# DEVELOPMENT OF A METABOLIC ACTIVATION SYSTEM FOR THE FROG EMBRYO TERATOGENESIS ASSAY: XENOPUS (FETAX)

By

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DEVELOPMENT OF A METABOLIC ACTIVATION

SYSTEM FOR THE FROG EMBRYO

TERATOGENESIS ASSAY:

XENOPUS

(FETAX)

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# PREFACE

The purpose of this study was to develop a metabolic activation system for the Frog Embryo Teratogenesis Assay: Xenopus (FETAX). Metabolism is a significant obstacle for most in vitro developmental toxicity assays and must not be overlooked in a proper screening test. Such a system when incorporated into the FETAX protocol will help eliminate potential false negative and false positive test results. In addition, it will provide meaningful insight into the effect of maternal metabolism on the developmental toxicity of compounds in the environment and workplace.

This thesis is divided into four chapters. An introduction to this work is presented in Chapter I, with a review of pertinent literature in Chapter II. Chapter III is a complete manuscript prepared and submitted for publication to the journal, Teratogenesis, Carcinogenesis, and Mutagenesis. The development of a metabolic activation system consisting of Aroclor 1254 induced rat liver microsomes is presented in Chapter III. Chapter IV describes preliminary validation of the metabolic activation system.

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# CHAPTER I

# INTRODUCTION

## Statement of Problem

Approximately 70,000-90,000 chemicals are currently available in the marketplace with some 600 new chemicals released each year. Prior to their release into the marketplace and ultimately the environment, the safety of these chemicals must be firmly established. The Environmental Protection Agency (EPA), under the Toxic Substance Control Act (TOSCA) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), has regulatory responsibility for the majority of these chemicals. Because of the large number of interactions possible when these chemicals are present in complex mixtures, *in vivo* assays employing mammals are not practical. Changing perceptions among the scientific, regulatory, and industrial communities on the issue of alternate methods were recently documented by the EPA's Office of Policy, Planning, and Evaluation (1). Of interest to the proposed study, the need for routine teratogenicity testing has led to the development of a number of *in vitro* teratogenesis assays that may prove useful in

prioritizing compounds for further testing (2-7). The use of the *in vitro* systems was identified in developmental effects test guidelines published by the EPA (8). In 1983, Dumont and coworkers (9) developed the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) and applied it to screening complex environmental mixtures as well as pure compounds. However, early *Xenopus* embryos do not have a functioning mixed-functional oxidase system (MFO). Many compounds that require metabolic activation in order to be teratogenic would presently be falsely scored as negative in the FETAX. Compounds that are deactivated by the MFO would be incorrectly scored as positives. The most straightforward approach to solving this deficiency is to provide an exogenous metabolic activation system (MAS). Once provided, the utility of the assay must be established with appropriate validation compounds. The purpose of this study was to develop a metabolic activation system for FETAX using Aroclor 1254 induced rat liver microsomes. This system when routinely used with FETAX may reduce the number of false positive and negative test results.

## Objectives

FETAX presently meets most of the criteria set forth by Kimmel et al. (10) for *in vitro* teratogenesis assay validation (9,11,12). Mortality, malformation, and

growth endpoints are easily quantifiable, generally exhibiting a dose-response relationship with the development of narrow confidence limits. Since many of the stages of the amphibian development are similar to mammalian development, the "developmental relevance" of FETAX is more significant (13) than many of the other teratogenesis assays available. Presently, FETAX lacks a MAS. Without such a system, it is doubtful that FETAX would be commonly used by research laboratories or approved for use by regulatory agencies. Because of this, an effective and efficient *in vitro* MAS will be developed using Aroclor 1254 induced rat liver microsomes.

## 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 (10). 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 (22) and a cultured whole rat embryo screen (23) employ rat liver S-9 supernatant. *Drosophila* have been successfully cultured with both *Drosophila* and

rat liver S-27 and S-9 supernatant (24). In addition, cultured mouse erythrocytes have been used to mediate the the metabolic activation of cyclophosphamide in the Saccharomyces cerevisiae mutagenicity test (25).

Noshiro and Omura (26) 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 (7). 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. (14) 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 risk of a given substance, a teratogenicity index (TI) was calculated by dividing the 96 h LC50 (median lethal concentration) by the EC50 (malformation) (median teratogenic concentration) (14). Compounds with TI values greater than 1.5 are considered to have strong teratogenic potential. Subsequently, several laboratories (11,12,13) have used FETAX for the screening of potential developmental toxicants.

## Design of the Metabolic Activation System

Rat liver microsomes were isolated from adult male Sprague-Dawley rats treated with 500 mg/kg Aroclor 1254 according to a modified procedure of Kitchin and Woods (15). Following isolation, purified microsome aliquots were immediately frozen in liquid nitrogen (16). Protein was determined by the method of Bradford (17). P-450 activity was inferred by the formaldehyde generated from the N-demethylation of aminopyrine by the method of Nash (18). Assay conditions were described by Lucier et al. (19). Several aliquots of microsomes were pretreated with carbon monoxide (CO) to selectively inactivate cytochrome P-450 (P-450) activity.

