro Teratogenicity Testing

Measurement of Teratogenic Potential. It has become

recently apparent that the current in vivo test procedures are overburdened by the large number of compounds requiring testing. Such a predicament has given rise to the development of short-term teratogenicity screens. To be successful, short-term in vitro screens must mimic the widely accepted in vivo mammalian developmental toxicity tests. However, until recently methods for validating in vitro systems were undefined. Obviously several questions must be considered (see 11 for a review). First, what is an acceptable measure of teratogenic potential for in vitro test systems? Obviously, an acceptable measure of teratogenic potential in vitro is an endpoint that would predict findings of generally accepted evaluations of teratogenic potential in vivo.

Second, should these endpoints be related to embryogenic events that occur in the intact mammalian system? In this case, the endpoints need not necessarily be related to embryogenic events, although an ontogenic system is desirable. Eventually all in vitro screens should predict human teratogenicity. The wide variety of in vitro developmental toxicity screens and the equally large number of endpoints used to assess potential risk makes evaluation difficult. For example, cell culture systems measure endpoints far removed from malformation and functional deformities. Rather, it measures an event that may or may not be relevant to the production of malformations.

Approaches. If a single test is to be used as a sole

indicator of teratogenic risk, the degree of developmental relevance to mammals must be great. Of those tests which bear little relevance to ontogeny, a battery of short-term assays are generally performed to form a composite answer. The battery approach is considered superior to the tier strategy (41). In the tier system, compounds initially are tested with inexpensive short-term tests. Those chemicals which test positive are then subjected to more rigorous testing. Since false negatives may occur during the initial screening, the possibility that a teratogen will evade detection is greater.

Assay Validation. In order to establish *in vitro* systems as an acceptable screening process, extensive validation must be performed. Generally compounds should be selected on the basis of their *in vivo* teratogenic activity from a list of agents selected by a panel of experienced investigators in the areas of teratology, pharmacokinetics, metabolism, and chemical classification. Validation in this manner facilitates interlaboratory comparisons. An overabudance of false-positive and negative test results detract from the validity of the test, depending on its use as part of a test battery or a single test, and its level in the overall toxicity assessment process.

Performance and Developmental Relevance of FETAX

Test performance and developmental relevance make FETAX

an attractive *in vitro* teratogenesis bioassay. Of 32 compounds tested with FETAX, 85 percent were similar to mammalian studies (42-43). Sabourin and Falk (44) have recently found that the total mammalian malformation (e.g., skeletal, visceral, nervous, etc.) caused by 17 teratogens were matched in *Xenopus* in 24/37 (65%) of the cases. The correlation between laboratories using FETAX indicates much promise for the assay as well. Courchesne and Bantle (20) found the teratogenic index of hydroxyurea to be 4.3 which is very similar to 4.5 obtained by Sabourin et al. (10). However, this was not always the situation, as was the case with 5-fluorouracil which tested strongly teratogenic in the laboratories of both Bantle and Sabourin, but the teratogenic index varied significantly. FETAX was found to be the most appropriate assay when compared to the *Planaria* (1) and the *Hydra* system (6). These results may be attributed to the multiple endpoints of the frog assay as well as its higher phylogenetic position. Dumont et al. (12) found meclizine induced hydrocephalia in both frogs and mammals. Courchesne and Bantle (20) found that several genotoxic compounds caused the same general malformations in both *Xenopus* and rodent embryos. In addition, anomalies produced by ethanol, caffeine, and 5-fluorouracil exposure were similar to those recorded in mammalian studies (27).

Teratogenic Assessment In Vivo

Assessment of teratogenic hazard *in vivo* centers

primarily around three endpoints: embryolethality, malformation, and growth inhibition (40). The relationship between these endpoints is extremely complex and typically varies with the type of agent tested (45). Obviously, different agents produce myriad of reponses, however, several patterns are characteristic of levels of teratogenic hazard.

Patterns of Response. Some developmental toxicants may induce abnormal development at doses which do not cause embryolethality. Increasing doses beyond that which malforms the entire litter may induce embryolethality but is often in conjuction with severe maternal toxicity. With such compounds, malformation is the most sensitive endpoint as terata is induced at doses below that which retards growth. This pattern of response although rare, is indicative of an agent with an extremely high teratogenic potential.

A much more common response involves embyolethality, malformation, and fetal growth inhibition. Exposure within an embryotoxic range may produce a combination of resorbed, deformed, stunted, and normal fetuses within a given litter. With this response pattern, a compound with high teratogenic potential may induce anomalies at lower doses. However, as the embryotoxic dose increases, embryolethality predominates as the major response. Agents with significant teratogenic potential would have malformation dose-response curves displaced to the left from the mortality curves at low

embryotoxic doses, but overlapping curves at higher doses. Compounds which are mainly embryolethal would display a converse dose-response relationship with the mortality curve displaced to the left. Typically, with these type of agents growth is the most sensitive endpoint. The majority of compounds producing such a response pattern pose moderate teratogenic hazard to developing organisms.

The third response involves only growth inhibition and embryolethality. Again, growth is generally the most sensitive endpoint. Agents producing this type of dose-response pattern would possess low teratogenic potential. However, only intermediate doses within the range of embryolethality and growth retardation may determine whether teratogenicity has been masked by lethality.

Teratogenic Assessment with FETAX

The teratogenic potential of chemicals assayed with FETAX is assessed based on TI values, growth endpoints, and the types and severity of induced malformations. Generally, TI values <1.5 indicate low teratogenic potential as there is little or no separation between concentrations which induce malformations, but cause no embryolethality, and concentration which are lethal. Greater TI values signify an increased potential for teratogenesis, as there is a greater chance for the embryos to be malformed in the absence of significant embryolethality (30). Since an adult to developmental (A/D) ratio (46) is not practicable with

FETAX, types and severity of malformations are also considered in test evaluations such that compounds with TI values <1.5 producing severe malformations in major organ systems may still be considered to pose a developmental hazard, i.e., as potent embryotoxins (27). The utility of the TI to evaluate potential teratogenic hazard will only become clear after a large data base is developed using FETAX.

The minimum concentration to inhibit growth (MCIG) expressed as a percent of the compound LC50 is also useful in assessing developmental toxicity. Typically, the MCIG of compounds posing a strong developmental hazard will be <30% of the compound LC50 (27,30).

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CHAPTER II

EVALUATION OF THE DEVELOPMENTAL TOXICITY OF FIVE COMPOUNDS WITH THE FROG EMBRYO TERATOGENESIS ASSAY: XENOPUS (FETAX) AND A METABOLIC ACTIVATION SYSTEM

Douglas J. Fort, Brenda L. James, and John A. Bantle*

Department of Zoology

Oklahoma State University

Stillwater, OK 74078

Running Head: Teratogenicity testing with Xenopus

*Author to whom correspondence should be addressed:

Dr. John A. Bantle

Department of Zoology

430 Life Sciences West

Oklahoma State University

Stillwater, OK 74078

ABSTRACT

The potential teratogenic hazard of five compounds was evaluated using the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) and a metabolic activation system. Embryos of the South African clawed frog, Xenopus laevis were exposed to three compounds suspected to be proteratogenic in mammalian test systems, [2-acetylaminofluorene (2-AAF), rifampicin (RA), and benzo(a)pyrene (BP)] for 96 h. One compound unaffected by mixed-functional oxidase (MFO) metabolism, $ZnSO_4$ (Zn) and one compound thought to be inactivated by cytochrome P-450, cytochalasin D (CD) were also tested. Two separate static renewal tests were conducted with and without the presence of an exogenous metabolic activation system (MAS). The metabolic activation system consisted of Aroclor 1254-induced rat liver microsomes. The teratogenic potential of each compound and the effects of metabolic activation were based on teratogenic indices [TI=96 h LC50/96 h EC50 (malformation)], types and severity of malformation, and effects on embryo growth. Metabolic activation increased the potential teratogenic hazard of 2-AAF, RA, and BP by TI factors of 1.3, 2.8, and 6.8, respectively. The teratogenic potential of Zn was virtually unaffected by the MAS. The MAS significantly reduced the teratogenic potential of CD by a TI factor of 2.7. These results demonstrate the utility and importance of a MAS for in vitro developmental toxicity screens such as FETAX.

Consistent use of a MAS with FETAX should reduce the number of potential false positive and false negative test results.

KEY WORDS: FETAX, developmental toxicity, 2-acetyl-aminofluorene, rifampicin, benzo(a)pyrene, zinc, cytochalasin D.

INTRODUCTION

Increasing demands for developmental toxicity screening necessitate the development, validation, and use of alternative screening systems to the traditional mammalian test systems[1]. In vitro teratogenesis screening systems may provide a rapid, cost-effective method of determining compounds and complex mixtures that may be potential teratogenic hazards. A fundamental problem shared by many in vitro developmental toxicity screening systems is the inability to metabolize xenobiotics[2]. This presents a major obstacle in the detection of proteratogens, as well as teratogens inactivated by the mixed-functional oxidase (MFO) system.

The Frog Embryo Teratogenesis Assay: Xenopus (FETAX) is a 96-h static renewal bioassay designed to screen potential developmental toxicants[3]. Since Xenopus embryos lack many metabolic enzyme systems through the first 96 h of development, an exogenous metabolic activation system was developed for FETAX using Aroclor 1254-induced rat liver microsomes[4]. In this study, the proteratogen cyclophosphamide (CP) was successfully biotransformed to its teratogenic metabolites. Recently, the developmental toxicity of nicotine and a primary metabolite cotinine on Xenopus embryos was evaluated using this MAS[5]. Bioactivation greatly reduced the teratogenicity and growth inhibiting effects of nicotine.

This report describes the evaluation of the developmental toxicity of three compounds suspected to be proteratogenic, 2-acetylamino-fluorene (2-AAF), rifampicin (RA), and benzo(a) pyrene (BP); one compound unaffected by MFO metabolism, $ZnSO_4$ (Zn); and one compound believed to be metabolically inactivated, cytochalasin D (CD). The results demonstrate the importance of a successful MAS for in vitro teratogenesis screening systems, such as FETAX, as well as the utility of these systems in evaluating the role MFO metabolism plays in teratogenesis.

EXPERIMENTAL

Chemicals and Biochemicals

All chemicals used in rat liver microsome preparation, test compounds, and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Aroclor 1254 was obtained from Monsanto Corporation (St. Louis, MO)

Microsome Preparation

Aroclor 1254-induced rat liver microsomes were prepared as previously described[4]. Adult male Sprague-Dawley C/D strain rats (200-300 g) were injected with 500 mg/Kg Aroclor 1254 in corn oil five days prior to microsome preparation[6]. Following cervical dislocation, the liver was exposed and perfused with 50 ml 0.02 M Tris-HCl buffer containing 1.15% KCl, pH 7.5. The liver was homogenized in 1.15% KCl-0.02 M Tris-HCl buffer containing 0.5% bovine serum albumin, pH 7.5. The homogenate was then centrifuged successively at 600 and 9,000 x g ave. The S-9 supernatant was further purified by two additional ultracentrifugation steps at 102,000 x g ave. The microsomal pellet was resuspended in 0.05 M Tris-HCl, pH 7.5, put into 1.5 ml aliquots, and immediately frozen in liquid nitrogen[7]. Protein content was determined by the method of Bradford[8]. Cytochrome P-450 (P-450) activity was inferred by the measurement of formaldehyde generated from the N-demethylation of aminopyrine (APD)[9] under standard assay conditions previously established[10]. Several aliquots of microsomes

were chemically reduced with dithionite and pretreated with carbon monoxide (CO-MAS) to selectively inactivate P-450 activity[11].

Animal Care and Breeding

Xenopus adult care, breeding, and embryo collection were performed according to Courchesne and Bantle[12].

Assay Procedure

Initial tests were conducted to determine the maximum amount of DMSO which could be used as diluent and the effect it had on P-450 activity. For unactivated tests, groups of 20 embryos were placed in 60 mm covered plastic Petri dishes with varying concentrations of the appropriate test compound. 2-AAF, RA, BP, and CD stock solutions were prepared by dissolving in 1% (v/v) DMSO diluted in FETAX solution, a reconstituted water medium[13]. $ZnSO_4$ was dissolved in appropriate volumes of FETAX solution. For each compound 8 to 13 concentrations were tested with replicates. Four separate dishes of 20 embryos were exposed to FETAX solution (designated FETAX solution controls). Forty embryos, 20 per dish, were exposed to 1% DMSO in FETAX solution and served as solvent controls. Each treatment contained a total of 8 ml of solution.

Stock Zn concentrations were determined by atomic absorption analysis. To facilitate the comparison of tests within this study and previous work with Zn[14], the concentration of each test dilution was normalized to 100 ug/ml hardness (as $CaCO_3$)[15,16].

The metabolically activated tests were also conducted in duplicate with 20 embryos per dish. Stock solutions were prepared as described for the unactivated tests. Each activated treatment received 0.04 U APD activity (units expressed as uM formaldehyde/min), an NADPH generating system, and 100 U/ml penicillin-100 ug/ml streptomycin. The NADPH generating system consisted of 3.5 mM glucose-6-phosphate, 0.31 IU/ml glucose-6-phosphate dehydrogenase, 0.1 mM NADP, and 7.0 uM NADPH. Each dish received no more than 0.06 mg/ml microsomal protein[4]. Controls for FETAX solution, DMSO, MAS, DMSO + MAS, CP (FETAX reference proteratogen)[5], CO-MAS + toxicant (negative control), and unactivated toxicant were also run with each test.

For each compound, one range-finder and two separate definitive tests were conducted with and without the MAS. The pH of all stock solutions was maintained between 7.0 and 8.0. Embryos were cultured at 23°C throughout the test. All solutions were removed every 24 h of the 4-d test and fresh solutions were added. Dead embryos were removed at this time. After 96 h of exposure, surviving embryos were fixed in 0.7% formalin, pH 7.0. The number of live malformed embryos and the stage of development[17] were determined using a dissecting microscope.

Data Analysis

Litchfield-Wilcoxon probit analysis was used to ascertain the 96-h LC50, the median lethal concentration and the 96-h EC50, the concentration inducing gross terata in 50

percent of the surviving embryos[18]. The 95 percent confidence limits were calculated as well. A Teratogenic Index (TI) which has proven useful in assessing teratogenic hazard[3-5,13,19] was determined by dividing the 96-h LC50 by the 96-h EC50 (malformation). Head-tail length (growth) was measured using a Radio Shack digitizer and a model 16 microcomputer.

RESULTS

Control Experiments

Preliminary tests indicated that upon exposure to 1% (v/v) DMSO, microsomal APD activity was 0.05 U/mg protein + 0.01 (n=6), which was not significantly different from FETAX solution controls, 0.05 U/mg protein + 0.03 (n=6) (grouped t-test, $p<0.05$). The lowest observable effect concentration (LOEC) (Dunnett's test) and the minimum concentration to inhibit growth (MCIG) (grouped t-test, $p<0.05$) for the embryos exposed to DMSO for 96 h were 1.4 and 1.2% (v/v), respectively.

In all tests conducted, FETAX solution control embryo mortality and malformation rates were less than 6%. Mortality and malformation rates in the MAS and DMSO controls were below 6 and 7%, respectivley. Mortality and malformation rates in control embryos exposed to the MAS and DMSO simultaneously were 15% or less. At least 90% of the embryos exposed to 4.0 mg/ml activated CP, died before 96 h. Survivors were severely malformed. Control embryos exposed to 4.0 mg/ml unactivated CP exhibited malformation and mortality rates less than or equal to 15%.

2-Acetylaminofluorene

Results from tests with 2-AAF are presented in Table I. The mean unactivated 96-h LC50 was 88.5 ug/ml. The mean 96-h EC50 (malformation) was 7.2 ug/ml, yielding a TI of 12.4. Metabolic activation reduced the 96-h LC50 at least 1.9 fold

to approximately 42.5 ug/ml. Activation reduced the 96-h EC50 (malformation) to approximately 2.6 ug/ml (2.7 fold). The average TI value was 16.7. Malformations induced by unactivated 2-AAF included improper gut coiling and pericardial edema at concentrations greater than 6 ug/ml. At concentrations greater than 10 ug/ml severe gut malformations, ophthalmic edema, and craniofacial abnormalities were also observed. Gut miscoiling, edema of the pericardium, ophthalmic region, and dorsal fin, and severe microophthalmia were elicited by activated 2-AAF at concentrations greater than 1.0 ug/ml. Concentrations greater than 10 ug/ml bioactivated 2-AAF produced severe skeletal kinking, malformations of the mouth, and microencephaly. The effects of unactivated and bioactivated 2-AAF on embryo growth is illustrated in Fig. 1. Exposure to metabolically activated 2-AAF had a greater growth inhibiting effect than did exposure to unactivated 2-AAF. Growth reduction as the result of 2-AAF bioactivation was also greater than the additive growth inhibition caused by the MAS + DMSO and unactivated 2-AAF treatments. Embryos exposed to CO-MAS and 85 ug/ml 2-AAF were 75.5% of FETAX solution control growth.

Rifampicin

Unactivated RA was not developmentally toxic at the limit of solubility in 1% (v/v) DMSO, thus the 96-h LC50 and EC50 (malformation) values are expressed as >2.0 mg/ml (Table II). Bioactivation decreased the 96-h LC50 to

approximately 1.4 mg/ml. The mean 96-h EC50 (malformation) for bioactivated rifampicin was 0.5 mg/ml and the TI was 2.8. Activated RA concentrations greater than 50 ug/ml caused miscoiling of the gut, craniofacial deformities, and microophthalmia. Severe microencephaly, ophthalmic and pericardial edema, and skeletal kinking were produced in concentrations above 1.0 mg/ml. The effect of metabolic activation of RA on embryo growth is presented in Figure 2. Activated RA concentrations less than 1 mg/ml caused slight growth inhibition. However, the reductions may have been caused by the sum of the actions of the MAS + DMSO and unactivated RA treatments. Unactivated RA concentrations greater than 1 mg/ml caused increased embryo growth compared to FETAX solution controls, whereas activated concentration greater than 1.0 mg/ml caused marked growth reduction. Embryonic growth in the FETAX solution controls and the CO-MAS + 2.0 mg/ml RA treatment was nearly identical.