## Incorporation into FETAX Protocol

Initial tests were conducted to determine the most suitable concentration of microsomal protein, NADPH generating system, and antibiotics using sublethal and subteratogenic concentrations of cyclophosphamide. For each clutch of embryos, four sets of 20 embryos were exposed to reconstituted water (FETAX solution) (20) and penicillin-streptomycin. These were designated FETAX controls. Treatments were performed in duplicate with 20 embryos per dish. Each test dish received microsomal protein, NADPH generating system, penicillin-streptomycin, and various concentrations of toxicant. Metabolic activation system, unactivated toxicant, and CO-gassed MAS controls plus toxicant were performed in addition. All solutions were removed every 24 hours of the test and new solutions added in a static-renewal fashion. The number of live-malformed embryos at 96 hours and the stage of development according to Nieuwkoop and Faber (21) were determined. Dose-response assays evaluated according to Litchfield-Wilcoxon were used to determine the 96 hour LC50 and EC50 (malformation). The head-tail length was then measured as an additional endpoint.

## The Use of Cyclophosphamide

Cyclophosphamide is a consensus compound listed

by Smith et al. (13) for use in validating *in vitro* teratogenesis assays. *In vitro* rodent embryo culture has demonstrated that cyclophosphamide must be bioactivated to be teratogenic (23,27). The metabolism of cyclophosphamide, mutagenic effects, and teratogenic potential have been thoroughly investigated, making it an excellent compound for the development of such a system. In addition, cyclophosphamide is soluble in water, thus eliminating the need for a carrier solvent.

## Alternate Metabolic Activation Systems

### *Cultured Hepatocytes*

One alternative is the use of *Xenopus* hepatocytes in a coculture with *Xenopus* embryos. One advantage of using cultured hepatocytes as a metabolic activation system would be the continuous production of the active metabolite. However, *Xenopus* hepatocytes have far less inducible P-450 than do rat liver microsomes, while rat liver microsomes have a similar basal level of P-450 activity to human (28). Rat liver hepatocytes cannot be used because of the osmotic differences between amphibian and mammalian cells. Cell culture is far more expensive and technically difficult than the use of rat liver microsomes. Furthermore, there are problems with plating the exact number of hepatocytes so that bioactivation is consistent and the test is repeatable.

## Whole-Liver Perfusion

Isolated adult *Xenopus* liver perfusion has also been considered as a possible method of converting proteratogens to teratogens. The perfusate containing the active metabolites would flow through culture dishes containing developing embryos. Again, an advantage of using liver perfusion would be the continuous generation of the active metabolite. The limited amount of *Xenopus* P-450 poses a significant disadvantage. This system is significantly more complex and expensive than an *in vitro* rat liver microsome/embryo coculture system. Inducing *Xenopus* liver P-450 with Aroclor 1254 offers no advantage, as reducing residual Aroclor 1254 exposed to the developing embryos would be technically difficult. Obviously, every *in vitro* metabolic activation system has advantages and disadvantages and no system has been universally accepted that simulates human metabolism. However, a metabolic activation system employing Aroclor 1254 induced rat liver microsomes appears to be the most straightforward approach. Implementing such a system into the FETAX protocol will increase the predictive value of the assay and increase the overall acceptance of FETAX as developmental toxicity screen.

# CHAPTER II

# LITERATURE REVIEW

## *In Vitro* Teratogenicity Testing

### Aspects of Successful In Vitro Assays

*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 10 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 (55). 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 (56-57). Sabourin and Falk (58) 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 (11) found the teratogenic index of hydroxyurea to be 4.3 which is very similar to 4.5 obtained by Sabourin et al. (12). 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 teratogenicity index varied significantly. FETAX (12) was found to be the most appropriate assay when compared to the Planaria (3) and the Hydra system (59). These results may be attributed to the multiple endpoints of the frog assay as well as its higher phylogenetic position. Dumont et al. (9) found meclizine induced hydrocephalia in both frogs and mammals. Courchesne and Bantle (11) 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 (20).

## The Role of P-450 in Xenobiotic Metabolism

### Importance of Xenobiotic Metabolism

Living organisms are exposed to a wide variety of substances, some necessary for the maintenance of life, some of medicinal value, and others of no functional value. The latter two classes are commonly called xenobiotics, or those compounds which are foreign to an organism. Whether a compound is a xenobiotic or not is species dependent since a compound which is foreign for one species may be essential for another. Anstrom and DePierre (29) suggest that xenobiotics have been present

on earth for $10^9$ years, formed either by natural processes or, more recently and ever increasing, by waste products of civilization.

Organisms may handle the problem of exposure to xenobiotics by two fundamental methods: either by preventing them from entering the body, and/or excreting them rapidly (29). Since highly evolved biological membranes provide no permeability barrier for hydrophobic compounds and since the vast majority of xenobiotics are hydrophobic, the evolution of systems for excretion has been fundamental to life. Water-soluble (lipophobic) chemicals may be easily excreted through the urine and bile. Lipid-soluble (lipophilic) substances are often excreted slowly and tend to remain in the body for long periods of time. This facilitates accumulation to potentially toxic concentrations. Therefore, the major goal of xenobiotic metabolism is to render lipophilic compounds more water-soluble so that they may more readily be excreted.

Since xenobiotics often have no structurally similar endogenous counterparts, unlike many pharmaceutical agents, the evolution of unspecific xenobiotic biotransforming enzymes was necessary. Consistent with the evolutionary trend of organ specialization, the highest activities of drug-metabolizing enzymes is found in the liver.

## The P-450 System

Xenobiotic metabolism can be generally divided into three steps: 1) activation of the compound; 2) inactivation (usually) of reactive groups by hydrolysis or conjugation with glutathione; and 3) conjugation with other hydrophilic moieties, such as glucuronic acid, sulfate, or acetate to make the compound more water-soluble (29). P-450 is involved in the first process called phase I metabolism. The latter conjugation steps are designated as phase II processes.

The P-450 system, also known as the mixed-functional oxidase system (MFO), has been found in different levels and forms in virtually all species (30-32). Within the cell, P-450 activity is chiefly located on the endoplasmic reticulum (30-32) but has been found in the nuclear membrane (33,34), and mitochondria (32,35). This system in microsomes consists of two proteins: the flavoprotein NADPH-P-450 reductase which reduces P-450 by a two electron transfer mechanism and cytochrome $b_5$ (36,37). The second electron may be transfered from cytochrome $b_5$ (38-41). P-450 usually catalyzes the insertion of one oxygen atom from molecular oxygen into the substrate, while the other is used in the formation of water. P-450 when reduced and bound to carbon monoxide exhibits a difference spectrum with a characteristic maximum at approximately 450 nm (42).

The broad substrate specificity of P-450 gives rise to numerous phase I reactions, including aromatic and aliphatic hydroxylation, epoxidation, N-demethylation, O-dealkylation, and N and S-oxidation (43). It should also be mentioned that P-450 is involved in the metabolism of several endogenous substrates, most notably steroids, fatty acids, and prostaglandins (44-47).

### Bioactivation

The metabolism of xenobiotics is not always a one-way street. Often the system gives rise to products more toxic than the parent compounds in the process. Reactive nucleophilic products formed during drug metabolism can react with electrophilic groups in the cell, usually localized in proteins, RNA and DNA (29). Such reactions may give rise to toxic and/or genotoxic effects, such as mutagenesis, carcinogenesis, and teratogenesis.

## Induction of P-450 Isozymes

### The Effect of Inducing Agents

In essense, ten major cytochrome P-450 isozymes from rat liver microsomes have been isolated and designated P-450a through P-450j (28). Immunochemical quantification of hepatic microsomes from rats treated with three inducers such as phenobarbital (PB), 3-methylcholanthrene (3-MC), and Aroclor 1254 (mixture of polychlorinated

biphenyls) have been recently studied (48-52). A 4-day treatment of rats with PB caused a 39-fold increase in P-450b and a doubling of P-450a with no change observed in the other isozymes. Treatment of rats with 3-MC caused a 71- to 74-fold increase in the amount of P-450c, a 10 fold increase in P-450d, and a 3- to 4-fold increase in P-450a, with no change in the other isozymes. Treatment of rats with Aroclor 1254 caused a 44 to 49 fold increase in the concentrations of P-450b and P-450c, a 19 fold increase in P-450d, and a 3 fold increase in P-450a. Although Aroclor 1254 greatly increases the quantity of several cytochrome P-450 isozymes, a few other isozymes may be repressed (50,51).

Types of Inducers

3-MC-type inducers are thought to bind to a cytosolic receptor that translocates into the nucleus and induces the synthesis of mRNA for a subset of P-450 isozymes (53). PB-type inducers, on the other hand, are mediated by a less direct process. Ortiz de Montellano and Costa (54) suggest that a receptor exists for an endogenous substance that is specifically turned over by the trace amounts of P-450$_b$ present in the uninduced liver. Changes in the concentration of this substance caused by the substances that inhibit P-450b would then trigger the induction response. PCBs are belived to display characteristics of both mechanisms.

## Metabolism and Teratogenicity of Cyclophosphamide

### Activation of Cyclophosphamide

Cyclophosphamide is one of the best studied teratogenic and antineoplastic agents (see 60 for a review). Activation of cyclophosphamide to its active teratogenic form is mediated by microsomal P-450 monooxygenases in which carbon-4 of the ring is oxidized to form 4-hydroxycyclophosphamide. This initial metabolite subsequently undergoes ring opening to form the amino-aldehyde aldophosphamide such that 4-hydroxycyclophosphamide is in equilibrium with the aldophosphamide. Two further metabolic fates are possible. One involves oxidation by soluble enzymes to yield 4-ketocyclophosphamide and carboxyphosphamide. This process constitutes the major detoxification pathway since neither of these two metabolites have cytotoxic activity and represent the major urinary excretory products of cyclophosphamide. The alternate pathway involves the spontaneous breakdown of 4-hydroxycyclophosphamide/aldophosphamide to yield phosphoramide mustard and acrolein. Phosphoramide mustard is the metabolite believed to be responsible for the teratogenic/mutagenic effects of cyclophosphamide, however, acrolein may also play a role.

## Target Molecules

Cyclophosphamide belongs to a class of chemical mutagens known as alkylating agents. Alkylating agents, in general, and cyclophosphamide, specifically, are capable of alkylating a variety of macromolecules including DNA, RNA, and proteins. It is generally assumed, however, that DNA molecules constitute the critical target in terms of cytotoxicity. Several lines of evidence suggest this assumption. First, cytotoxic levels of cyclophosphamide inhibit DNA synthesis (61,62) and this inhibition seems to be due to damage of the DNA template (63-67). Second, DNA contains electron-rich targets for alkylation such as nitrogen and oxygen atoms of the nirogenous bases, as well as the phosphoryl oxygens and sugar-phosphate backbone, all of which have been shown to be alkylated by a variety of agents (68). Mehta et al. (69) have shown that phosphoramide mustard readily alkylates the N-7 position of guanosine and deoxyguanosine. Third, studies have shown that DNA cross-linking by bifunctional alkylating agents correlates with cytotoxicity and that drug-resistant cells do not exhibit cross-linked DNA. Finally, cyclophosphamide is also a carcinogen and a mutagen, and the critical target for these processes is DNA.