Benzo(a)pyrene

Results from tests with BP are shown in Table III. The 96-h LC50 for both unactivated and metabolically activated BP is expressed as greater than 10 ug/ml since the median lethal concentration exceeded the limit of solubility in 1% (v/v) DMSO. The 96-h EC50 (malformation) for unactivated BP was about 11 ug/ml. Bioactivation reduced the 96-h EC50 (malformation) approximately 6.7 fold to 1.7 ug/ml. The TI increased at least 5.6 fold upon activation. At concentrations greater than 2.5 ug/ml, unactivated BP

elicited moderate gut malformations while concentrations greater than 7.5 ug/ml produced severe gut abnormalities and malformations of the mouth. Bioactivation induced ophthalmic edema, severe gut miscoiling, and mouth malformation at concentrations greater than 0.5 ug/ml. In addition, microencephaly, severe eye malformations, and skeletal kinking were observed above 5 ug/ml activated BP. The effect of bioactivated BP on embryo growth is shown in Fig 3. Activated BP concentrations greater than approximately 5 ug/ml caused greater growth reduction than could be explained by the additive effect of the MAS + DMSO and unactivated BP treatments. Embryos exposed to 10 ug/ml BP and CO-MAS were 87% of FETAX solution control growth.

$\underline{ZnSO_4}$

In tests without the MAS, the average normalized 96- h LC50 was 34.4 ug/ml (Table IV). The 96-h EC50 (malformation) was aproximately 2.7 ug/ml in tests without the MAS. In tests with the MAS the mean 96-h LC50 was 36.7 ug/ml. The activated LC50 values standardized per ug of microsomal protein was approximately 1.6 ug Zn/ug protein. With the MAS the 96- h EC50 (malformation) was approximately 2.9 ug/ml. The TI values for unactivated Zn and Zn-MAS experiments were 13.3 and 12.7, respectively. Embryos exposed to Zn alone at concentrations above 1.5 ug/ml caused mild gut malformations and pericardial edema. Above 4.0 ug/ml, Zn induced severe edema of the pericardium and eye, gut miscoiling, and head and mouth malformations. Higher

concentrations caused severe skeletal kinking, microophthalmia, microencephaly, and craniofacial malformations, as well. Zn-MAS exposure produced similar malformations to those induced by the unactivated treatments. The severity of the gut miscoiling and edema was slightly greater at the lower concentrations (<4.0 ug/ml) when cultured with the MAS. At higher concentrations (>5.0 ug/ml) skeletal kinking was observed less and was generally less severe than that produced in the unactivated treatments. The effect of Zn on embryo growth is shown in Fig. 4. Addition of the MAS had little effect on the embryo growth inhibiting potential of Zn. Embryos exposed to 85 ug/ml Zn (30.5-36.3 ug/ml normalized) were 81.7% of FETAX solution control growth.

Cytochalsin D

Table V shows the results of tests conducted with CD. The mean 96-h LC50 for unactivated CD experiments was 450 ng/ml. The unactivated 96-h EC50 (malformation) was approximately 100 ng/ml. In tests conducted with the MAS, the 96-h LC50 averaged 800 ng/ml. The bioinactivated 96-h EC50 (malformation) was approximately 600 ng/ml. The TI for unactivated CD was approximately 3.9 whereas the TI for bioinactivated CD was about 1.5. Unactivated CD concentrations greater than 50 ng/ml induced severe impairment of eye formation, gut miscoiling, and craniofacial malformations. At concentrations greater than 75 ng/ml, microencephaly, muscular kinking, and skeletal

defects were observed. In tests with the MAS, severe miscoiling of the gut, pericardial edema, and mouth malformations were induced at concentrations greater than 250 ng/ml. Additional anomalies found above 900 ng/ml were skeletal kinking, moderate craniofacial malformations, and microencephaly. The effect of metabolic inactivation of CD on embryo growth is presented in Fig. 5. Addition of the MAS increased embryo growth compared to embryos exposed to CD alone. Embryos exposed to CD and the CO-MAS were 87.1% of FETAX solution control growth.

DISCUSSION

Results obtained in this study suggest the importance of metabolism in in vitro teratogenesis screening. Metabolic activation increased the developmental toxicity of 2-AAF, RA, and BP; reduced the effect of CD; and had no significant effect on Zn. Generally, in FETAX, TI values less than 1.5 indicate low teratogenic potential whereas greater values signify an increase in the potential hazard[4,5,19]. However, types and severity of malformations, growth inhibition, and results with CO-MAS are also considered. Some compounds with TI values less than 1.5 produce severe malformations of major organ systems. These compounds may still pose a hazard for the developing embryo (possibly as an embryo toxin). CO-MAS controls provide needed information in assessing the role of P-450 mediated metabolism in teratogenesis.

Bioactivation increased the TI of 2-AAF 1.3 fold from approximately 12.4 to 16.7. Even without activation 2-AAF is a significant teratogenic hazard in FETAX. Several additional factors suggest the importance of biotransformation in 2-AAF teratogenesis. First, activated 2-AAF induced different types of terata with greater severity than unactivated 2-AAF produced. Second, embryos exposed to 2-AAF and the CO-MAS exhibited similar malformation responses including growth reduction to embryos exposed to unactivated 2-AAF. Metabolic activation of 2-AAF

primarily caused severe malformations of the brain, eye, and skeletal system in Xenopus. These malformations were not observed in unactivated treatments. Activation also caused a slight decrease in embryo growth and was not as marked as rates for RA and BP. These anomalies are similar to those found in in vivo and in vitro mammalian teratogenesis test systems. Izumi[20] produced primarily skeletal malformations by administering 2-AAF to mice between the 8th and 15th day of gestation. Faustman-Watts et al.[21] exposed cultured whole-rat embryos to 2-AAF, activated 2-AAF, and two metabolites N-hydroxy-2-acetylaminofluorene (N-OH-AAF) and N-acetoxy-2-acetylaminofluorene (N-AAAF). No malformations and minimal decreases in viability and growth were observed with unactivated 2-AAF at concentrations up to 75 ug/ml. Concentrations above 60 ug/ml bioactivated 2-AAF caused a decrease in embryo viability, incomplete closure of the neural tube, and a decrease in embryo growth. In the present study, the concentration of unactivated 2-AAF required to malform all Xenopus embryos was 7.5 times greater than activated 2-AAF. Thus, it may be possible that concentrations greater than 75 ug/ml unactivated 2-AAF were required to elicit malformations in cultured rat embryos. These concentrations were not tested, however. N-OH-AAF and N-AAAF produced ventrolateral protrusion and hypoplasia of the prosencephalon. Although ventrolateral protrusion of the brain was not observed, hypoplasia of the prosencephalon appears to be similar to microencephaly detected with

Xenopus.

Bioactivation increased the teratogenic hazard of RA as indicated by the rise in TI. Unactivated RA was not developmentally toxic at concentrations up to 2 mg/ml. In addition, embryos exposed to high concentrations of unactivated RA grew to lengths greater than that of the FETAX solution controls. This response may be the result of hormesis and/or sterilization of the assay environment. Metabolic activation of rifampicin caused severe malformations of the gut, brain, eye, and skeletal system at low RA concentrations. Concentrations greater than 1 mg/ml had a detrimental effect on embryo growth. Embryos exposed to the CO-MAS and RA developed normally and growth was not inhibited, thus implying the role of P-450 in teratogenesis. Malformations induced by RA exposure in Xenopus are similar to deformaties found in mammalian models and possibly the human. Oral doses of greater than 150 mg/kg in mice and rats caused spina bifida in both species and cleft palate malformations in the mouse fetus[22]. Similar treatment of rabbits had no apparent effect on the developing fetus. Greenaway et al.[23] produced open neural tubes in rats grown in vitro and exposed to RA. The response was only observed in the presense of an exogenous microsomal monooxygenase system. Embryo growth was also reduced but was not dependent on the presence of a MAS. In addition, RA has been determined to be more problematic during pregnancy than several other antituberculosis drugs, such as isoniazid and

ethambutol[24]. Steen and Stainton-Ellis[25] reported 9 malformations among 202 exposed newborns (4.5%). The malformations observed were anencephaly (1), hydrocephalus (2), genitourinary anomalies (2), dislocated hip (1), and skeletal reduction deformaties (3). However, evaluation of this information is difficult since no information about patient selection was included in the report[26].

Metabolic activation of BP decreased the 96-h EC50 (malformation) by 5 to 6 fold. Embryo lethality was not affected up to the maximum soluble concentration. Thus, bioactivation significantly increased the potential teratogenic hazard of BP. Unactivated BP induced primarily gut, mouth, and skeletal malformations. Bioactivation increased the severity of skeletal deformities, but caused serious brain (microencephaly) and eye malformations at low BP concentrations. Activation also caused decreased Xenopus embryo growth at concentrations greater than 5.0 ug/ml. Some of the malformations induced by BP in mammalian test systems are similar to those observed with Xenopus. Shum et al.[27] found that B-naphthoflavone-enhanced BP metabolism in AKR inbred mice injected (i.p.) with BP between 50 and 300 mg/kg was associated with increased in utero toxicity and terata (club foot, cleft palate and lip, kinky tail, hemangioma, anophthalmia, and scoliosis). Skeletal kinking in Xenopus may bear some relationship to skeletal limb defects in mammals[13]. Similar terata were also observed in B6[27] and C57BL/6[28] strain mice after i.p. injection, but

occurred more frequently than in the AKR strain. Greater incidence of anomalies found in the B6 strain has been attributed to genetic variability in the rate of BP biotransformation (i.e. C57BL/6N and B6 strain mice have a highly inducible P-450 isozyme (AAH) responsible for BP metabolism).

FETAX can not pharmacologically represent the fetal-maternal system, however it may provide an opportunity to compare the developmental toxicity of parent compounds and metabolites. Such studies have already been performed with cyclophosphamide and one of its metabolites 4-hydroxycyclophosphamide[4], and nicotine and a primary metabolite cotinine[5].

Metabolic activation did not significantly change the developmental toxicity of Zn, as indicated by the similarity of the TI values. Malformations in activated and unactivated experiments were similar as well. Differences in the LC50 values for activated experiments initially suggested that some other factor, such as microsomal metallothionine, might be responsible for the slight detoxification of Zn. However, normalization for microsomal protein did not significantly change the variability between the two experiments. If detoxification was the result of protein binding, normalization should correct this variation. Thus, differences were probably due to only genetic variability of the embryos used and minor procedural variations. Similar results obtained with and without the

MAS seem to indicate that the MAS does not appreciably affect the $CaCO_3$ hardness of the test solution. If the MAS significantly contributed to the hardness of the test solution, a decrease in the developmental toxicity of Zn would have been observed. Growth, which is often the most sensitive endpoint measured by FETAX, was not significantly reduced upon activation. Much emphasis placed on MAS studies concerns the ability to detect proteratogens. However, a successful MAS must not alter the results of direct-acting teratogens. Established direct-acting teratogens, such as Zn in Xenopus, should be tested in parallel with a metabolic activation system to show that results are not affected. Eventually all compounds selected for validation of in vitro teratogenesis screening systems should be tested with and without the MAS.

Results from experiments performed with the MAS indicate that the developmental toxicity of CD was reduced. Bioinactivation of CD decreased the TI at least 2.6 fold from approximately 3.9 to 1.5. Embryos exposed to metabolically inactivated CD developed markedly better which was reflected in the absence of several different types of terata observed in unactivated CD and in embryo growth. Whereas 50 ng/ml of unactivated CD caused malformations of the eye, craniofacial region, and gut, an inactivated concentration 5 fold greater induced pericardial edema and mouth anomalies. These malformations were not observed in any concentration of unactivated CD. A 12 fold increase in

inactivated CD was required to elicit brain, craniofacial, and skeletal defects. At such high concentrations the possibility of malformations caused by unmetabolized CD may be significant. In general, the severity of malformations induced by inactivated CD was significantly less than that caused by unactivated CD. Previous studies with CD in other animal models are similar to results obtained with FETAX. Shepard and Greenaway[29] found that C-57 and BALB-C, but not Swiss-Webster, strain mice injected on days 7 through 11 produced exencephaly, hypognathia, and skeletal reduction effects. Exencephaly was found to be the predominant malformation in hamsters[30]. In the rat, CD was non-teratogenic *in vivo*; however, impaired neural tube closure was observed *in vitro*[31]. Interestingly, the majority of *Xenopus* embryos that died at concentrations greater than 2.5 ug/ml were arrested during neurulation. The addition of a MAS to the cultured whole-rat embryo system caused significant inhibition of CD teratogenesis[29]. Inhibition of teratogenesis was reduced with the addition of CO. In FETAX, CO-MAS restored much of the developmentally toxic effects of CD including growth inhibition. Based on the present evaluation scheme using the TI, inactivated CD has the potential to be developmentally hazardous. However, differences in the types and severity of malformations and inhibition of growth reduction upon inactivation, as well as reversal of the effects by CO, indicate that CD teratogenesis may be inhibited by the P-450 system. These

results suggest the importance of *in vitro* developmental toxicity test systems, including FETAX, in evaluating discrepancies between *in vivo* and *in vitro* models.

DMSO seems to be an effective carrier solvent for FETAX at concentrations not exceeding 1%. Several additional cosolvents have been tried with FETAX including acetone and triethylene glycol. However, preliminary results indicate that acetone is not compatible with the MAS. Bioactivated acetone proved to be extremely toxic. Further evaluation of metabolic activation in teratogenesis with FETAX may necessitate the use of other carrier solvents. Formamide, ethanol, and glycerol formal have also been studied for use as cosolvents, but have not been used in formal validation studies (T. H. Dresser, personal communication). We are presently investigating the effect of carrier solvent-toxicant interactions with FETAX.

Without the ability to metabolize xenobiotics it is doubtful that *in vitro* test systems will be suitable for screening human developmental toxicants. Results of tests performed with CP[4], nicotine[5], 2-AAF, RA, BP, Zn, and CD all indicate that with an exogenous MAS, FETAX may be able to overcome metabolic incompetance and successfully serve as a screen in developmental toxicity hazard assessment. Such a system should also provide information on the role of MFO metabolism in teratogenesis. By implementing a MAS into the FETAX protocol, we may increase the predictive value by decreasing the number of false-positive and false-negative

test results.

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Table I. Effect of 2-acetylaminofluorene on Xenopus embryo development.

Treatment	Unactivated		Activated	
	Trial 1	Trial 2	Trial 1	Trial 2
FETAX SOLUTION CONTROL[a]:				
Mortality	5.0	0	1.3	0
Malformation	3.5	2.5	2.5	5.0
MAS[b] CONTROL[a]:				
Mortality	-	-	5.0	0
Malformation	-	-	3.4	5.0
1% (V/V) DMSO CONTROL[a]:				
Mortality	2.5	0	0	0
Malformation	5.1	5.0	5.0	5.0
MAS + 1% (V/V) DMSO[a]:				
Mortality	-	-	7.5	0
Malformation	-	-	15.0	12.5
4.0 mg/ml CP[ac]:				
Mortality	0	0	100.0	100.0
Malformation	20.0	10.0	-	-
CO-MAS[d] + 75 ug/ml 2-AAF[ae]:				
Mortality	0	0	0	0
Malformation	100.0	85.0	100.0	100.0
LC50[f] (ug/ml)	87.0	90.0	45.0	40.0
	(84.0-89.0)	(88.0-93.0)	(26.0-78.0)	(32.0-58.0)
EC50[f] (ug/ml)	6.9	7.4	2.6	2.5
	(6.5-7.3)	(7.0-8.0)	(0.4-9.0)	(2.0-3.0)
TI[g]	12.6	12.2	17.3	16.0

[a] Data presented as percent effect.
[b] Metabolic activation system.
[c] Cyclophosphamide.
[d] Carbon monoxide inactivated MAS.
[e] Unactivated column represents data for embryos exposed to 75 ug/ml 2-AAF alone.
[f] Determined by Litchfield-Wilcoxon probit analysis with 95% percent confidence interval in parenthesis.
[g] 96 h LC50/96 h EC50 (malformation).

Table II. Effect of rifampicin on Xenopus embryo development.

Treatment	Unactivated		Activated	
	Trial 1	Trial 2	Trial 1	Trial 2
FETAX SOLUTION CONTROL[a]:				
Mortality	1.9	1.9	2.5	0
Malformation	3.4	5.1	5.1	0
MAS[b] CONTROL[a]:				
Mortality	-	-	2.5	0
Malformation	-	-	5.1	5.0
1% (V/V) DMSO CONTROL[a]:				
Mortality	0	0	0	0
Malformation	5.0	2.5	5.0	0
MAS + 1% (V/V) DMSO[a]:				
Mortality	-	-	2.5	0
Malformation	-	-	10.3	5.0
4.0 mg/ml CP[ac]:				
Mortality	0	0	100.0	100.0
Malformation	5.0	5.0	-	-
CO-MAS[d] + 2.0 mg/ml RA[ae]:				
Mortality	0	13.3	0	0
Malformation	5.0	15.4	15.4	10.0
LC50[f] (mg/ml)	>2.0[g]	>2.0	1.40 (1.24-1.56)	1.35 (1.26-1.46)
EC50[f] (mg/ml)	>2.0	>2.0	0.53 (0.45-0.63)	0.47 (0.39-0.55)
TI[h]	-	-	2.6	2.9

[a] Data presented as percent effect.
[b] Metabolic activation system.
[c] Cyclophosphamide.
[d] Carbon monoxide inactivated MAS.
[e] unactivated column represents data for embryos exposed to 2.0 mg/ml RA alone.
[f] Determined by Litchfield-Wilcoxon probit analysis with 95 percent confidence interval in parenthesis.
[g] Limit of solubility in 1% (v/v) DMSO.
[h] 96 h LC50/96 h EC50 (malformation).

Table III. Effect of benzo(a)pyrene on Xenopus embryo development.