## Developmental Anomalies

Cyclophosphamide primarily produces central nervous system and skeletal anomalies in rats, mice, rabbits, monkeys, and humans. Intraperitoneal injection of CP into Wistar and S/D rats produced fetuses characterized by brain malformations (anencephaly, exencephaly, and microencephaly), deformed palate and lip, open eyes, and defective limbs (retarded, club, ectrodactylic, syndactylic, and polydactylic) (70-72). Deformities reported in mice (73-74), rabbits (75), and monkeys (76) were similar to those found in rats and included kinky tail. The effects of metabolically activated CP on cultured whole rat embryos included abnormal brain development, hypoplasia of the mandibular arches, limb buds, and tail (77). Decreased growth was also noted (77).

CHAPTER III

# DEVELOPMENT OF A METABOLIC ACTIVATION SYSTEM FOR THE FROG EMBRYO TERATOGENESIS ASSAY: *XENOPUS* (FETAX)

by

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Running Head: Teratogenic action of bioactivated cyclophosphamide on *Xenopus* embryos

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ABSTRACT

FETAX (Frog Embryo Teratogenesis Assay: Xenopus) is a 96 h teratogenesis screening assay using embryos of the South African clawed frog, Xenopus laevis. Since Xenopus embryos have limited xenobiotic metabolism through 96 hours of development, we have developed an in vitro metabolic activation system employing Aroclor 1254 induced rat liver microsomes. By adding an exogenous source of mixed functional oxidase (MFO) activity, we may more accurately assess the teratogenic risk of proteratogenic compounds. Xenopus embryos were cocultured with varying concentrations of cyclophosphamide (CP), Aroclor 1254 induced microsomal protein, NADPH generating system, and antibiotics in a static renewal fashion for 96 hours. Residual Aroclor 1254 remaining in the microsomes was successfully reduced during purification to levels which had no significant effect on embryo survival and development. The results of three definitive dose-response tests performed with CP revealed that activation reduced the 96 h LC50 from 8.0 to 1.4 mg/ml (5.7 fold). The 96 h EC50 (malformation) was reduced from 6.2 to 0.4 mg/ml (15.5 fold). Activation also increased the types and severity of malformation, and reduced embryonic growth. Aroclor 1254 induced rat liver microsomes may be used as an acceptable in vitro metabolic activation system for FETAX.

Key Words: metabolic activation, cyclophosphamide, FETAX, proteratogen, Xenopus

# INTRODUCTION

*Xenopus* embryos have been used to determine the teratogenic and toxic effects of complex environmental mixtures and pure compounds (1-7). The Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) has been routinely used by several laboratories (8-13) since its development by Dumont and coworkers (7).

In its present form, FETAX complies with most of the criteria required for *in vitro* teratogenesis assay validation (14). However, early *Xenopus* embryos have little or no ability to biotransform proteratogenic compounds to teratogenic metabolites via the microsomal mixed-functional oxidase system (MFO). Bantle and Dawson (15) recently have employed uninduced rat liver microsomes as an exogenous metabolic activation system for FETAX based on that of Kitchin and Woods (16) using cultured whole rat embryos. Such an activation system should increase the predictive accuracy of FETAX so that it can be used to detect human developmental toxicants. We have continued the development of an exogenous metabolic activation system for FETAX by using an Aroclor 1254 induced rat liver microsomal activating/embryo coculture system with cyclophosphamide (CP). CP alone is neither antineoplastic nor mutagenic without metabolic activation (17). Since CP is one of the best studied proteratogens, it is an excellent candidate compound for

the development of such a system. Smith et al. (18) listed CP as a consensus compound for use in the validation of *in vitro* teratogenesis assays.

This report describes the successful use of Aroclor 1254 induced rat liver microsomes as an *in vitro* metabolic activation system for FETAX. A marked decrease in 96 h LC50 and EC50 (malformation) and the identification of 4-hydroxycyclophosphamide/aldophosphamide (4-OHCP/AP), a cytochrome P-450 (P-450) catalyzed metabolite of CP, indicated that cyclophosphamide was successfully metabolized to its actively teratogenic forms. Standardization of the P-450 content supplied to each CP treatment using aminopyrine N-demethylase activity as a basis allowed for repetitive dose-response curves. Less than 120 ng/ml residual Aroclor 1254 was present in the culture dishes. These concentrations did not affect *Xenopus* embryo development nor did they potentiate CP toxicity. The employment of an enhanced metabolic activation system protocol should greatly increase the predictive value and the overall acceptance of FETAX as an *in vitro* screening assay.

## MATERIALS AND METHODS

### Chemicals and Biochemicals

All chemicals used in rat liver microsome preparation and FETAX were purchased from Sigma (St. Louis, MO.). Reagents used in Aroclor 1254 analysis were purchased from Burdick and Jackson (Muskegon, MI.). Aroclor 1254 was obtained from Monsanto (St. Louis, MO.).

### Rat Liver Microsome Preparation

Five days prior to microsome preparation, an adult Sprague-Dawley (S/D) C/D strain male rat (200-280 g) was injected with 500 mg/Kg Aroclor 1254 in corn oil (19). A modified procedure for microsomal isolation described by Kitchin and Woods (16) was followed. The 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 (20). Protein was determined by the method of Bradford (21) (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 (22). Assay conditions were described by Lucier et al. (23). Several aliquots of microsomes were pretreated with carbon monoxide (CO) to selectively inactivate P-450 activity (15).

Animal Care and Breeding

*Xenopus* adult care, breeding, and embryo collection was performed according to Courchesne and Bantle (8).