Treatment	Unactivated		Activated	
	Trial 1	Trial 2	Trial 1	Trial 2
FETAX SOLUTION CONTROL[a]:				
Mortality	0	0	0	0
Malformation	0	3.4	0	3.4
MAS[b] CONTROL[a]:				
Mortality	-	-	0	3.4
Malformation	-	-	2.5	3.4
1% (V/V) DMSO CONTROL[a]:				
Mortality	2.5	0	2.5	0
Malformation	5.0	0	3.4	0
MAS + 1% (V/V) DMSO[a]:				
Mortality	-	-	0	0
Malformation	-	-	15.0	3.4
4.0 mg/ml CP[ac]:				
Mortality	0	0	100.0	100.0
Malformation	15.0	15.0	-	-
CO-MAS[d] + 10 ug/ml BP[ae]:				
Mortality	0	0	0	0
Malformation	-	-	40.0	33.3
LC50[f] (ug/ml)	>10.0[g]	>10.0	>10.0	>10.0
EC50[f] (ug/ml)	12.0	10.0	1.5	1.8
	(6.0-20.0)	(8.0-12.0)	(1.0-2.4)	(1.2-3.0)
TI[h]	>0.8	>1.0	>6.7	>5.6

[a] Data presented as percent effect.
[b] Metabolic activation system.
[c] Cyclophosphamide.
[d] Carbon monoxide inactivated MAS.
[e] Unactivated column represents data for embryos exposed to 10 ug/ml BP alone.
[f] Determined by Litchfield-Wilcoxon probit analysis with 95 percent confidence interval in parenthesis.
[g] Limit of solubility in 1% (v/v) DMSO.
[h] 96 h LC50/96 h EC50 (malformation).

Table IV. Effect of $ZnSO_4$ on *Xenopus* embryo development.

Treatment	Unactivated		Activated	
	Trial 1	Trial 2	Trial 1	Trial 2
FETAX SOLUTION CONTROL[a]:				
Mortality	0	0	0	0
Malformation	5.0	2.5	1.3	2.5
MAS[b] CONTROL[a]:				
Mortality	-	-	2.5	3.4
Malformation	-	-	0	3.6
4.0 mg/ml CP[ac]:				
Mortality	0	0	100.0	90.0
Malformation	0	0	-	100.0
CO-MAS[d] + 85 ug/ml[e] $ZnSO_4$[af]:				
Mortality	0	0	0	0
Malformation	100.0	100.0	100.0	100.0
LC50[g] (ug/ml)	35.0 (33.9-36.1)	33.8 (33.2-33.4)	40.0 (39.7-40.3)	33.4 (32.7-34.1)
LC50 (ug Zn/ug protein)	-	-	1.72 (1.72-1.75)	1.39 (1.36-1.42)
EC50[g] (ug/ml)	2.22 (1.79-2.76)	3.15 (2.80-3.54)	2.92 (2.55-3.35)	2.85 (2.64-3.09)
TI[h]	15.8	10.7	13.7	11.7

[a] Data presented as percent effect.

[b] Metabolic activation system.

[c] Cyclophosphamide.

[d] Carbon monoxide inactivated MAS.

[e] Concentration not normalized to 100 ug/ml hardness (as $CaCO_3$).

[f] Unactivated column represents data for embryos exposed to 85 ug/ml Zn alone.

[g] Determined by Litchfield-Wilcoxon probit analysis with 95 percent confidence interval in parenthesis. Concentrations normalized to 100 ug/ml hardness (as $CaCO_3$)[14,15].

[h] 96 h LC50/96 h EC50 (malformation).

Table V. Effect of Cytochalasin D on Xenopus embryo development.

Treatment	Unactivated		Activated	
	Trial 1	Trial 2	Trial 1	Trial 2
FETAX SOLUTION				
CONTROL[a]:				
Mortality	3.8	2.5	1.3	1.3
Malformation	1.3	3.8	3.8	5.1
MAS[b] CONTROL[a]:				
Mortality	-	-	2.5	2.5
Malformation	-	-	2.6	5.2
1% (V/V) DMSO[a]:				
Mortality	2.5	6.7	0	0
Malformation	5.1	3.6	5.0	5.0
MAS + 1% (V/V) DMSO[a]:				
Mortality	-	-	0	0
Malformation	-	-	7.5	10.0
4.0 mg/ml CP[ac]:				
Mortality	0	3.4	100.0	100.0
Malformation	0	3.4	-	-
CO-MAS[d] + 1.0 ug/ml CD[ae]:				
Mortality	75.0	80.0	96.7	95.0
Malformation	100.0	100.0	100.0	100.0
LC50[f] (ng/ml):	490 (376-638)	433 (313-599)	883 (765-1020)	728 (637-831)
EC50[f] (ng/ml):	121 (86-170)	121 (77-199)	541 (458-638)	551 (486-625)
TI[g]:	4.1	3.6	1.6	1.3

[a] Data presented as percent effect.
[b] Metabolic activation system.
[c] Cyclophosphamide
[d] Carbon monoxide inactivated metabolic activation system.
[e] Unactivated column represents data for embryos exposed to 1 ug/ml CD alone.
[f] Determined by Litchfield-Wilcoxon probit analysis with 95 percent confidence interval in parenthesis.
[g] 96 h LC50/96 h EC50 (malformation).

FIGURE LEGENDS

FIGURE 1 - Representative growth curves, presented as percent of FETAX solution control, for Xenopus embryos exposed to unactivated (■) and bioactivated (✸) 2-AAF for 96 h. (●) represents the additive growth inhibiting effects of the MAS + DMSO and unactivated 2-AAF treatments.

FIGURE 2 - Representative growth curves, presented as percent of FETAX solution control, for Xenopus embryos exposed to unactivated (■) and bioactivated (✸) RA. (●) represents the additive growth inhibiting effects of the MAS + DMSO and unactivated RA treatments.

FIGURE 3 - Representative growth curves, presented as percent of FETAX solution control, for Xenopus embryos exposed to unactivated (■) and bioactivated (✸) BP for 96 h. (●) represents the additive growth inhibiting effects of the MAS + DMSO and unactivated BP treatments.

FIGURE 4 - Representative growth curves, presented as percent of FETAX solution control, for Xenopus embryos exposed to unactivated (■) and Zn plus the MAS (✸) for 96 h. (●) represents the additive growth inhibiting effects of the MAS and unactivated Zn treatments.

Figure 5 - Representative growth curves, presented as percent of FETAX solution control, for Xenopus embryos exposed to unmetabolized (■) and bioinactivated CD (✸) for 96 h. (●) represents the additive growth inhibiting effects of the MAS + DMSO and unmetabolized CD treatments.

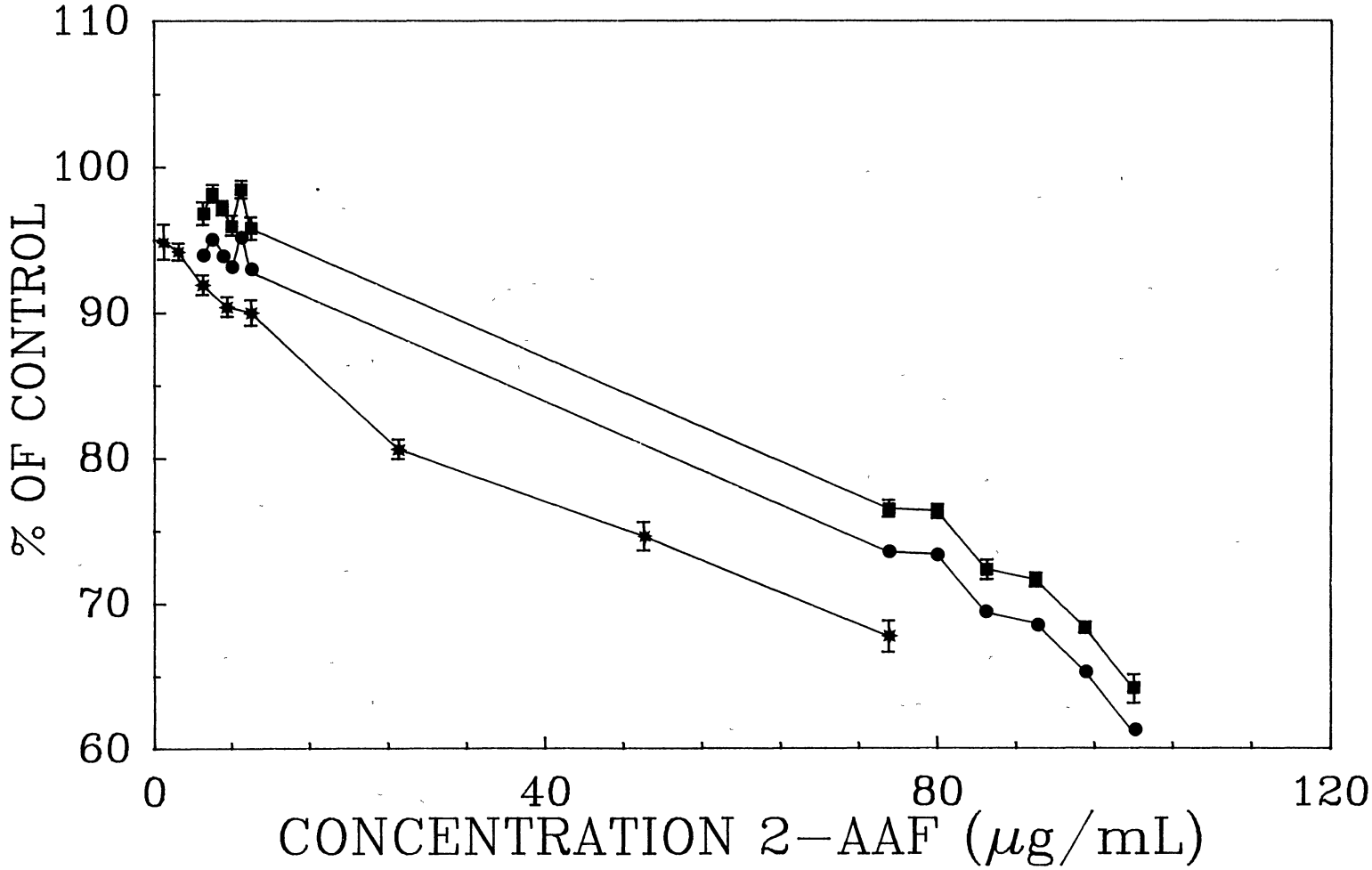
110
100
90
80
70
60
% OF CONTROL
0
40
80
120
CONCENTRATION 2-AAF (μg/mL)

Figure 1

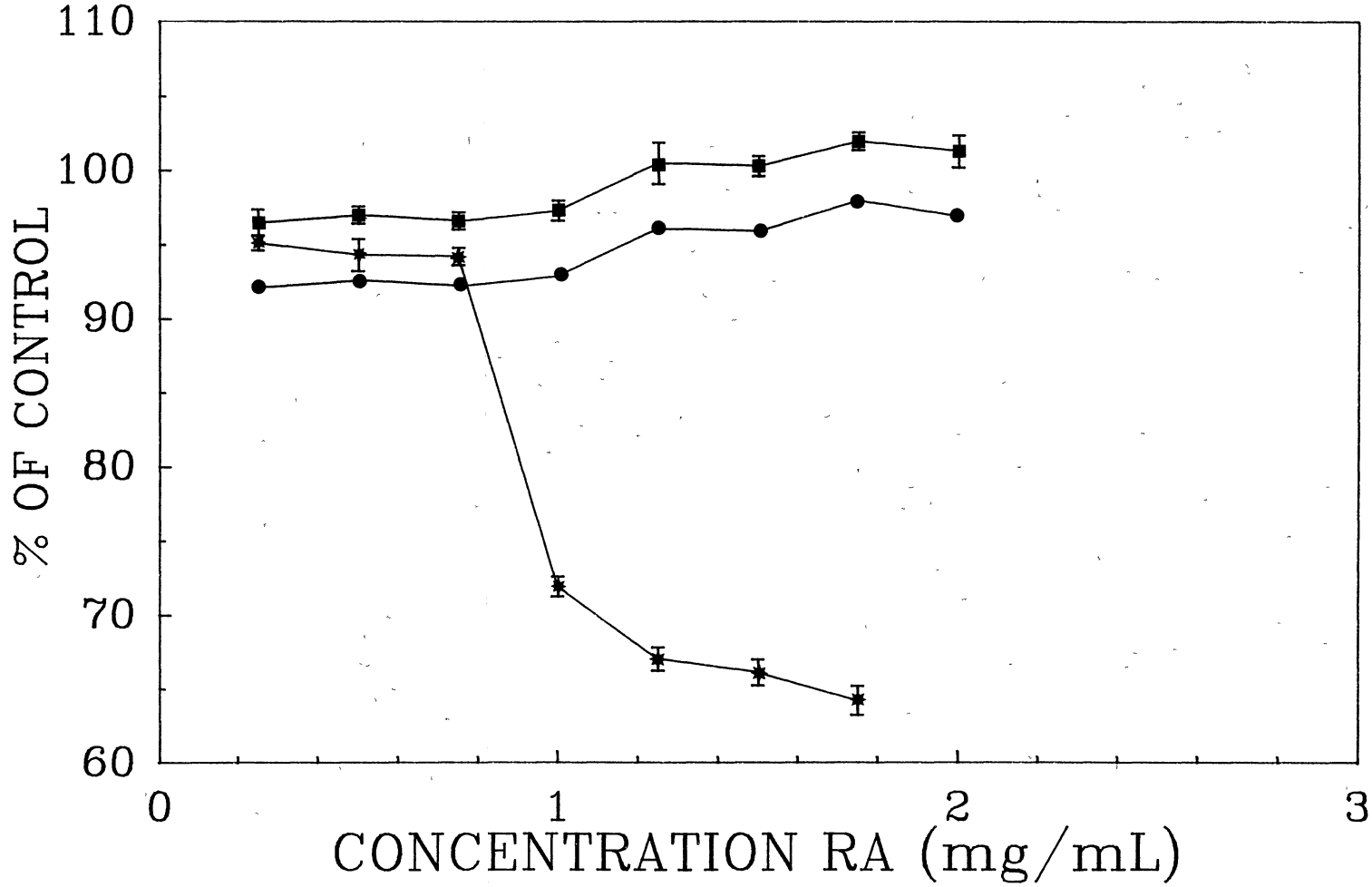

Figure 2

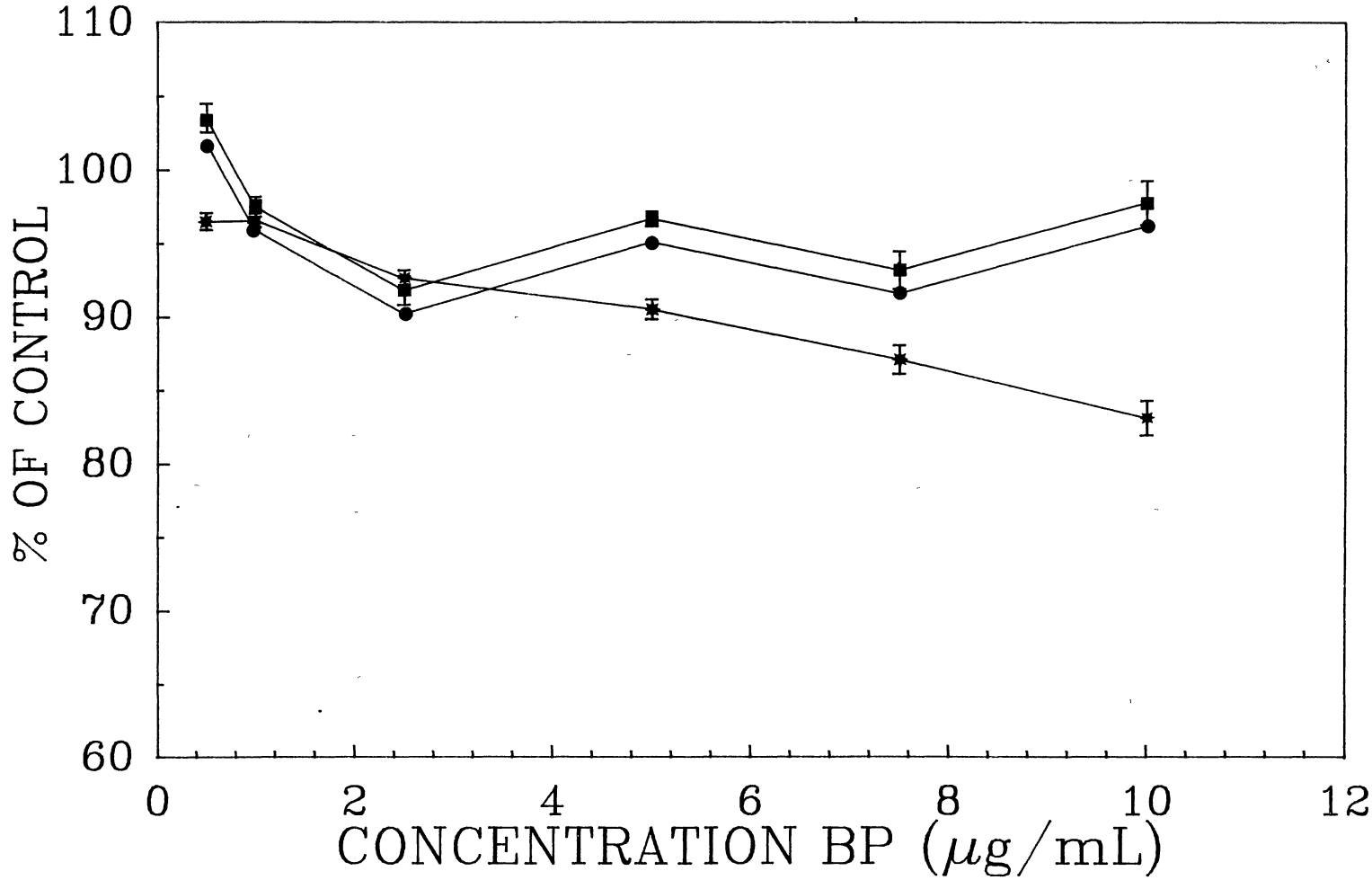
110
100
90
80
70
60
% OF CONTROL
0
2
4
6
8
10
12
CONCENTRATION BP (μg/mL)

Figure 3

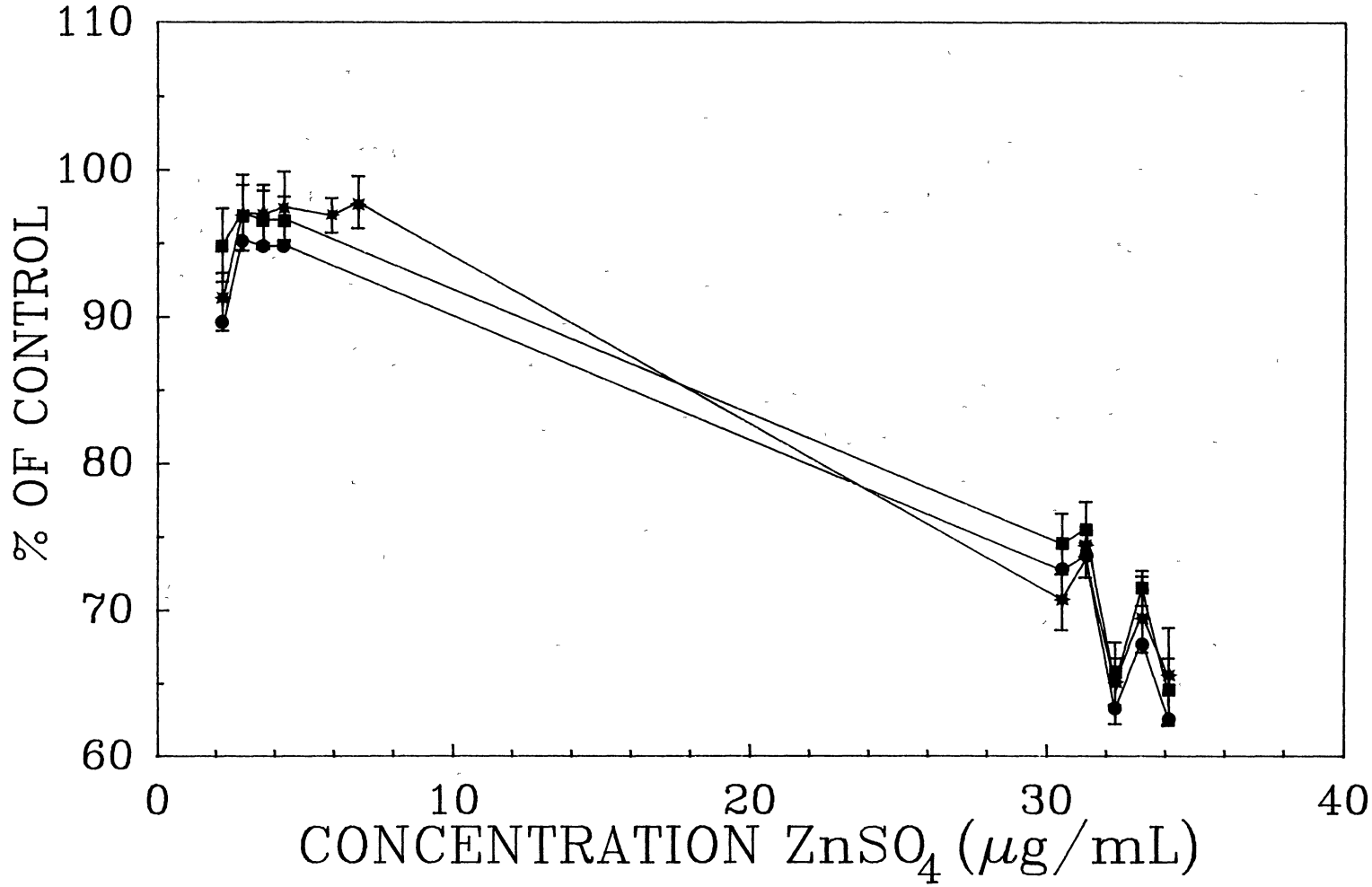
110
100
90
80
70
60
% OF CONTROL
0
10
20
30
40
CONCENTRATION $ZnSO_4$ (μg/mL)

Figure 4

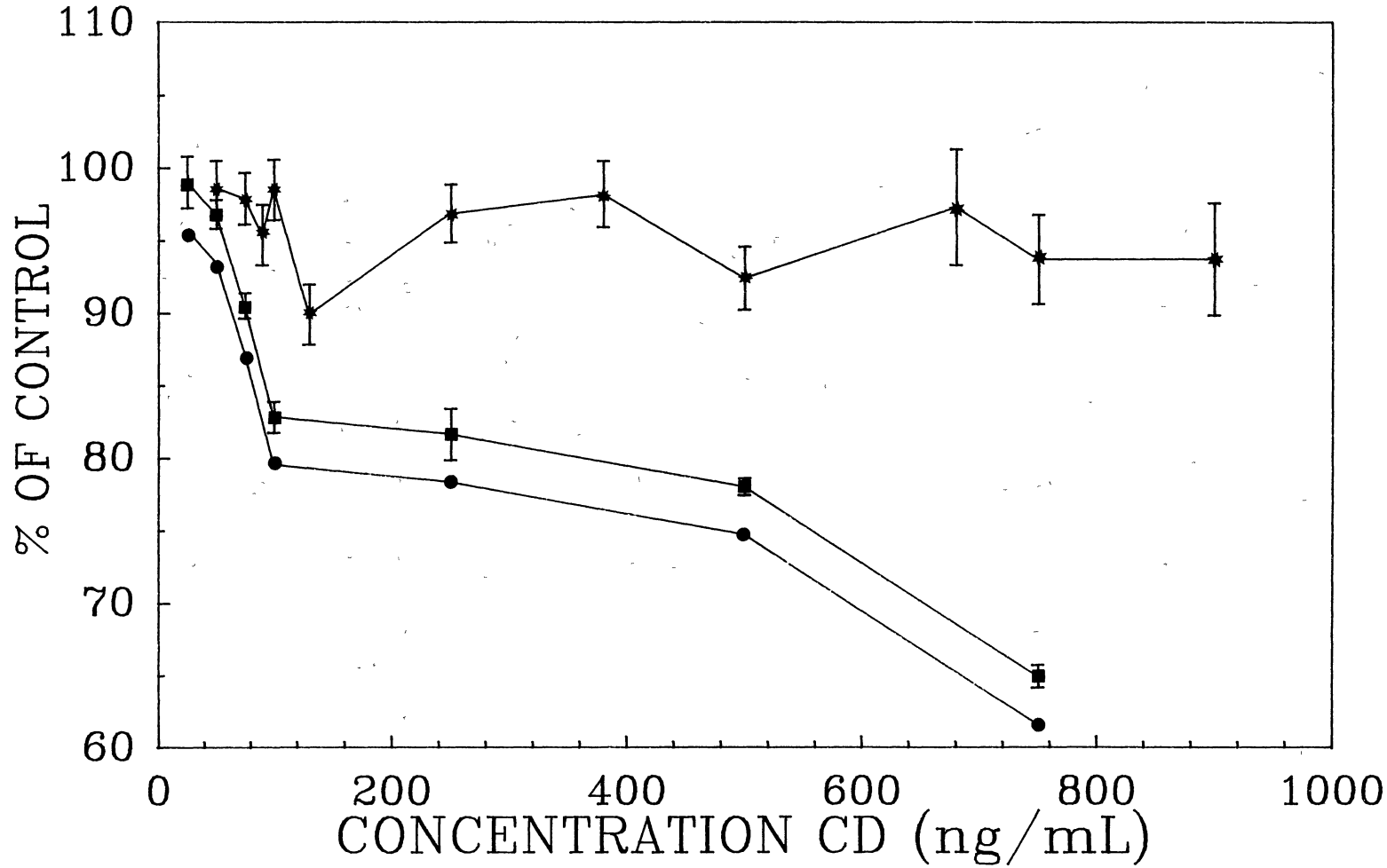
110
100
90
80
70
60
% OF CONTROL
0
200
400
600
800
1000
CONCENTRATION CD (ng/mL)

Figure 5

CHAPTER III

ANALYSIS OF THE DEVELOPMENTAL TOXICITY OF ISONIAZID USING EXOGENOUS METABOLIC ACTIVATION SYSTEMS WITH FROG EMBRYO TERATOGENESIS ASSAY - *XENOPUS* (FETAX)

Douglas J. Fort and John A. Bantle*

Department of Zoology

430 Life Sciences West

Oklahoma State University

Stillwater, OK 74078

Running Head: Teratogenic activity of isoniazid *in vitro*

*Author to whom correspondence should be addressed

ABSTRACT

The developmental toxicity of isoniazid (INH) and metabolites acetylhydrazide (AH) and isonicotinic acid (INA) were examined with Frog Embryo Teratogenesis Assay - Xenopus (FETAX). Late Xenopus laevis blastulae were exposed to INH, AH, and INA for 96-h in two separate static-renewal tests with and without the presence of three differently induced metabolic activation systems (MAS). The MAS consisted of uninduced, Aroclor 1254-induced, and INH-induced rat liver microsomes. Addition of the INH-induced MAS decreased the 96-h LC50 of INH and AH approximately 1.6-fold and 6.9-fold, respectively. The 96-h EC50 (malformation) of INH increased slightly (ca. 1.5-fold), decreasing the Teratogenic Index [TI=96-h LC50/96-h EC50 (malformation)] nearly 2.3-fold. The 96-h EC50 (malformation) of AH increased approximately 2.3-fold, decreasing the TI value 17.5-fold. INA yielded a TI value of approximately 2.6. Neither the uninduced MAS nor the Aroclor 1254-induced MAS had an effect on any of the compounds tested and none of the MAS affected the developmental toxicity of INA. Based on TI values, embryo growth, and types and severity of induced malformations, INH, AH, and INA all scored as potential developmental toxicants. Results from this study suggest that mixed functional oxidase metabolism may alter the developmental toxicity of INH in vitro by producing a more embryolethal, but less teratogenic species than INH or AH themselves. Results are indicative of the utility and versatility of

FETAX in the rapid screening of developmental toxicants.

Key Words: bioactivation, developmental toxicity, teratogenesis, cytochrome P-450, Xenopus

INTRODUCTION

Several alternative developmental toxicity screening systems to traditional tests employing pregnant mammals have been developed and utilized to minimize burdens associated with time, cost, and concern for animal welfare (1). One such alternative system is the Frog Embryo Teratogenesis Assay - Xenopus (FETAX) (2). FETAX is a 96-h, whole-embryo, static-renewal bioassay designed to screen for potential developmental toxicants in the environment and the workplace. To increase the predictability of FETAX an exogenous metabolic activation system (MAS) consisting of Aroclor 1254-induced rat liver microsomes was developed (3) and evaluated (4). The developmental toxicity of nicotine and primary metabolite cotinine (5); as well as, diphenylhydantoin and hydroxylated phenytoin metabolites (6) have also been assessed with this system.

Isoniazid (INH) is one of the most widely used tuberculostatic drugs available today. Although only a few studies with mammalian systems (7) and occasional cases in humans (8-10) have suggested INH to have teratogenic activity in vivo, INH was recently shown to have a high teratogenic potential in FETAX (11) and the Hydra assay (12) when tested without an in vitro MAS.

The primary metabolic pathway for the metabolism of INH is via acetylation to acetylisoniazid and subsequent hydrolysis to acetylhydrazide (AH) and isonicotinic acid (INA) (13). AH is further oxidized by cytochrome P-450j to

N-hydroxyl- acetylhydrazide, an unstable derivative fragment capable of forming an acetyl radical (carbonium ion) (14).

In the present investigation, we attempt to determine if the positive results obtained by the in vitro assay systems are related to the lack of maternal metabolism. Specifically, the developmental toxicity of INH and metabolites AH and INA is evaluated with and without the addition of various exogenous bioactivation systems. Results of this study support the continued use of FETAX and an exogenous MAS in the rapid screening of potential developmental toxicants, as well as its utility in investigating possible toxicological mechanisms of teratogenesis.

MATERIALS AND METHODS

Microsome Isolation

Rat liver microsomes were prepared as described previously (3). Adult male Sprague-Dawley rats (100-150 g) were treated with either Aroclor 1254 (Monsanto, St. Louis, MO) or INH (Sigma, St. Louis, MO). Aroclor 1254 (500 mg/Kg) in corn oil was injected intraperitoneally 5 d prior to microsome preparation (15). INH (0.1% w/v, pH 7.4) was administered via drinking water for 10 consecutive days prior to microsome isolation (13). All rats were fasted 24 h prior to microsome preparation.

Protein content was determined by the method of Bradford (16) (BioRad, Richmond, CA). Cytochrome P-450 activity was inferred by measurement of formaldehyde generated from the N-demethylation of aminopyrine (17) and N-nitrosodimethylamine (18) under standard assay conditions described previously (19). Units are expressed as uM formaldehyde generated/min. Several aliquots of microsomes were chemically reduced with dithionite and pretreated with carbon monoxide (CO-MAS) to selectively inactivate P-450 activity (20).

Animal Care and Breeding

Xenopus adult care, breeding, and embryo collection were performed as described by Dawson and Bantle (21).

Assay Protocol

For experiments without the MAS, groups of 20 embryos were placed in 60-mm covered plastic Petri dishes

(Fisher Scientic, Houston, TX) with varying concentrations of the appropriate test compound. INH, AH (Aldrich, Milwaukee, WI), INA, cyclophosphamide (CP) (both from Sigma, St. Louis, MO) were dissolved in appropriate volumes of FETAX solution, a reconstituted water medium suitable for the culture of Xenopus embryos (21). For each compound 10-16 concentrations were tested with replicates. Four separate dishes of 20 embryos were exposed to FETAX solution alone and designated FETAX solution controls. Each treatment dish contained a total of 8 ml of solution.

Since the purchased INH was not anhydrous, stock concentrations were measured spectrophotometrically at 266 nm.

Tests including the MAS were also conducted in duplicate with 20 embryos per replicate concentration. Each activated treatment received 60 ug/ml of either uninduced, Aroclor 1254-, or INH-induced microsomal protein, an NADPH generating system, and a penicillin-streptomycin mixture to control bacterial growth (3-6). For each compound 8-12 concentrations were tested. Controls including FETAX solution, each MAS, CO-gassed MAS + toxicant (negative control), CP [FETAX reference proteratogen (4-6)] and AH (positive controls), and unactivated toxicant were run simultaneously with each experiment.

For each compound, one range-finder and two definitive dose-response experiments were conducted with and without the MAS. The pH of all stock solutions was 7.0. Embryos

were cultured at 23±1°C throughout each test. All solutions were changed every 24 h of the 4-d test and fresh solutions were added. Dead embryos were removed at this time. Following 96-h of exposure, surviving embryos were fixed in 0.7% formalin (pH 7.0). The number of live malformed larvae and the stage of development (22) were ascertained using a dissecting microscope.

Data Analysis

Litchfield-Wilcoxon probit analysis (23) was used to determine the 96-h median lethal concentration (LC50) and the concentration inducing gross terata in 50% of the surviving embryos (EC50). The 95% confidence limits were calculated as well. A measure of the teratogenic potential, the Teratogenic Index (TI) was calculated by taking the ratio of the 96-h LC50 to the 96-h EC50 (malformation) (3-6,11). Head-tail length of surviving embryos was measured as an index of growth using an IBM-AT compatible computer and Sigma Scan (Jandel Scientific, Corte Madera, CA) digitizing software. Minimum concentrations to inhibit growth (MCIG) (11,20) were determined for each experiment with the t-Test ($p<0.05$).

RESULTS

Microsomal N-Demethylase Activity

Preliminary results determined that Aroclor 1254-induced microsomal aminopyrine-N-demethylase activity (6.38±0.11 U/mg protein) was significantly greater than either the uninduced or the INH-induced microsomal protein (3.50±0.11 and 3.41±0.25 U/mg protein, respectively). However, INH-induced microsomal N-nitrosodimethylamine-N-demethylase activity (3.67±0.95 U/mg protein) was significantly elevated compared to either the uninduced or the Aroclor 1254-induced protein (1.34±0.39 and 0.55±0.92 U/mg protein, respectively) [N=6 for all treatments; P=0.05 for both].

FETAX Control Results

In all tests conducted, FETAX solution control embryo mortality and malformation rates were <2.5%. Mortality and malformation rates for each MAS control were <4.0%. Control embryos exposed to 4.0 mg/ml unactivated CP exhibited mortality and malformation rates <8%. Mortality and malformation rates for 3.0 mg/ml AH were <2% and 100%, respectively. Embryos subjected to 4.0 mg/ml CP and either the uninduced MAS or the INH-induced MAS exhibited mortality and deformity rates <25%, whereas no less than 95% of those exposed to 4.0 mg/ml CP and the Aroclor 1254-induced MAS died. Survivors of the latter treatment were severely malformed. Exposure of control embryos to 3.0 mg/ml AH and the INH-induced MAS produced mortality and malformation rates of >87.5% and 100%, respectively.

Isoniazid

Results from tests with INH are presented in Table VI. The mean unactivated 96-h LC50 and EC50 (malformation) of both definitive trials were 9.99 mg/ml and 0.28 mg/ml, respectively, producing an average TI value of 35.7. Bioactivation with the uninduced-MAS and Aroclor 1254-MAS produced a mean 96-h LC50 of 8.76 mg/ml and 8.90 mg/ml, respectively. Inclusion of the INH-MAS reduced the mean 96-h LC50 approximately 1.6-fold to 6.34 mg/ml, however, the 96-h EC50 (malformation) was increased to a mean value of 0.42 mg/ml. The TI value was reduced nearly 2.3-fold to a mean value of 15.3 by the addition of the INH-MAS. Average MCIG values for INH were 2.3, 2.5, and 3.0% of the 96-h LC50 for unactivated, uninduced-MAS, and Aroclor 1254-MAS activated treatments, respectively, and 2.0% of the 96-h LC50 for those exposed to the INH-MAS. Concentration ranges and teratological effects of the various INH treatments are presented in Table IX.

Acetylhydrazide

The results of experiments performed with AH are shown in Table VII. The mean 96-h LC50 of both unactivated AH trials was 12.42 mg/ml. The average 96-h EC50 (malformation) was 0.05 mg/ml, yielding a mean TI value of 202.0. Addition of the uninduced-MAS and Aroclor 1254-MAS produced a mean 96-h LC50 value of 10.63 mg/ml and 11.14 mg/ml, respectively. The 96-h mean EC50 (malformation) was 0.06 mg/ml with the addition of either the uninduced system

or the Aroclor 1254-MAS. The TI values for the uninduced-MAS and Aroclor 1254-MAS treatments ranged from 170.5 to 220.6 and 162.0 to 218.6, respectively. Inclusion of the INH-MAS decreased the 96-h LC50 approximately 7-fold to an average of 1.8 mg/ml, but increased the 96-h EC50 (malformation) 2.7-fold to mean of 0.12 mg/ml. The TI was reduced nearly 18-fold to 16.0. MCIG values varied only slightly in the unactivated and metabolically activated treatments ranging from 0.3% to 1.0% of the 96-h LC50. Teratological concentration ranges and biological effects are shown in Table IX.