Assay Protocol

Initial tests were conducted to optimize the cytochrome P-450 activity and determine the most suitable concentration of microsomal protein, NADPH generating system, and antibiotics using sublethal and subteratogenic levels of unactivated CP. For each separate clutch of embryos, four sets of 20 were placed in 60 mm covered plastic Petri dishes containing FETAX solution 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 CP. 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, unactivated CP controls, and CO-gased microsomes controls 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 (24) were determined. Dose-response bioassays evaluated according to Litchfield-Wilcoxon (25) were used to determine the 96 h LC50 and EC50 (malformation). The 96 h LC50 was divided by the EC50 (malformation) yielding a teratogenicity index (TI) which has proven useful in assessing teratogenic risk (7,12). Head-tail length was measured using a Radio Shack digitizer and model 16 microcomputer.

Detection of Residual Aroclor 1254

Residual Aroclor 1254 remaining in the microsomal fraction was determined by gas-liquid chromatography (GLC) in accordance with standard methods (26). Three successive methylene chloride extractions were performed

on samples of purified microsomes. 1 ml of isooctane was added and the samples concentrated to 500 ul in a tube heater. Analysis of the extracts was performed with a Tracor model 560 gas chromatograph operated under the following conditions:

Column: 5% OV-1 on 80/100 Supelcoport (Supelco, Bellefonte, Pa) 6' x 0.25'' ID glass packed

Detector: $^{63}$Ni Electron Capture

Carrier Gas: Argon-Methane (95%:5%) flowing 45 ml/min

Temperatures ($^{o}$C): Oven-250

Injection Port-255

Detector-240

Integratator: Hewlett Packard model 3390A

Sample peak retention times were compared to Aroclor 1254 reference standards. Identification of Aroclor 1254 was determined by the presence of five major peaks. Three differing concentrations of Aroclor 1254 standards were analyzed in triplicate. Samples were analyzed in duplicate. Environmental Protection Agency (EPA) quality control standards were performed to confirm the accuracy of quantification. Concentration of Aroclor 1254 in extracts was computed by regression analysis. Efficiency of extraction was determined by spiking uninduced microsomal preparations with known quantities of Aroclor 1254.

Detection of CP Metabolism

Qualitative analysis of CP metabolism was determined

by GLC. The following treatments were prepared in Petri dishes and incubated at 24 °C similar to those subjected to the embryos: 4.0 mg/ml CP, MAS control, CO-gassed MAS and 4.0 mg/ml CP, and 4.0 mg/ml induced microsome activated CP. At 0, 0.5, 3, 6, and 24 h the incubations were stirred, a sample removed, and immediately injected onto a Tenax GC (60/80) 6' x 0.25'' ID glass packed column (Applied Science Laboratory, State College, Pa.) under the following conditions:

Detector: Flame Ionization Detector

Carrier Gas: Helium flowing 22 ml/min

Air flow: 400 ml/min

Hydrogen gas flow: 20 ml/min

Temperatures (°C): Oven-200

Injection Port-240

Detector-250

Retention times of the samples relative to the water solvent were then compared to a 4-OOHCP standard which spontaneously hydrolyzed to 4-OHCP, the primary metabolite of CP. No quantitative assessment was sought.

# RESULTS

## Optimization of the Metabolic Activation System

Preliminary optimization experiments were conducted to maximize CP bioactivation without increasing the metabolic activation system components to toxic levels. Tests were performed to determine the optimum concentration of aminopyrine N-demethylase (APD) activity added to each CP treatment. The optimum level of APD activity was determined to be 0.04 U/ml which caused signigicant malformation, but only moderate mortality. Subsequent dose-response tests received 0.04 U/ml APD. No further adjustments in the NADPH generating system or antibiotics were found necessary from that previously described (15). The concentration of induced microsomal protein which caused a <5% reduction in growth was defined as the maximum which could be subjected to the embryos. Figure 1 shows that under these constraints, the induced microsomal protein content of each co-culture could not exceed 0.06 mg/ml.

## Effect of Bioactivation on CP Toxicity and Teratogenicity

The effect of metabolic activation on CP toxicity and teratogenicity is presented in Figure 2. Activation reduced the 96 h LC50 5.7 fold from 8.0 to 1.4 mg/ml. The 96 h EC50 (malformation) was reduced 15.5 fold from 6.2 to 0.4 mg/ml. The 96 hour TI was increased 2.7 fold upon activation from 1.3 to 3.5. The malformations

observed in unactivated CP at concentration <7.0 mg/ml were generally slight to moderate skeletal disorders, edema, and improper gut coiling. At unactivated CP concentrations greater than 7.0 mg/ml moderate to severe skeletal kinking, edema, and improper gut coiling occurred. Activation increased both the number of different types and severity of malformation. Severe skeletal disorders, head and eye malformations, edema, and impairment of gut coiling were characteristic malformations found with activated CP (Figure 3). Table I shows that carbon monoxide treatment of induced microsomes successfully inactivated P-450, indicated by the marked increase in embryo survival and normal development in the presence of 4.0 mg/ml CP. All embryos exposed to 4.0 mg/ml normally activated CP died.

Success of Cytochrome P-450 Standardization

To determine the success of P-450 standardization, three dose-response assays were performed, each with a different clutch of embryos (Table I). The repetitive nature of the results and generally narrow confidence limits indicate that P-450 content had been successfully standardized by the measurement of APD activity.

Effect of Activation on Embryo Growth

The effect of CP activation on embryo growth is represented in Figure 4. The reduction in growth of those embryos exposed to activated CP occurred at markedly lower concentrations than those exposed to

unactivated CP. The growth reduction (% of control growth) observed in embryos exposed to 0.5 mg/ml activated CP was similar to the effect observed in 6.0 mg/ml unactivated CP. Embryos exposed to 4.0 mg/ml CP plus CO-gassed microsomes, and 4.0 mg/ml unactivated CP were 90.6 and 95.5% of control growth, respectively.