Isonicotinic Acid

Results from tests conducted with INA are presented in Table VIII. Mean unactivated, uninduced-MAS, Aroclor 1254-MAS, and INH-MAS activated 96-h LC50 values of INA were 3.13, 3.28, 3.29, and 3.29 mg/ml, respectively. The average unactivated 96-h EC50 (malformation) was 1.26 mg/ml yielding a mean TI value of 2.6. The mean 96-h EC50 (malformation) for the uninduced-, Aroclor 1254-, and INH-MAS activated treatments were 1.57, 1.52, and 1.53 mg/ml. Respective mean TI values for the uninduced-MAS, Aroclor 1254-MAS, and INH-MAS bioactivated treatments were 2.1, 2.2, and 2.2. MCIG values ranged from a low of 30.4% recorded with the Aroclor 1254 activated treatment to 39.6% (% of the 96-h LC50 for both) observed in embryos exposed to INA alone. Table IX lists the types of malformations and terata-inducing concentration ranges of the INA treatments.

DISCUSSION

Results from this study suggest that MFO metabolism may have an impact on the developmental toxicity of INH *in vitro* by producing a more embryolethal, but less teratogenic species than INH or acetylated metabolite, AH, themselves. Data obtained from the preliminary enzyme assays, as well as, the two positive FETAX controls CP for the Aroclor 1254-induced and AH for the INH-induced system, were indicative of an active P-450 metabolic system. Furthermore, the similarity of results between unactivated INH or unactivated AH treatments and the same treatments accompanied by CO-inactivated MAS, including rates of mortality and malformation, types and severity of deformities, and embryo growth, further suggests that P-450 may affect the developmental toxicity of INH.

Typically, the teratogenic potential of chemicals evaluated with FETAX is based on TI values, embryo growth, and the types and severity of induced terata. Generally, TI values <1.5 signify low teratogenic potential because there is little separation between concentrations which induce malformations, but do not cause embryolethal effects and concentrations which are lethal. Greater TI values indicate an increased potential for teratogenesis since a greater chance exists for embryos to be malformed in the absence of significant embryolethality (11). Since an adult to developmental (A/D) toxicity ratio (24) is not practicable with FETAX, the types and severity of malformations are

considered so that compounds with TI values <1.5 that produce severe deformities in major organ systems may still be considered to pose a developmental hazard, usually as embryo toxins (21).

The MCIG expressed as a percent of the toxicant LC50 has also proven useful in evaluating developmental toxicity (5,7,11). Dawson et al. (11) suggest that the MCIG of compounds posing a high developmental hazard will be <30% of the 96-h LC50.

Based on this evaluation scheme, unmetabolized INH and AH possess significant teratogenic potential, whereas unmetabolized INA has a lesser potential to induce terata. Results obtained with unactivated INH are similar to those obtained by Dawson et al. (11). Addition of the INH-MAS decreased the 96-h LC50, however, it also increased the concentration required to elicit malformations. INH (11) and AH (25) have been previously shown to cause osteolathyrogenic malformations in Xenopus which is consistent with the defects observed in this study. Osteolathyrogens disrupt the polymerization of connective tissue which are important in the development of the axial skeleton (25). Metabolism of INH and AH reduced the incidence of osteolathyrogenic malformations and increased the concentration of both compounds required to induce skeletal kinking (see Table IX). Greater concentrations of test material were, therefore, required to induce skeletal malformations in the presence of the INH-MAS. Defects of

this type at higher concentrations may have been due to a greater proportion of the unmetabolized drug compared to the lower concentration treatments. In addition, no skeletal defects were associated with INA treatment, either with or without metabolic supplementation. The reduction of skeletal defects appears to be the result of metabolic alteration of the hydrazino moiety [$-H_2N-N(H)-C(=O)-$] of INH and AH. Schultz et al. (26) determined that this molecular substructure is correlated with the induction of osteolathyrism and alteration of the hydrazino group sharply reduces skeletal defects, whereas alteration of the carbonyl moeity elicits a graded reduction in effect. Similar rates of skeletal defects in CO-inactivated MAS and INH, AH and identical unactivated treatments further suggest the role of MFO metabolism in the reduction of the teratogenic potential of INH.

Typically, the MCIG is a sensitive endpoint in FETAX. Both INH and AH inhibited embryo growth at concentrations <4% and <1% of the compound LC50, respectively. INA did not have as much of an impact on embryo growth reduction (approximately <40% of LC50). Inclusion of the various MAS had no significant effect on the growth inhibition associated with any of the compounds.

With the widespread use of isoniazid as an antituberculosis drug, it is surprising that evaluation of the developmental toxicity has not been more rigorously pursued with mammalian models. Of the few reported cases of

INH-induced terata in mammals including the human, the malformations recorded were similar to those observed in FETAX. Dluzniewski and Gasol-Lewinska (7) found INH to have teratogenic activity in the rat, but not in the rabbit. Wistar rats injected with 0.31 to 3.1 mg/Kg INH on d 6 to 14 yielded fetuses with abnormally developed cranial bones. Rabbits treated with 1.5 to 5.0 mg/Kg INH produced no malformed fetuses. In addition, no anomalies were detected in Swiss mice subjected to approximately 50 mg/Kg INH during d 1 to 4 and 10 to 13 of pregnancy (27). Conflicting results have been reported in humans as well. An examination of 74 exposed infants (28) and 19 children (29) found no increase in congenital defects. However, Heinonen (8) observed 10 malformed children from 85 mothers (11.8%) exposed to INH during the first four months of pregnancy. Severe encephalopathies were reported in five children from mothers exposed to INH (9). Warkany (10) has extensively reviewed the effects of tuberculostatic drugs in human pregnancy suggesting a small association of defects with INH. Variable metabolizing capacities may in part explain the diverse array of results obtained with mammalian species.

Differences in the developmental toxicity of metabolized INH and AH probably reflect differing complexities in the routes of metabolism. INH requires acetylation prior to microsomal oxidation, whereas AH may be directly oxidized by P-450j. Thus, metabolic conversion of

INH to N-hydroxylacetylhydrazide is less efficient *in vitro* than is the conversion of AH to the same metabolic endproduct.

Neither the uninduced or the Aroclor 1254-induced MAS had any significant effect on any of the compounds tested in this study. The lack of effect with these two systems is consistent with the finding that Aroclor 1254 significantly decreased N-nitrosodimethylamine-N-demethylase activity [a measure of P-450j activity] compared to the uninduced and INH-induced systems. Repression of P-450j activity by Aroclor 1254 has been shown previously, as well (30). Several drugs and compounds besides INH and AH have been shown to be metabolized by P-450j including pyrazole, imidazole, acetone, trichloroethylene, N-nitroso-dimethylamine, and ethanol (18). Thus, Aroclor 1254-induced liver microsomes may prove to be inefficacious as exogenous metabolic activation systems for *in vitro* developmental toxicity screening systems in these situations. To increase the predictability of the MAS routinely used by FETAX, we are currently testing mixtures of microsomes induced by a broad-spectrum of P-450 inducing agents.

Without the ability to metabolize xenobiotics, it is doubtful that *in vitro* tests, including FETAX, would be suitable for screening human developmental toxicants. Results from previous studies with FETAX and an exogenous MAS (3-6) suggest that FETAX may be able to overcome the obstacle of maternal metabolism. Since FETAX is an *in vitro*

system providing abiotic exposure of toxicants including metabolites, the pharmacological relationship between the fetal and maternal systems has been oversimplified. However, FETAX provides an opportunity to compare the developmental toxicity of parent compounds to metabolites and possibly generate information concerning toxicological mechanisms of action (6).

CONCLUSIONS

Tests of INH, AH, and INA with FETAX indicated that each has the potential to be teratogenic. However, reduction in TI values and induced malformations for both INH and AH upon metabolism compared to these compounds alone may indicate this compound and its metabolite could be more embryotoxic than teratogenic in mammalian systems. Metabolic conversion of INH and AH to reactive metabolites increased the concentration required to induce terata. INA possessed a much lower teratogenic potential than INH or AH, as well. These results further suggest that positive results obtained in several *in vitro* developmental toxicity assays may be the result of metabolic incompetence emphasizing the importance of exogenous MAS for *in vitro* teratogenesis assays. This study is indicative of the utility and the versatility of FETAX in screening developmental toxicants.

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Table VI. Analysis of the Developmental Toxicity of Isoniazid (INH) with FETAX.[a]

TREATMENT	% DEAD (N)	% MALFORMED (N)	LC50 (mg/ml)		EC50 (mg/ml)		TI		MCIG (mg/ml) [% of LC50]	
			TEST NO.							
			1	2	1	2	1	2	1	2
FETAX Solution	0 (320)	1.6 (320)	-	-	-	-	-	-	-	-
Uninduced-MAS	0 (80)	2.5 (80)	-	-	-	-	-	-	-	-
Aroclor 1254-MAS	1.3 (80)	2.6 (79)	-	-	-	-	-	-	-	-
INH-MAS	0 (80)	3.8 (80)	-	-	-	-	-	-	-	-
4.0 mg/ml CP	0 (80)	0 (80)	-	-	-	-	-	-	-	-
3.0 mg/ml AH	0 (80)	100.0 (80)	-	-	-	-	-	-	-	-
Uninduced-MAS + 4.0 mg/ml CP	2.5 (80)	20.5 (78)	-	-	-	-	-	-	-	-
Aroclor 1254-MAS + 4.0 mg/ml CP	100.0 (80)	-	-	-	-	-	-	-	-	-
INH-MAS + 3.0 mg/ml AH	92.5 (80)	100.0 (6)	-	-	-	-	-	-	-	-
CO-Uninduced-MAS + 9.5 mg/ml INH	47.5 (80)	100.0 (42)	-	-	-	-	-	-	-	-
CO-Aroclor 1254-MAS + 9.5 mg/ml INH	56.3 (80)	100.0 (35)	-	-	-	-	-	-	-	-
CO-INH-MAS + 6.5 mg/ml INH	12.5 (80)	100.0 (70)	-	-	-	-	-	-	-	-
INH	-	-	9.86 (9.55-10.19)	10.11 (9.76-10.47)	0.27 (0.24-0.30)	0.29 (0.26-0.32)	36.5	34.9	0.2 [2.0]	0.25 [2.5]
Uninduced-MAS + INH	-	-	8.90 (8.67-9.14)	8.61 (8.30-8.93)	0.30 (0.27-0.32)	0.28 (0.25-0.30)	29.7	30.8	0.2 [2.2]	0.23 [2.7]
Aroclor 1254-MAS + INH	-	-	9.15 (8.91-9.39)	8.65 (8.40-8.91)	0.29 (0.27-0.32)	0.30 (0.28-0.33)	31.6	28.8	0.25 [2.7]	0.28 [3.2]
INH-MAS + INH	-	-	6.14 (5.82-6.48)	6.54 (6.21-6.89)	0.39 (0.35-0.44)	0.44 (0.40-0.48)	15.7	14.9	0.1 [1.6]	0.15 [2.3]

[a] Represents the results of two definitive dose-response experiments with and without each of the metabolic activation systems (uninduced, Aroclor 154-induced, and isoniazid-induced [INH]. FETAX solution, metabolic activation system (MAS), cyclophosphamide (CP) [positive control (4-6)], acetylhydrazide (AH) [positive control], and carbon monoxide inactivated MAS (CO-MAS) + toxicant [negative control] controls were performed concurrently with each experiment. Median lethal (LC50) and teratogenic (EC50) concentrations were determined by Litchfield-Wilcoxon probit analysis (23) with 95% confidence intervals in parentheses. Teratogenic Indices (TI) were calculated by taking the ratio of the 96-h LC50 to the 96-h EC50 (malformation). Minimum concentrations to inhibit growth (MCIG) were determined by the T-test ($p<0.05$).

Table VII. Analysis of the Developmental Toxicity of Acetylhydrazide (AH) with FETAX.[a]

TREATMENT	% DEAD (N)	% MALFORMED (N)	LC50 (mg/ml)		EC50 (mg/ml)		TI		MCIG (mg/ml) [% of LC50]	
			TEST NO.							
			1	2	1	2	1	2	1	2
FETAX Solution	0.3 (320)	2.2 (319)	-	-	-	-	-	-	-	-
Uninduced-MAS	0 (80)	2.5 (80)	-	-	-	-	-	-	-	-
Aroclor 1254-MAS	1.3 (80)	2.6 (79)	-	-	-	-	-	-	-	-
INH-MAS	1.3 (80)	1.3 (80)	-	-	-	-	-	-	-	-
4.0 mg/ml CP	1.3 (80)	3.8 (79)	-	-	-	-	-	-	-	-
3.0 mg/ml AH	0 (80)	100.0 (80)	-	-	-	-	-	-	-	-
Uninduced-MAS + 4.0 mg/ml CP	11.3 (80)	23.6 (71)	-	-	-	-	-	-	-	-
Aroclor 1254-MAS + 4.0 mg/ml CP	95.0 (80)	100.0 (4)	-	-	-	-	-	-	-	-
INH-MAS + 3.0 mg/ml AH	90.0 (80)	100.0 (8)	-	-	-	-	-	-	-	-
CO-Uninduced-MAS + 12.0 mg/ml AH	61.3 (80)	100.0 (31)	-	-	-	-	-	-	-	-
CO-Aroclor 1254-MAS + 12.0 mg/ml AH	60.0 (80)	100.0 (32)	-	-	-	-	-	-	-	-
CO-INH-MAS + 2.5 mg/ml AH	3.8 (80)	100.0 (77)	-	-	-	-	-	-	-	-
AH	-	-	12.39 (11.98-12.81)	12.44 (12.11-12.77)	0.05 (0.04-0.07)	0.04 (0.03-0.07)	247.8	311.0	0.04 [0.3]	0.05 [0.4]
Uninduced-MAS + AH	-	-	11.03 (10.68-11.39)	10.23 (9.98-10.48)	0.05 (0.04-0.06)	0.06 (0.04-0.07)	220.6	170.5	0.05 [0.5]	0.07 [0.7]
Aroclor 1254 MAS + AH	-	-	11.34 (11.11-11.58)	10.93 (10.62-11.24)	0.07 (0.05-0.08)	0.05 (0.03-0.06)	162.0	218.6	0.03 [0.3]	0.04 [0.4]
INH-MAS + AH	-	-	2.01 (1.73-2.32)	1.58 (1.34-1.87)	0.12 (0.09-0.15)	0.11 (0.09-0.13)	17.6	14.4	0.02 [1.0]	0.01 [0.6]

[a] Represents the results of two definitive dose-response experiments with and without each of the metabolic activation systems (uninduced, Aroclor 154-induced, and isoniazid-induced [INH]. FETAX solution, metabolic activation system (MAS), cyclophosphamide (CP) [positive control (4-6)], acetylhydrazide (AH) [positive control], and carbon monoxide inactivated MAS (CO-MAS) + toxicant [negative control] controls were performed concurrently with each experiment. Median lethal (LC50) and teratogenic (EC50) concentrations were determined by Litchfield-Wilcoxon probit analysis (23) with 95% confidence intervals in parentheses. Teratogenic Indices (TI) were calculated by taking the ratio of the 96-h LC50 to the 96-h EC50 (malformation). Minimum concentrations to inhibit growth (MCIG) were determined by the T-test ($p<0.05$).

Table VIII. Evaluation of the Developmental Toxicity of Isonicotinic Acid (INA) with FETAX.[a]

TREATMENT	% DEAD (N)	% MALFORMED (N)	LC50 (mg/ml)		EC50 (mg/ml)		TI		MCIG (mg/ml) [% of LC50]	
			TEST NO.							
			1	2	1	2	1	2	1	2
FETAX Solution	0.6 (320)	1.9 (319)	-	-	-	-	-	-	-	-
Uninduced-MAS	0 (80)	0 (80)	-	-	-	-	-	-	-	-
Aroclor 1254-MAS	0 (80)	0 (80)	-	-	-	-	-	-	-	-
INH-MAS	0 (80)	1.3 (80)	-	-	-	-	-	-	-	-
4.0 mg/ml CP	3.8 (80)	7.8 (77)	-	-	-	-	-	-	-	-
3.0 mg/ml AH	1.3 (80)	100.0 (79)	-	-	-	-	-	-	-	-
Uninduced-MAS + 4.0 mg/ml CP	7.5 (80)	21.5 (74)	-	-	-	-	-	-	-	-
Aroclor 1254-MAS + 4.0 mg/ml CP	97.5 (80)	100.0 (2)	-	-	-	-	-	-	-	-
INH-MAS + 3.0 mg/ml AH	87.5 (80)	100.0 (70)	-	-	-	-	-	-	-	-
CO-Uninduced-MAS + 3.0 mg/ml INA	62.5 (80)	100.0 (30)	-	-	-	-	-	-	-	-
CO-Aroclor 1254-MAS + 3.0 mg/ml INA	52.5 (80)	100.0 (38)	-	-	-	-	-	-	-	-
CO-INH-MAS + 3.0 mg/ml INA	61.3 (80)	100.0 (31)	-	-	-	-	-	-	-	-
INA	-	-	3.16 (3.04-3.28)	3.20 (3.08-3.32)	1.24 (1.16-1.34)	1.28 (1.19-1.37)	2.6	2.5	1.25 [39.6]	1.0 [31.3]
Uninduced-MAS + INA	-	-	3.22 (3.14-3.39)	3.33 (3.17-3.50)	1.51 (1.39-1.64)	1.62 (1.38-1.75)	2.1	2.1	1.35 [41 9]	1.25 [37 5]
Aroclor 1254-MAS + INA	-	-	3.28 (3.03-3.47)	3.30 (3.15-3.49)	1.57 (1.43-1.72)	1.46 (1 33-1.71)	2.0	2.3	1.0 [30 4]	1.25 [37.9]
INH-MAS + INA	-	-	3.26 (3.05-3.52)	3.32 (3.12-3.53)	1.44 (1.32-1.59)	1.62 (1.44-1.82)	2.3	2.1	1.25 [38.3]	1.3 [39.2]

[a] Represents the results of two definitive dose-response experiments with and without each of the metabolic activation systems (uninduced, Aroclor 154-induced, and isoniazid-induced [INH]. FETAX solution, metabolic activation system (MAS), cyclophosphamide (CP) [positive control (4-6)], acetylhydrazide (AH) [positive control], and carbon monoxide inactivated MAS (CO-MAS) + toxicant [negative control] controls were performed concurrently with each experiment. Median lethal (LC50) and teratogenic (EC50) concentrations were determined by Litchfield-Wilcoxon probit analysis (23) with 95% confidence intervals in parentheses. Teratogenic Indices (TI) were calculated by taking the ratio of the 96-h LC50 to the 96-h EC50 (malformation). Minimum concentrations to inhibit growth (MCIG) were determined by the T-test ($p<0.05$).

Table IX. Terata Induced in Xenopus by Exposure to Isoniazid (INH), Acetylhydrazide (AH), and Isonicotinic Acid (INA).

Treatment	Concentration[a] (mg/ml)	Terata Induced
INH[b]	>0.2	Skeletal Kinking, Incomplete Mouth Development, Gut Miscoiling
	>0.8	Eye Malformations
	>1.0	Incomplete Heart Development, Pericardial Edema
INH-MAS + INH	>0.2	Visceral Edema, Gut Miscoiling
	>0.4	Craniofacial Defects, Muscular Kinking[c]
	>0.8	Skeletal Kinking, Eye Malformations, Incomplete Mouth Development, Microencephaly
AH[b]	>0.02	Gut Miscoiling, Skeletal Kinking
	>0.05	Abnormal Mouth Development
	>0.15	Eye Malformations, Visceral Edema, Abnormal Heart Development, Craniofacial Defects
INH-MAS + AH	>0.04	Gut Miscoiling
	>0.06	Skeletal Kinking, Microencephaly
	>0.15	Hydrocephalus, Craniofacial Defects, Blistering of the Dorsal Fin, Eye Malformations
INA[d]	>1.5	Gut Miscoiling, Craniofacial Defects, Pericardial Edema
	>1.8	Eye Malformations, Hypognathia, Muscular Kinking[c]
	>3.0	Ophthalmic Edema, Visceral Edema, Microencephaly, Hydrocephalus

[a] Defines concentration range of teratogenicity.

[b] Treatments with INH or AH and either the uninduced or Aroclor 1254-induced MAS produced similar malformations at the given concentrations.

[c] Term muscular kinking, caused most likely by abnormal somite development, is used to differentiate from that finding termed skeletal kinking in which the spine (including the notocord) is affected.

[d] Treatments with INA and any of the MAS produced similar malformations at the given concentrations.

CHAPTER IV

USE OF FROG EMBRYO TERATOGENESIS ASSAY-*XENOPUS* (FETAX) AND AN EXOGENOUS METABOLIC ACTIVATION SYSTEM TO EVALUATE THE DEVELOPMENTAL TOXICITY OF DIPHENYLHYDANTOIN

Douglas J. Fort and John A. Bantle*

Department of Zoology

Oklahoma State University

Stillwater, OK 74078

(405) 744-9683

Running Head: Developmental Toxicity of Diphenylhydantoin

*Author to whom correspondence should be addressed

ABSTRACT

Use of the Frog Embryo Teratogenesis Assay - Xenopus (FETAX) and an Exogenous Metabolic Activation System to Evaluate the Developmental Toxicity of Diphenylhydantoin. Fort, D.J. and Bantle, J.A. (1989). Fundam. Appl. Toxicol. ___ , _____.

The teratogenic potential of diphenylhydantoin (DPH) and hydroxylated metabolites (HPPH) was evaluated with the Frog Embryo Teratogenesis Assay - Xenopus (FETAX). Embryos of the South African clawed frog, Xenopus laevis were exposed to DPH and HPPH in two separate static-renewal experiments with and without the presence of an exogenous metabolic activation system (MAS) for 96 hr. Two separate dose-response tests were also conducted with DPH and HPPH with a MAS modulated by various mixed functional oxidase inhibitors [carbon monoxide (CO) (broad spectrum cytochrome P-450), cimetidine (mainly cytochrome P-450), and ellipticine (cytochrome P-448)] and an epoxide hydrolase inhibitor [cyclohexene oxide]. Assessment of the potential teratogenic hazard was based on teratogenic indices [TI=96-hr LC50/96-hr EC50 (malformation)], types and severity of malformations, and embryo growth endpoints. Addition of the intact MAS to DPH increased the 96-hr LC50 and EC50 (malformation) from approximately 74.5 and 32.4 mg/L to 126.4 and 62.9 mg/L, repectively. The TI was reduced 1.2 fold. Both p-HPPH and m-HPPH were much less developmentally toxic than DPH. CO and cimetidine inhibition of cytochrome

P-450 maintained much of the developmental toxicity of DPH, whereas ellipticine inhibition of cytochrome P-448 was much less effective in maintaining the developmental toxicity of DPH. Cyclohexene oxide inhibition of epoxide hydrolase markedly increased DPH-induced embryotoxicity decreasing the 96-hr from approximately 74.5 to 38.6 mg/L. These results suggest that unmetabolized DPH and an embryotoxic epoxide intermediate may serve as the teratogenic species in FETAX.

INDEX TERMS

Xenopus, Metabolic Activation System, FETAX, Mixed Functional Oxidase, Epoxide Hydrolase, Developmental Toxicity, Teratogenicity, Diphenylhydantoin, 5-(4-hydroxyphenyl)-5-phenylhydantoin, 5-(3-hydroxyphenyl)-5-phenylhydantoin, Carbon monoxide, Cimetidine, Ellipticine, Cyclohexene Oxide.

INTRODUCTION

Demand for routine developmental toxicity hazard assessment necessitates the development, validation, and use of alternate test systems to traditional mammalian test systems (Brown, 1987). *In vitro* test systems, such as the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) provide a rapid, cost-effective method of determining compounds and complex mixtures which may be potential teratogenic hazards, and offer insight into toxicological mechanisms of teratogenesis. FETAX is a 96-hr, whole-embryo developmental toxicity bioassay employing embryos of the South African clawed frog *Xenopus laevis* (Dumont et al., 1983). Because *Xenopus* embryos lack many metabolic enzyme systems through the first 96 hr of development, an *in vitro* metabolic activation system (MAS) was developed (Fort et al., 1988) and evaluated (Fort et al., 1989) using Aroclor 1254-induced rat liver microsomes. The developmental toxicity of nicotine and a primary metabolite cotinine was also evaluated using FETAX and MAS (Dawson et al., 1988).

Elucidating possible toxicological mechanisms of teratogenesis in *in vivo* test systems is difficult due to the complexity of the placental relationship between mother and embryo. Fetal and maternal drug pharmacokinetics and xenobiotic metabolism also compound the problem as exemplified by previous studies with the anticonvulsant drug, diphenylhydantoin (DPH) (Flint and Brown, 1987). Results from *in vivo* mammalian studies provide evidence for

DPH as a proteratogen (Harbison, 1978; Martz et al., 1977), as well as the ultimate teratogen (McClain and Rohrs, 1985; Hansen and Hodes, 1983; Harbison and Becker, 1970). Recent in vitro studies with the whole-rat embryo (Bruckner et al., 1983) and differentiating rat embryo mid-brain and limb culture (Flint and Orton, 1984) seem to indicate that DPH is a direct-acting teratogen not requiring exogenous bioactivation. Probable metabolic pathways for DPH (see Fig. 7) have been studied by a number of investigators (Barcellona et al., 1987; Glazko, 1987; Martz et al., 1977; Chang et al., 1970; Maynert, 1960; Butler, 1957). Steps involving the formation and detoxification of highly embryotoxic arene oxide intermediates by cytochrome P-450 (P-450) and epoxide hydrolase or spontaneous isomerization appear to be the most important (Martz et al., 1977; Jerina and Daly, 1974). Meta and para isomers of 5-(hydroxyphenyl)-5-phenylhydantoin (HPPH) and 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (dihydrodiol) are generated via spontaneous isomerization or by enzymatic inactivation by epoxide hydrolase (Barcellona et al., 1987). Pantarotto et al. (1982) further hypothesized that an additional arene oxide intermediate may be generated by MFO oxidation of the major metabolite, HPPH.

In this report we evaluate the developmental toxicity of DPH and HPPH using an exogenous MAS and various inhibitors of the microsomal mixed-functional oxidase system (MFO) and epoxide hydrolase. The results presented show the utility

of FETAX as a screen for developmental toxicants and further demonstrate the value of FETAX in elucidating potential toxicological mechanisms of teratogenesis.

METHODS

Microsome Preparation

Aroclor 1254-induced rat liver microsomes were prepared as described previously (Fort et al., 1988). Five days prior to microsome preparation, adult male Sprague-Dawley CD strain rats (125-200 g) were injected with 500 mg/Kg Aroclor 1254 in corn oil (Freireich, 1977). Following cervical dislocation, the liver was perfused with 0.02 M Tris-HCl buffer containing 1.15% KCl, pH 7.5, and subsequently homogenized in 1.15% KCl-0.05 M Tris-HCl containing 0.5% bovine serum albumin, pH 7.5. Homogenate was then successively centrifuged at 600 and 9,000 x g. Purification of the crude S-9 supernatant was achieved by ultracentrifugation at 120,000 x g. The microsomal pellet was resuspended in 0.05 M Tris-HCl, pH 7.5, put into 2 ml aliquots, and immediately frozen in liquid nitrogen (Dent et al., 1981). Protein content was determined by the method of Bradford (1976). P-450 activity was inferred by the measurement of formaldehyde generated from the N-demethylation of aminopyrine (APD) (Nash, 1955) under standard assay conditions previously established by Lucier et al. (1971). Specific aliquots were pretreated with microsomal enzyme inhibitors (Bantle and Dawson, 1987; Brown et al., 1986): carbon monoxide (CO-MAS) [broad spectrum P-450 inhibitor], cimetidine (mainly P-450 inhibitor), ellipticine (P-448 inhibitor), and cyclohexene oxide (Guenthner, 1986; Oesch et al., 1971) (epoxide hydrolase

inhibitor) to modulate metabolic activity. Inhibition by CO was obtained by chemically reducing designated aliquots with dithionite and pretreating with CO for 3 min. Cimetidine, ellipticine, and cyclohexene oxide were added directly to the appropriate microsome aliquots.

Animal Care and Breeding

Xenopus adult care, breeding, and embryo collection were performed according to Dawson and Bantle (1987).

Assay Protocol

For unactivated tests (no MAS), groups of 20 embryos were placed in 60 mm covered plastic Petri dishes with varying concentrations of DPH (Sigma Chemical Co., St. Louis, MO), p-HPPH, and m-HPPH (Aldrich Chemical Co., Milwaukee, WI). Stock solutions were prepared by dissolving in 1% (v/v) DMSO diluted in FETAX solution, a reconstituted water media suitable for culturing Xenopus embryos (Dawson and Bantle, 1987). FETAX solution contains 625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 60 mg $CaSO_4 2\ H_2O$, 30 mg KCl, and 15 mg $CaCl_2$ per 1 of solution, pH 7.9. DMSO not in excess of 1% (v/v) was previously found to have no significant effect on microsomal APD activity, as well as embryo growth and development (Fort et al., 1989). The DPH stock solution concentration was confirmed spectrophotometrically at 35 nm (Sunshine, 1981). For each compound 8 to 16 concentrations were tested in duplicate. DPH and HPPH concentrations ranged from 20 to 100 mg/L and 80 to 150 mg/L, respectively. For tests with DPH and the cyclohexene oxide-MAS, DPH

concentrations ranged from 5 to 75 mg/L. Four separate dishes of 20 embryos were exposed to FETAX solution (designated FETAX solution controls). Forty embryos, 20 per dish, were exposed to 1% DMSO and served as solvent controls. Each treatment contained a total of 8 ml of solution.

Experiments conducted with MAS and inhibited MAS were performed in duplicate with 20 embryos per dish. Preliminary experiments were conducted to determine the maximum concentration of inhibitor which did not significantly affect embryo development. Each activated treatment received 0.04 U APD activity (expressed as uM formaldehyde generated/min), an NADPH generating system, and 100 U/ml penicillin-100 ug/ml streptomycin. The NADPH generating system consisted of 3.5 mM glucose-6-phosphate, 0.31 IU glucose-6-phosphate dehydrogenase, 0.1 mM NADP, and 0.7 uM NADPH. Each dish received no more than 60 ug/ml microsomal protein (Fort et al., 1988). Controls for FETAX solution, DMSO, MAS, inhibitor-MAS, MAS + DMSO, inhibitor-MAS + DMSO, cyclophosphamide (CP) [FETAX reference proteratogen] (Dawson et al., 1988), and unactivated toxicant were also run with each individual test.

Two separate definitive tests were conducted following establishment of concentration ranges in preliminary experiments for each particular treatment. For experiments performed with metabolically activated HPPH and HPPH plus cyclohexene oxide-MAS, one range and one definitive test

were run. The pH of all stock solutions was maintained between 8.2 and 8.5. Embryos were cultured at 23 + 1°C throughout the test. All solutions were removed every 24 hr of the 4-d test and fresh solutions were added. Dead embryos were removed when solutions were chnaged. Following 96 hr of exposure, surviving embryos were fixed in 0.7% formalin, pH 7.0. The number of live-malformed embryos and the stage of development (Nieuwkoop and Faber, 1975) was ascertained using a dissecting microscope.

Data Analysis

Litchfield-Wilcoxon probit analysis (Tallarida and Murray, 1980) was used to determine the 96-hr median lethal concentration (96-hr LC50), the 96-hr EC50, the concentration inducing gross terata in 50 percent of the surviving larvae, and their respective 95 percent confidence limits. Comparison of dose-response data was performed by the method of Sprague and Fogels (1977). A Teratogenic Index (TI) which has proven useful in assessing teratogenic hazard (Dumont et al., 1983; Courchesne and Bantle, 1985; Dawson and Bantle, 1987; Fort et al., 1988; Dawson et al., 1988; Dawson et al., 1989) was determined by the ratio of the 96-hr LC50 to the 96-hr EC50 (malformation). Head-tail length was measured as an indicator of embryo growth using Sigma Scan digitizing software (Jandel Scientific, Corte Madera, CA) and an IBM-AT equivalent personal computer.

Results and Discussion

Control Data

Maximum non-developmentally toxic concentrations of cimetidine, ellipticine, and cyclohexene oxide determined by preliminary dose-response experiments were 2.4, 0.04, and 0.1 mM, respectively (Dunnett's test). These concentrations have been previously shown to inhibit P-450, P-448 (Brown et al., 1986), and epoxide hydrolase activity (Oesch et al., 1971). Marked reduction in the developmental toxicity of bioactivated CP (FETAX reference proteratogen) by CO- and cimetidine-MAS indicates that these concentrations were adequate to inactivate P-450 activity. The ellipticine-MAS failed to reduce the developmental toxicity of bioactivated CP. These results are consistent with the concept that a phenobarbital-inducible P-450 isozyme is responsible for the conversion of CP to an ultimately teratogenic metabolite(s) in the rat (Hales and Jain, 1980b; Hales, 1981; Greenaway et al., 1982). In addition, neither B-naphthoflavone (Hales and Jain, 1980b; Hales, 1981) nor 3-methylcholanthrene (Greenaway et al., 1982) [P-448-type inducers] had an effect on the hepatic bioactivation of CP *in vivo* or *in vitro*. Control data for experiments performed with DPH, DPH and MAS; HPPH, HPPH and MAS, and HPPH and cyclohexene oxide-MAS; as well as DPH and selectively inhibited MAS are presented in Tables X-XII, respectively.

Developmental Toxicity of DPH

Results of tests conducted with DPH and DPH with MAS

are presented in Table XIII. The 96-hr LC50, 96-hr EC50 (malformation), and TI values were 77.9 and 71.0 mg/L, 33.0 and 31.8 mg/L, and 2.4 and 2.2, respectively. These results are consistent with those obtained by Sabourin and Faulk (1987) with Xenopus (TI = 1.5-2.6). Exogenous bioinactivation significantly (P=0.05) increased the 96-hr LC50 of DPH at least 1.8-fold to 122.1 and 130.6 mg/L. Addition of the MAS significantly (P=0.05) increased the 96-hr EC50 (malformation) to 62.9 and 75.4 mg/L (at least 2.4-fold). Bioinactivation produced TI values of 1.9 and 1.8. Concentration ranges and teratological effects of DPH on Xenopus embryo development are presented in Table XIV, as well as illustrated in Fig. 7. The effect of DPH and bioactivated DPH on 96-hr embryo growth is presented in Figure 8. Inclusion of MAS with DPH reduced some growth inhibiting effects caused by DPH exposure.

Developmental Toxicity of HPPH

Table XIII shows the results of tests performed with HPPH, HPPH and intact MAS, and HPPH and cyclohexene oxide-MAS. None of the embryos exposed to either p-HPPH or m-HPPH with or without the addition of the intact MAS or cyclohexene oxide-MAS died at maximum soluble concentrations in 1% (v/v) DMSO (150 mg/L). At a concentration of 150 mg/L p-HPPH and m-HPPH induced malformation rates of 17.5% and 16.3% (N=160 for both), respectively. Thus, 96-hr LC50 and EC50 (malformation) for all three treatments is expressed as >150.0 mg/L. Types of terata and appropriate concentration

ranges are presented in Table XIV. The effect of HPPH on 96-hr Xenopus embryo growth is shown in Fig. 8. Neither isomer had a pronounced effect on embryo growth with or without MAS.