Effect of Residual Aroclor 1254

The effect on embryo development and growth of residual Aroclor 1254 remaining in the microsomal preparation is shown in Table II. The Aroclor 1254 extraction efficiency was estimated to be approximately 70%. Addition of 0.5% BSA to the liver homogenization buffer reduced the Aroclor 1254 concentration from 21.2 to 6.2 ug/ml. Each Petri dish was estimated to contain from 30 to 120 ng/ml Aroclor 1254 depending on the amount of added microsomal protein. Whole embryo exposure to Aroclor 1254 spiked microsomal protein indicated that this range had little adverse effect on embryonic development and growth. A single experiment revealed that the 96 h lethal lowest observable effect concentration (LOEC) for CP activated by both uninduced microsomes and uninduced microsomes spiked with 1 ug Aroclor 1254 was 1.0 mg/ml. Additionally, the embryo-lethal effect generated by 2 and 3 mg/ml CP activated by uninduced and Aroclor 1254 spiked uninduced microsomes was consistent. Therefore, the residual Aroclor 1254 appeared not to potentiate CP nor did interaction appear

to affect toxicity and teratogenicity.

Identification of Metabolites

Table III lists the absolute retention times from the GLC analysis of bioactivated CP. The 2.58, 5.58, 5.71; and 3.61, 6.81, 10.16, 15.42 min peaks corresponded to the CP and metabolic activation system (MAS), respectively. The 9.06 min peak present in the 4-OOH CP standard was also present in the metabolically activated CP sample, but absent in each of the other treatments. Since 4-OOHCP spontaneously hydrolyzed to 4-OHCP, the MFO-derived metabolite of CP, we believe that this peak was the result of CP metabolism. Detectable quantities of 4-OHCP/AP were not present until 30 min, but were detected throughout the remainder of the 24 h assay. The lack of a 9.06 min peak in the CO-gassed MAS and 4.0 mg/ml CP culture gave further indication that the carbon monoxide treatment preferentially inactivated P-450.

## DISCUSSION

The results of this study indicate that the Aroclor 1254 induced rat liver microsome metabolic activation system successfully bioactivated CP into highly toxic and teratogenic species. The greater than 2.5 fold increase in the TI upon activation suggests that metabolism has a greater effect on the teratogenicity than the toxicity of the active metabolites. We consider nonteratogens to be those compounds that have TI values <1.5. As teratogenic risk increases, the TI rises to >1.5. CP was not teratogenic (TI=1.3) unless it was metabolically activated, whereupon it became a strong teratogen (TI=3.5). The decrease in line slope found with bioactivated CP further indicates that the range of teratogenic concentrations increases as well. Bantle and Dawson (15) reported a TI value of 1.4 for activated CP using uninduced microsomes, 2.5 fold less than we report with Aroclor 1254 induced microsomes. The detection of 4-OHCP in the activated CP cultures and the greater TI value seem to indicate that the enhanced system more effectively converted CP into active teratogenic metabolites. The induction of the proper P-450 isozymes required for CP bioactivation may account for the greater developmental toxicity we achieved. Although the TI may not always indicate whether a compound is a teratogen, the severity of the malformation produced was a strong

indication that activated CP was teratogenic. The rate of growth inhibition and overall reduction in growth increased with bioactivation and the subsequent increase in teratogenicity. This trend was consistent with findings of Dawson and Bantle (12) for ethanol, saccharin, caffeine, and 5-fluorouracil.

The malformations observed Xenopus embryos due to activated CP exposure appear similar to those recorded in mammalian studies. Intraperitoneal injection of CP into Wistar and S/D rats produced fetuses characterized by brain malformations (anencephaly, exencephaly, and microencephaly), deformed palate and lip, open eyes, and defective limbs (retarded, club, ectrodactyly, syndactyly, and polydactyly) (27-29). Deformities reported in mice (30-31), rabbits (32), and monkeys (33) were similar to those found in rats and included kinky tail. The effects of metabolically activated CP on cultured whole rat embryos included abnormal brain development, hypoplasia of the mandibular arches, limb buds, and tail (34-35). Decreased growth was also noted (35).

Anomalies in Xenopus as the result of activated CP exposure were severe to moderate head malformations (microencephaly and anencephaly), opthalmic abnormalities, impairment of gut coiling, and severe skeletal kinking. Embryo growth also was significantly impaired. Since FETAX is a 96 h test, it is incapable of

detecting limb malformations. Skeletal kinking observed in Xenopus may bear some relationship to the skeletal limb defects observed in mammals. Dawson and Bantle (12) have observed the same relationship with ethanol (36), caffeine (37-38), and 5-fluorouracil (39-40).

The use of BSA in the liver homogenization buffer and additional purification by ultracentrifugation reduced the residual Aroclor 1254 concentration to acceptable levels. Initial attempts to employ commercially available Aroclor 1254 induced rat liver S-9 supernatant (Litton Bionetics) failed due to high toxicity thereby prompting this investigation of the use of purified microsomes. In future validation work Aroclor 1254-toxicant potentiation and interaction will need to be closely investigated. An additional method of reducing residual Aroclor 1254 would be to use only those congeners responsible for P-450 induction. We are actively pursuing this possibility.