Effect of MFO and Epoxide Hydrolase Inhibitors on DPH Teratogenicity

Results from tests conducted with CO-, cimetidine-, ellipticine-, and cyclohexene oxide-MAS and DPH are presented in Table XIII. The 96-hr LC50, 96-hr EC50 (malformation), and TI values for embryos exposed to CO-MAS and DPH were 69.3 and 68.8 mg/L, 30.6 and 33.7 mg/L, and 2.3 and 2.0, respectively. The 96-hr LC50 of cimetidine-MAS and DPH treatment was 83.7 and 78.8 mg/L while the 96-hr EC50 (malformation) was 36.0 and 41.8 mg/L. The TI values for both trials were 2.3 and 1.9, respectively. The 96-hr LC50 and EC50 (malformation) values for both the CO-MAS and cimetidine-MAS plus DPH treatments were not significantly different from values for DPH alone, but were significantly different from the DPH and intact-MAS treatment (P=0.05 for both). Exposure to the ellipticine-MAS and DPH resulted in a 96-hr LC50 of 98.6 and 95.7 mg/L. The 96-hr EC50 (malformation) for the same treatment was 59.2 and 53.4 mg/L, yielding TI values of 1.7 and 1.8. Ellipticine-MAS and DPH 96-hr LC50 and EC50 (malformation) values were not significantly different from the DPH plus the intact-MAS treatment. These endpoints were, however, significantly different from those of DPH alone (P=0.05 for both). The

96-hr LC50 for cyclohexene oxide-MAS and DPH was 31.5 and 45.6 mg/L. The 96-hr EC50 (malformation) and TI values were 24.7 and 39.7 mg/L, and 1.3 and 1.1, respectively. The median lethal concentration of the cyclohexene oxide-MAS and DPH treatment was significantly different from that of DPH alone, but not significantly different from intact-MAS and DPH treatment (P=0.05 for both). The 96-hr median teratogenic concentration was significantly different from the DPH and intact-MAS treatment; however, it was not significantly different from DPH alone (P=0.05 for both). The effects of DPH and modulation of MAS activity by each specific inhibitor on Xenopus embryo development and growth are presented in Table XIV and Figure 9, respectively. CO-MAS, cimetidine-MAS, and cyclohexene oxide-MAS and DPH caused a slightly greater reduction in growth than was observed by DPH alone. However, embryos exposed to ellipticine-MAS and DPH showed less growth reduction than those exposed to DPH alone.

The potential teratogenic hazard of compounds tested with FETAX are based on Teratogenic Index (TI) values, embryo growth, and the types and severity of induced anomalies. Typically, TI values <1.5 indicate low teratogenic potential since there is little or no separation between concentrations that cause malformation, but cause no embryolethal effects; and concentrations which are embryolethal. Greater TI values signify a larger separation between the two aforementioned responses and thus, a greater

possibility of embryos being malformed in the absence of significant embryolethality. Because calculation of an adult to developmental (A/D) ratio is not practicable with FETAX, types and severity of induced malformations must also be considered for compounds with TI values <1.5 which produce severy deformaties in major organ systems. To assess the utility of the TI we are presently establishing a large data base with FETAX. Several conclusions about DPH-induced teratogenesis in vitro may be drawn from the results of this study. First, DPH seems to be at least partially detoxified by MAS as evidenced by the increase in the 96-hr LC50 and EC50 (malformation), as well as the reduction in TI. Inhibition of MAS with known inhibitors of P-450 prevented the loss of much of the developmental toxicity of unactivated DPH as indicated by the higher TI values for the CO- and cimetidine-inhibited MAS and DPH treatments compared to bioactivated DPH. Also, similarities between terata observed with the P-450-inhibited MAS and DPH to DPH itself (see Table XIV) and the marked inhibition of embryo growth in these treatments seem to suggest that unmetabolized DPH could participate in DPH-induced teratogenesis.

Similar results were obtained in mammalian studies both in vivo and in vitro. Harbison and Becker (1970) found that pretreatment of Swiss-Webster mice with phenobarbitone significantly decreased the teratogenicity of DPH supposedly due to enhanced metabolism. By lowering the basal drug metabolic rate via dietary modulation in Swiss-Webster mice,

McClain and Rohrs (1985) showed that the incidence of DPH related abnormalities was increased. Similar results have also been reported in different strains of mice with specific interstrain variablity in metabolic capacity (Hansen and Hodes, 1983). Inhibition of P-448 activity by ellipticine has less effect on the developmental toxicity of DPH suggesting that metabolism may be mainly P-450 mediated in FETAX. Barcellona et al. (1987) drew similar conclusions using primipara Swiss CD-1 mice in in vivo developmental toxicity tests. However, Brown et al. (1986) found ellipticine-inhibition of P-448 to increase the developmental toxicity of DPH in the differentiating rat limb mesenchymal cell culture assay. This discrepancy may be the result of differences in pathways utilized in xenobiotic metabolism by various cell lines and bioactivation systems.

Second, the primary metabolites (HPPH) of DPH were not as developmentally toxic to Xenopus at soluble levels as DPH itself. These results would also seem to support unaltered DPH or the epoxide intermediate as the most important teratogenic species. Harbison and Becker (1974) report similar results in Swiss Webster mice in which 87.5 mg/Kg DPH induced 85% orofacial anomalies whereas equimolar doses of the metabolites diphenylhydantoic acid, p-HPPH, and aminodiphenylacetic acid did not produce a significant incidence of these defects. However, conflicting results were obtained in vitro in the differentiating rat embryo

limb cell assay in which DPH was found to be nearly equitoxic with both isomers of HPPH (Brown et al., 1986).

Third, potentiation of DPH embryotoxicity caused by epoxide hydrolase inhibition with cyclohexene oxide implicates the possibility of an embryotoxic arene oxide intermediate. The 1.9-fold decrease in the 96-hr LC50 of DPH, as well as the reduction in TI with the epoxide hydrolase-inhibited MAS indicates that the arene oxide is significantly more embryolethal than DPH itself in FETAX. However, the severity of malformations induced and the significant reduction in embryo growth suggests that the epoxide intermediate poses a teratogenic threat to the surviving embryos. Thus, the role epoxide intermediates play in the developmental toxicity of DPH appears to be of major importance which is consistent with several in vivo findings (Martz et al., 1977; Blake and Martz, 1980; Hansen and Hodes, 1983; Barcellona et al., 1987). Brown et al. (1986), however, found that trichloropropene oxide (TCPO), a potent inhibitor of epoxide hydrolase, treatment of cultured differentiating rat limb cells exposed to DPH had no effect, thus arguing against arene oxide formation being responsible for the toxicity observed. Since we were not able to use TCPO because of the lack of availability from commercial vendors, no direct comparison can be made. We are presently unable to explain the descrepancy in results. In addition, the concentrations of TCPO used in the Brown et al. (1986) study may not have provided sufficient inhibition of epoxide

hydrolase. Because no embryolethal and limited (<20%) teratogenic effects were generated by exposing Xenopus embryos to HPPH and the intact or epoxide hydrolase-inhibited MAS, a second route of arene oxide formation from HPPH does not seem to play a role in DPH teratogenesis in FETAX (see Fig. 7).

Unactivated DPH induced malformations of the craniofacial region, heart and brain in Xenopus. Similar malformations were observed in the inhibited-MAS and DPH treatments. Embryos exposed to DPH and intact MAS also exhibited body flexure and skeletal malformations. Skeletal kinking observed in Xenopus embryos may bear some relationship to skeletal limb anomalies observed in mammals (Dawson and Bantle, 1987). Although body flexure malformations and skeletal kinking were observed in a small percentage (<20% at 150 mg/L) of HPPH treated embryos, they were the primary anomalies observed with HPPH. Skeletal defects were observed in the epoxide hydrolase-inhibited MAS and DPH treatment. The occurrence of these malformations in the DPH and intact MAS treatments further suggests that DPH metabolism was taking place in the MAS supplemented treatments. Similarity in the types of malformations induced by DPH with and without MAS may be due in part to residual unmetabolized DPH. Several of the malformations caused by DPH in Xenopus are similar to those found in mammals including humans. Single injections of DPH on days 10-12, 14 and 15 in Swiss-Webster mice produced open eye,

ectrodactyly, cleft lip and palate, hydronephrosis, internal hydrocephalus, and skeletal defects (Harbison and Becker, 1969). Cases of cleft palate (McClain and Rohrs, 1985) and orofacial defects (Harbison and Becker, 1974) have also been reported in Swiss-Webster and A/J mice (Massey, 1966). Open eyes, cleft palate, and various limb defects were observed in New Zealand white rabbits exposed to DPH (McClain and Langhoff, 1980). Bruckner et al. (1983) found cranial defects, abnormal body curvature, and craniofacial defects in cultured whole-ICR mice exposed to DPH. Similar malformations have been observed in the rat, however, it appears less suceptible than the mouse to the teratogenic action of DPH. DPH-induced in utero toxicity and fetal growth inhibition are similar in both species, however (Harbison and Becker, 1972). Since DPH is extensively p-hydroxylated in the rat (Butler, 1957), Harbison and Becker (1972) suggest that sensitivity may be due to species variation in the temporal arrangement of development on the genetic regulation of morphogenesis. Exposure to serum from Fischer 344 rats chronically exposed to phenytoin produced severe cardiovascular defects in cultured rat embryos (Clapper et al., 1986). Abnormalities (commonly referred to as fetal hydantoin syndrome) including craniofacial defects, nail and digital hypoplasia, and prenatal-onset growth deficiency have been found in children born to epileptic mothers treated with hydantoin anticonvulsants (Hanson and Smith, 1975).

Evaluating possible interactions between DPH or HPPH, DMSO, and the inhibitors was virtually impossible in this study due to the complexity of the exogenous MAS. However, we are presently investigating the effect of solvent interactions on the developmental toxicity of a variety of compounds with FETAX.

Since FETAX is an in vitro system providing abiotic exposure of toxicants including metabolites, establishment of precise toxicological mechanisms may be subject to simplification. The complex pharmacological and toxicological effects of DPH are not as apparent as with compounds such as CP. However, in cases where mechanistic descrepancies occur in vivo, in vitro systems may help provide information. Results of FETAX tests with DPH, the primary metabolite HPPH, and an exogenous metabolic activation system modulated by various microsomal inhibitors suggest that DPH-induced teratogenesis may be the result of both unmetabolized DPH and a highly embryotoxic arene oxide intermediate. This study further demonstrates the capacity of FETAX to determine differences in the teratogenic potential of a toxicant due to metabolism and ellucidate possible mechanisms of teratogenesis in vitro. Such results are indicative of the versatility of FETAX in the rapid screening of developmental toxicants.

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Table X. Control Data for Experiments Performed with Diphenylhydantoin (DPH) and DPH with the Metabolic Activation System (MAS).

Treatment	N	# Dead (%)	# Malformed (%)	Growth[a]
FETAX SOLUTION	320	2 (0.6)	14 (4.4)	100.0 ± 1.1
DMSO[b]	160	2 (1.3)	4 (2.6)	99.4 ± 2.2
MAS	80	1 (1.3)	3 (3.8)	98.2 ± 1.6
MAS + DMSO	80	2 (2.5)	5 (6.4)	96.8 ± 1.7
4.0 MG/ML CP[c]	80	80 (100.0)	-	-

[a] Expressed as percent of mean FETAX solution control length ± SE.

[b] 1% (v/v).

[c] Cyclophosphamide.

Table XI. Control Data for Experiments Performed with Hydroxylated Metabolites of Diphenylhydantoin (HPPH), HPPH and the Metabolic Activation System (MAS), and HPPH and the Cyclohexene Oxide-Inhibited MAS.

Treatment	[Inhibitor][a] (mM)	N	# Dead (%)	# Malformed (%)	Growth[b]
FETAX SOLUTION		480	1 (0.2)	15 (3.2)	100.0 ± 0.8
DMSO[c]		240	1 (0.4)	7 (2.9)	98.5 ± 1.5
MAS		80	2 (2.5)	1 (1.3)	96.7 ± 1.3
CYCLOHEXENE OXIDE-MAS	0.1	80	2 (2.5)	2 (2.6)	97.6 ± 1.0
MAS + DMSO[c]		80	0 (0)	3 (3.8)	95.6 ± 2.2
CYCLOHEXENE OXIDE-MAS + DMSO[c]	0.1	80	0 (0)	7 (8.8)	95.3 ± 2.3
4.0 MG/ML CP[d] + MAS		80	80 (100.0)	-	-

[a] Represents concentration in microsomal preparation. No Observable Effect Concentration (malformation) as determined by Dunnett's test.

[b] Expressed as percent of mean FETAX solution control length ± SE.

[c] 1% (v/v).

[d] Cyclophosphamide.

Table XII. Control Data for Experiments Performed with Diphenylhydantoin (DPH) and Selectively Inhibited Bioactivation System (MAS).

Treatment	[Inhibitor][a] (mM)	N	# Dead (%)	# Malformed (%)	Growth[b]
FETAX SOLUTION		640	2 (0.3)	15 (2.4)	100.0 ± 0.7
DMSO[c]		320	2 (0.6)	9 (2.8)	99.1 ± 0.9
CO-MAS		80	1 (1.3)	4 (5.1)	98.9 ± 1.0
CIMETIDINE-MAS	2.4	80	1 (1.3)	2 (2.6)	97.6 ± 0.8
ELLIPTICINE-MAS	0.04	80	0 (0)	3 (3.8)	97.9 ± 0.9
CYCLOHEXENE OXIDE-MAS	0.1	80	0 (0)	3 (3.8)	97.8 ± 0.9
CO-MAS + DMSO[c]		80	2 (2.5)	3 (3.8)	97.5 ± 0.7
CIMETIDINE-MAS + DMSO[c]	2.4	80	1 (1.3)	4 (5.1)	96.7 ± 0.7
ELLIPTICINE-MAS + DMSO[c]	0.04	80	1 (1.3)	4 (5.1)	97.5 ± 1.6
CYCLOHEXENE OXIDE-MAS + DMSO[c]	0.1	80	0 (0)	6 (7.5)	94.0 ± 0.8
4.0 MG/ML CP[a] + CO-MAS		80	3 (3.8)	6 (7.8)	95.0 ± 3.1
4.0 MG/ML CP[a] + CIMETIDINE-MAS	2.4	80	10 (12.5)	18 (25.7)	92.8 ± 1.0
4.0 MG/ML CP[a] + ELLIPTICINE-MAS	0.04	80	77 (96.3)	3 (100.0)	82.5 ± 4.2
4.0 MG/ML CP[a] + CYCLOHEXENE OXIDE-MAS	0.1	80	79 (98.7)	1 (100.0)	41.2

[a] Represents concentration in microsomal preparation. No Observable Effect Concentration (malformation) as determined by Dunnett's test.

[b] Expressed as percent of mean FETAX solution control length ± SE.

[c] 1% (v/v).

[d] Cyclophosphamide.

Table XIII. Effect of Exogenous Bioactivation System (MAS) on the Developmental Toxicity of Diphenylhydantoin (DPH) and Primary Metabolites (HPPH).

Treatment	[Inhibitor][a] (mM)	Trial	Unactivated			MAS		
			96 h LC50[b] (mg/L)	96 h EC50[b] (mg/L)	TI[c]	96 h LC50[b] (mg/L)	96 h EC50[b] (mg/L)	TI[c]
DPH		1	77.9 (70.9-85.7)	33.0 (28.6-38.1)	2.4	122.1[d] (90.2-165.2)	62.9[d] (52.6-75.3)	1.9
		2	71.0 (62.4-80.9)	31.8 (27.9-36.4)	2.2	130.6[d] (114.4-149.1)	75.4[d] (66.0-86.1)	1.8
p-HPPH		1	>150.0[e]	>150.0[e]	-	>150.0[ef]	>150.0[ef]	-
		2	>150.0[e]	>150.0[e]	-	>150.0[eg]	>150.0[eg]	-
m-HPPH		1	>150.0[e]	>150.0[e]	-	>150.0[ef]	>150.0[de]	-
		2	>150.0[e]	>150.0[e]	-	>150.0[eg]	>150.0[eg]	-
CO-MAS + DPH		1	-	-	-	69.3[hi] (60.5-79.3)	30.6[hi] (26.5-35.3)	2.3
		2	-	-	-	68.8[hi] (60.8-77.9)	33.7[hi] (27.5-41.2)	2.0
CIMETIDINE-MAS+DPH	2.4	1	-	-	-	83.7[hi] (72.4-96.8)	36.0[hi] (31.3-41.3)	2.3
		2	-	-	-	78.8[hi] (68.7-90.4)	41.8[hi] (37.0-47.2)	1.9
ELLIPTICINE-MAS+DPH	0.04	1	-	-	-	98.6[dj] (93.3-104.2)	59.2[dj] (54.7-64.0)	1.7
		2	-	-	-	95.7[dj] (87.9-104.2)	53.4[dj] (49.4-57.9)	1.8
CYCLOHEXENE OXIDE-MAS +DPH	0.1	1	-	-	-	31.5[di] (25.0-39.6)	24.7[hi] (18.9-32.4)	1.3
		2	-	-	-	45.6[di] (41.7-49.9)	39.7[hi] (34.4-45.9)	1.1

[a] Represents concentration in microsomal preparation. No Observable Effect Concentration (malformation) as determined by Dunnett's test.

[b] Determined by Litchfield-Wilcoxon probit analysis (Tallarida and Murray, 1980) with 95% confidence interval in parenthesis.

[c] 96-hr LC50/96-hr EC50 (malformation).

[d] Significantly different from DPH at P=0.05.

[e] Limit of solubility in 1% (v/v) DMSO.

[f] Performed with standard MAS. Summarization of results from two definitive experiments.

[g] Performed with cyclohexene oxide-MAS. Summarization of results from two definitive experiments.

[h] Not significantly different from DPH at P=0.05.

[i] Significantly different from DPH and the MAS at P=0.05.

[j] Not significantly different from DPH and the MAS at P=0.05.

Table XIV. Terata Induced in *Xenopus* by exposure to DPH and HPPH.