Aroclor 1254 induction may present one primary disadvantage. Aroclor 1254 has been thought to reduce the amount of particular P-450 isozymes in the liver as was suggested with thalidomide (41). However, Aroclor 1254 induced microsomes offer several advantages over uninduced preparations. The deleterious background effects of the microsomal activation system must be minimal. This was not the case with the uninduced microsomal protein/Xenopus embryo coculture system.

Bantle and Dawson (15) pointed out that much of the reduction in Xenopus growth observed in the activated CP treatments was due to the uninduced metabolic activation system itself. By using induced microsomes, less than half of the uninduced protein originally required was necessary to achieve CP activation. To achieve similar assay data, Bantle and Dawson (15) were required to use 0.1-0.12 mg/ml of uninduced microsomal protein. This level retarded embryo growth to 8% of the control. In the present study, the growth inhibiting effect of the Aroclor 1254 induced metabolic activation system including bacterial contamination was reduced to <5%. The effects generated by induced microsomal protein were mainly the result of bioactivation and not the metabolic activation system itself.

The evaluation of P-450 content by measuring APD activity is rapid and inexpensive. Differential spectrophotometry used previously (15,42) is tedious and often compromised by sample turbidity. The repetitive results obtained in this study give a fair indication that P-450 was successfully standardized. However, for each validation compound tested in the future the success of standardization will need to be evaluated in a similar manner.

The large number of new chemicals manufactured and released each year, many of which pose a human health risk, warrants the use of short-term in vitro

teratogenesis screening assays such as FETAX. Without metabolic capabilities, the success of these assays as screens for human developmental toxicants is doubtful. Proteratogenic compounds that evade detection may be converted to teratogenic forms in humans and cause birth defects. Biotransformation of proteratogenic compounds is a recognized obstacle in most in vitro assay systems, but must not be overlooked in a proper teratogenesis screening test (14). We are presently conducting further validation work with a variety of direct-acting and proteratogenic compounds. By employing Aroclor 1254 induced rat liver microsomes as a metabolic activation system for FETAX the number of false negative and positive results may be minimized.

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Table I. Effect of Activated CP on Xenopus Embryo Mortality and Malformation.

| Treatment | Mortality (%) | Malformation (%) | LC50 (mg/ml) | EC50 (mg/ml) | TI |
|---|---|---|---|---|---|
| FETAX Solution (w/Pen-Strep) | 0 | 1.3 | - | - | - |
| Metabolic Activation System | 0 | 4.2 | - | - | - |
| CO-gassed Metabolic Activation System[1] and 4.0 mg/ml CP | 0.8 | 14.3 | - | - | - |
| Activated CP[1]: | | | | | |
| 1 | - | - | 1.36 (0.78-2.33) | 0.44 (0.12-1.64) | 3.1 |
| 2 | - | - | 1.29 (1.08-1.54) | 0.33 (0.11-1.11) | 3.9 |
| 3 | - | - | 1.46 (1.26-1.39) | 0.39 (0.29-0.53) | 3.7 |

[1] Cyclophosphamide

Results from three 96 h tests in which 80 embryos were exposed to FETAX solution and 40 exposed to each additional treatment. 96 h LC50 and EC50 (malformation) values presented with respective 95% confidence limits were determined by the Litchfield-Wilcoxon test. The Teratogenicity Index (TI)=96 h LC50/96 h EC50 (malformation).

Table II. Effect of Residual Aroclor 1254 in the Rat Liver Metabolic Activation System on 96 h *Xenopus* Embryo Mortality, Malformation, and Growth.

| Treatment | Microsomal protein (mg/ml) | Aroclor 1254 spike (ug/ml) | Mortality (%) | Malformation (%) | Growth (% of control) |
|---|---|---|---|---|---|
| Control (w/Pen-Step) | - | - | 2.5 | 7.7 | - |
| Uninduced Metabolic Activation System | 0.1 | - | 10.0 | 11.1 | 98.9* |
| Aroclor Spiked Metabolic Activation System | 0.1 | 0.26 | 7.5 | 10.6 | 97.2* |
| | 0.1 | 0.18 | 10.0 | 16.7 | 96.6* |
| | 0.1 | 0.13 | 5.0 | 10.5 | 97.0* |
| | 0.1 | 0.05 | 2.5 | 5.1 | 97.8* |
| | 0.1 | 0.02 | 0 | 5.0 | 100.4* |
| | 0.1 | 0.01 | 0 | 5.0 | 99.6* |

*Not significant (P=0.05, Student's t-test).

Results from a 96 h test in which embryos were subjected to varying concentrations of Aroclor 1254 spiked into an uninduced metabolic activation system. 80 embryos were designated controls and 40 embryos were subjected to each particular treatment.

Table III. Qualitative Analysis of 4-Hydroxycyclophosphamide in Metabolically Activated CP Cultures using GLC.

| Absolute Retention Time (min) | MAS[1] | CP[2] | Activated CP[3] | | | | | | 4-OOHCP[4] | CO-MAS[5] & CP |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0h | 0.5h | 1h | 3h | 6h | 24h | | |
| 1.27 | - | - | - | - | - | - | - | - | + | - |
| 2.58 | - | + | + | + | + | + | + | + | - | + |
| 3.12 | - | - | - | - | - | - | - | - | + | - |
| 3.61 | + | - | + | + | + | + | + | + | - | + |
| 5.58 | - | + | + | + | + | + | + | + | + | - |
| 5.71 | - | + | + | + | + | + | + | + | - | + |
| 6.81 | + | - | - | - | - | - | - | - | - | - |
| 9.06 | - | - | - | + | + | + | + | + | + | - |
| 10.16 | + | - | - | - | - | - | - | - | - | - |
| 15.42 | + | - | + | + | + | + | + | + | - | + |
| 27.54 | - | - | - | + | + | + | + | + | - | - |

1 Metabolic activation system

2 Cyclophosphamide (CP)

3 Metabolically activated cyclophosphamide

4 4-Hydroperoxycyclophosphamide

5 Carbon monoxide gassed metabolic activation system and CP

Results from GLC analysis in which various cultures were analyzed for the presence of 4-OHCP. Retention times are relative to water solvent. (+) indicates the presence of a particular peak and (-) the absence. Sample peaks were compared to a 10 mg/ml 4-OOHCP standard. The activated CP culture contained the induced MAS and 4.0 mg/ml CP.