Treatment	Concentration (mg/L)[a]	Terata Induced
DPH	>20	gut miscoiling, pericardial edema and craniofacial defects
	>40	microencephaly, microophthalmia and incomplete mouth development
	>60	heart miscoiling (tubular heart) and muscular kinking[b]
DPH + MAS	>33	gut miscoiling and hypognathia
	>66	muscular kinking[b]
	>100	ophthalmic edema, microophthalmia, craniofacial defects, skeletal kinking and lateral body flexure
HPPH[c]	>100	muscular kinking[b] and lateral body flexure
CO-MAS + DPH	>20	gut miscoiling, mouth anomalies and craniofacial defects
	>50	pericardial edema and muscular kinking
	>80	heart miscoiling and muscular kinking[b]
CIMETIDINE-MAS + DPH	>20	gut miscoiling
	>40	pericardial edema, microophthalmia and craniofacial defects
	>70	muscular[b] and skeletal kinking, microencephaly, mouth defects
ELLIPTICINE-MAS + DPH	>50	gut miscoiling
	>70	microphthalmia, skeletal kinking and body flexure abnormalities
	>100	ophthalmic edema and microencephaly
CYCLOHEXENE OXIDE-MAS + DPH	>5	gut miscoiling
	>10	severe skin blistering
	>20	skeletal and muscular kinking[b], craniofacial defects, pericardial edema and microophthalmia
	>50	microencephaly and hydrocephalus

[a] Defines concentration range for teratogenicity.

[b] Term muscular kinking, caused most likely by abnormal somite development, is used to differentiate from that finding termed skeletal kinking in which the spine (including the notocord) is affected.

[c] Treatments with HPPH and MAS or cyclohexene oxide-MAS produced similar malformations at the given concentrations.

FIGURE LEGEND

FIGURE 6 - Probable route of diphenylhydantoin metabolism in mammals.

FIGURE 7 - Malformations and growth reduction caused by 96-hr exposure to diphenylhydantoin (DPH). FETAX solution control embryo (top), embryo exposed to 50 mg/L DPH alone (middle), and embryo exposed to 100 mg/L DPH with the MAS (bottom).

FIGURE 8 - Representative growth curves, presented as the percent of the mean FETAX solution controls + SE, for Xenopus embryos exposed to diphenylhydantoin (DPH) (——), DPH with MAS (—··—), 5-(p-hydroxyphenyl)-5-phenylhydantoin (—·—) and 5-(m-hydroxyphenyl)-5-phenylhydantoin (– – –) for 96 hr.

FIGURE 9 - Representative growth curves, presented as the percent of the mean FETAX solution control + SE, for Xenopus embryos exposed to diphenylhydantoin (DPH) (– – –), DPH and carbon monoxide-inhibited MAS (—··—), DPH and cimetidine-inhibited MAS (—·—), DPH and ellipticine-inhibited MAS (·····) and DPH and cyclohexene oxide-inhibited MAS (——) for 96 hr.

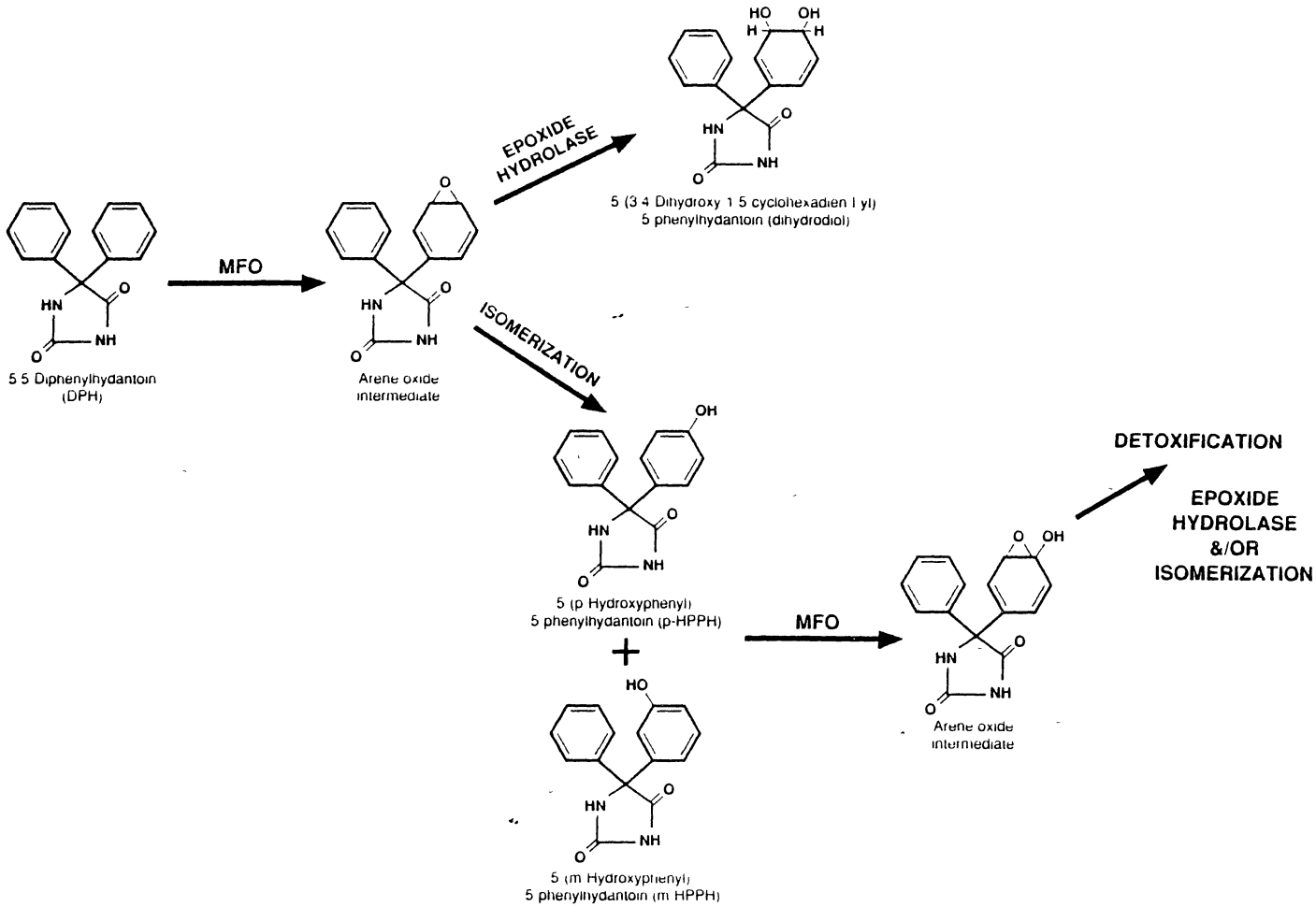
5 5 Diphenylhydantoin
(DPH)
MFO
Arene oxide
intermediate
EPOXIDE
HYDROLASE
5 (3 4 Dihydroxy 1 5 cyclohexadien 1 yl)
5 phenylhydantoin (dihydrodiol)
ISOMERIZATION
5 (p Hydroxyphenyl)
5 phenylhydantoin (p-HPPH)
+
5 (m Hydroxyphenyl)
5 phenylhydantoin (m HPPH)
MFO
Arene oxide
intermediate
DETOXIFICATION
EPOXIDE
HYDROLASE
&/OR
ISOMERIZATION

Figure 6

Figure 7

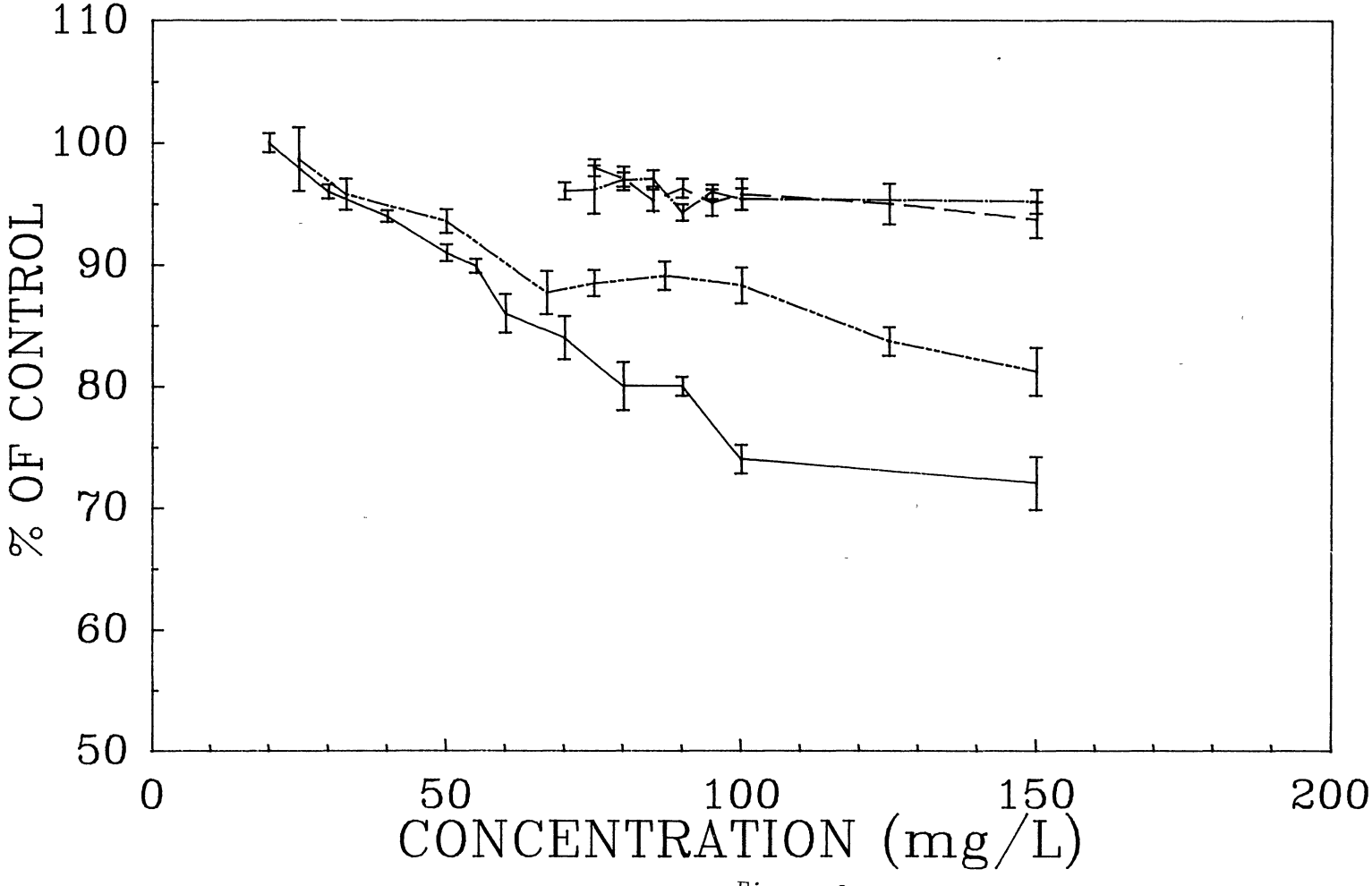
110
100
90
80
70
60
50
% OF CONTROL
0
50
100
150
200
CONCENTRATION (mg/L)

Figure 8

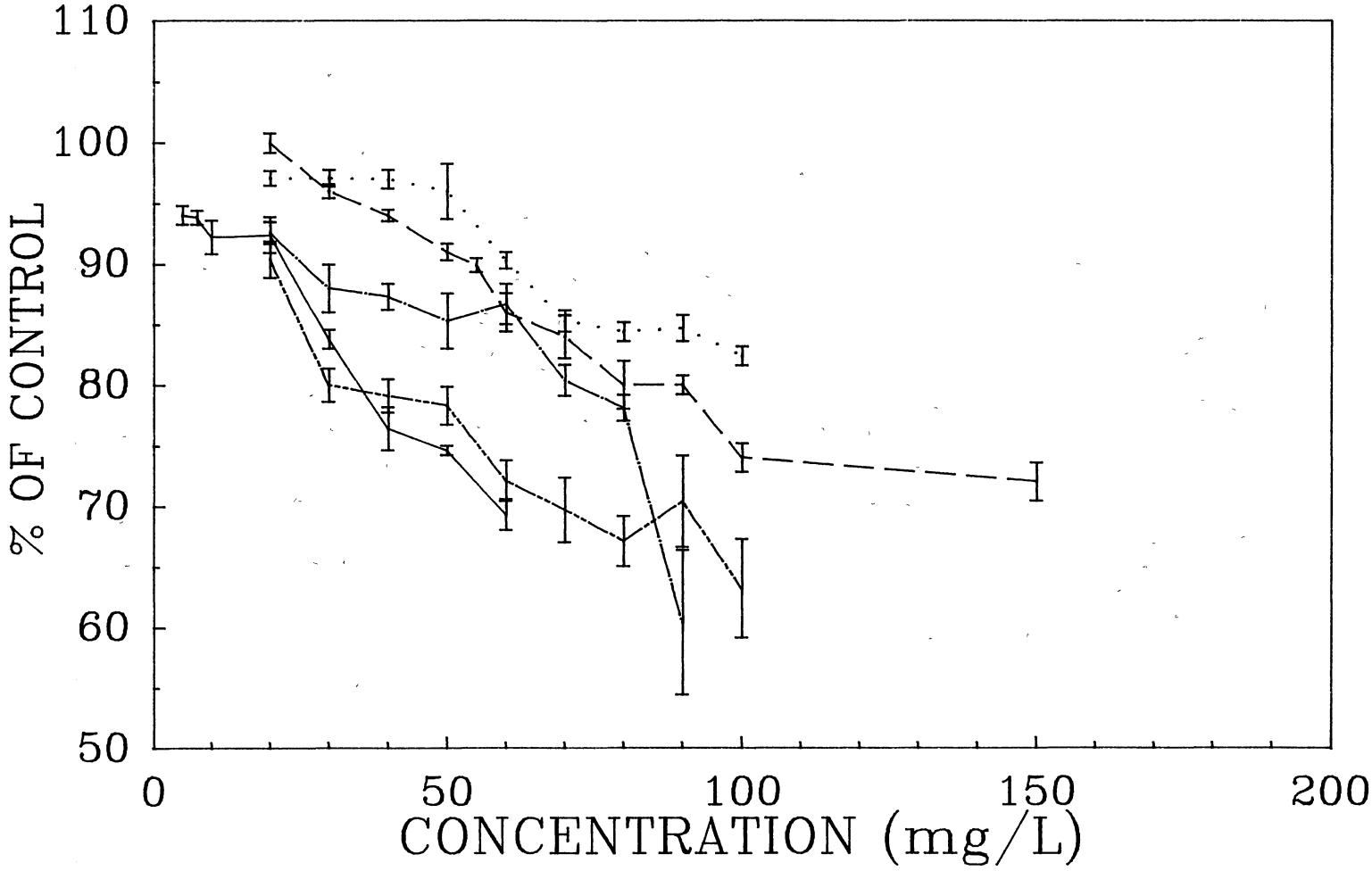
110
100
90
80
70
60
50
% OF CONTROL
0
50
100
150
200
CONCENTRATION (mg/L)

Figure 9

CHAPTER V

CONCLUSIONS

The primary objective of this research project was to combine the evaluation of the metabolic activation system with creative applications of the system. In an effort to validate the Aroclor 1254-induced metabolic activation system on a preliminary basis, the developmental toxicity of four compounds thought to be modulated by cytochrome P-450-mediated metabolism and one compound unaffected by the P-450 system were tested. With the inclusion of the exogenous metabolic activation system, FETAX correctly predicted the teratogenicity of each compound. However, without the bioactivation system FETAX only correctly predicted the developmental toxicity of two of the five compounds. Thus, by implementing a MAS into the FETAX protocol, the predictive value of the assay may be increased by decreasing the number of potential false-positive and false-negative test results.

The developmental toxicity of two compounds, isoniazid and acetylhydrazide, metabolized by Aroclor 1254-repressed P-450 isozymes (P-450j) was assessed in an effort to evaluate the overall efficacy of Aroclor 1254 induction. The Aroclor 1254-induced metabolic activation system had no

significant effect on the developmental toxicity of either compound. However, an isoniazid-induced bioactivation system significantly altered the developmental toxicity of isoniazid producing a more embryolethal, but less teratogenic species. Several chemicals have been shown to be metabolized by P-450j including, pyrazole, imidizole, acetone, trochloroethylene, N-nitrosodimethylamine, and ethanol. Thus, Aroclor 1254-induced liver microsomes may prove to be counter-productive for in vitro developmental toxicity screening systems in these situations. To increase the predictability of the metabolic activation system routinely used by FETAX, mixtures of microsomes induced by a broad-spectrum of P-450 inducing agents are currently being tested. In addition, these results suggest that false-positive test results obtained with isoniazid using several in vitro test systems may be the result of metabolic incompetence.

To demonstrate the utility and versatility of FETAX and the exogenous metabolic activation system, the developmental toxicity of diphenylhydantoin was evaluated with a bioactivation system modulated by various metabolic inhibitors to identify toxicological mechanisms of teratogenesis. Results suggest that the parent compound and a highly embryotoxic arene oxide intermediate were responsible for diphenylhydantoin-induced developmental toxicity in FETAX.

Without the ability to metabolize xenobiotics, it is

doubtful that in vitro tests, including FETAX, would be suitable for screening potential human developmental toxicants. Results from this research suggest that FETAX may be able to overcome the obstacle of maternal metabolism. Since FETAX is an in vitro system, providing abiotic exposure of toxicants and metabolites, the pharmacological relationship between the fetal and maternal systems has been oversimplified. However, FETAX provides an opportunity to compare the developmental toxicity of parent compounds to metabolites and possibly generate information concerning toxicological mechanisms of teratogenesis.

VITA

Douglas James Fort

Candidate for the Degree of

Doctor of Philosophy

Thesis: DEVELOPMENTAL TOXCITY TESTING WITH THE FROG EMBRYO TERATOGENESIS ASSAY: XENOPUS (FETAX) AND AN EXOGENOUS METABOLIC ACTIVATION SYSTEM: EVALUATION AND APPLICATIONS

Major Field: Zoology

Biographical:

Personal Data: Born in Denver, Colorado, June 9, 1964, the son of Donald J. and Sandra S. Fort.

Education: Graduated from Dodge City Senior High School, Dodge City, Kansas, in May 1982; received Bachelor of Science Degree in Biology-Chemistry from Southwestern College, Winfield, Kansas, in May 1986; received Master of Science Degree in Zoology from Oklahoma State University, Stillwater, Oklahoma, in July 1988; completed requirements for the Doctor of Philosophy Degree at Oklahoma State University in December, 1990.

Professional Experience: Teaching Assistant, Department of Zoology, Oklahoma State University, August 1986, to December 1988; Research Fellow in Water Resources, Oklahoma State University, January 1988 to present.