## FIGURE LEGENDS

Figure 1 - Effect of increasing protein concentration on 96 h *Xenopus* embryo growth. All other components of the MAS were as described in Methods and Materials.

Figure 2 - Dose-Response curves for *Xenopus* embryos exposed to activated CP (mg/ml) (■) mortality, (△) malformation; and unactivated CP (mg/ml) (□) mortality, (▲) malformation after 96 h exposure.

Figure 3 - Malformations and growth reduction caused by 96 h exposure to activated CP. Control embryo (top) and *Xenopus* embryo exposed to 3.0 mg/ml microsomal activated CP (bottom).

Figure 4 - Representative *Xenopus* embryo growth curves for 96 h exposure to (■) activated and (△) unactivated CP (mg/ml).

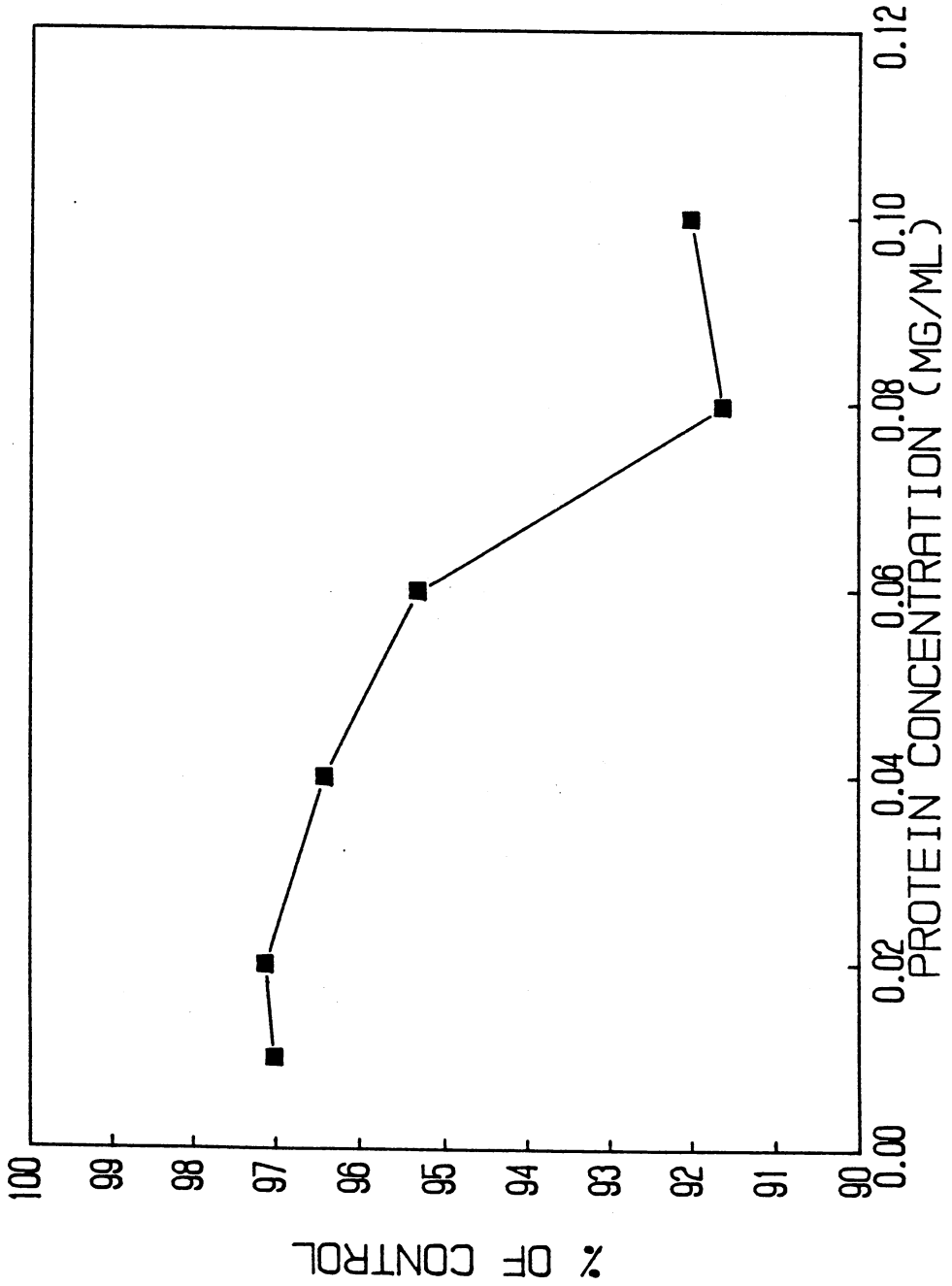
% OF CONTROL
100
99
98
97
96
95
94
93
92
91
90
0.00
0.02
0.04
0.06
0.08
0.10
0.12
PROTEIN CONCENTRATION (MG/ML)

Figure 1

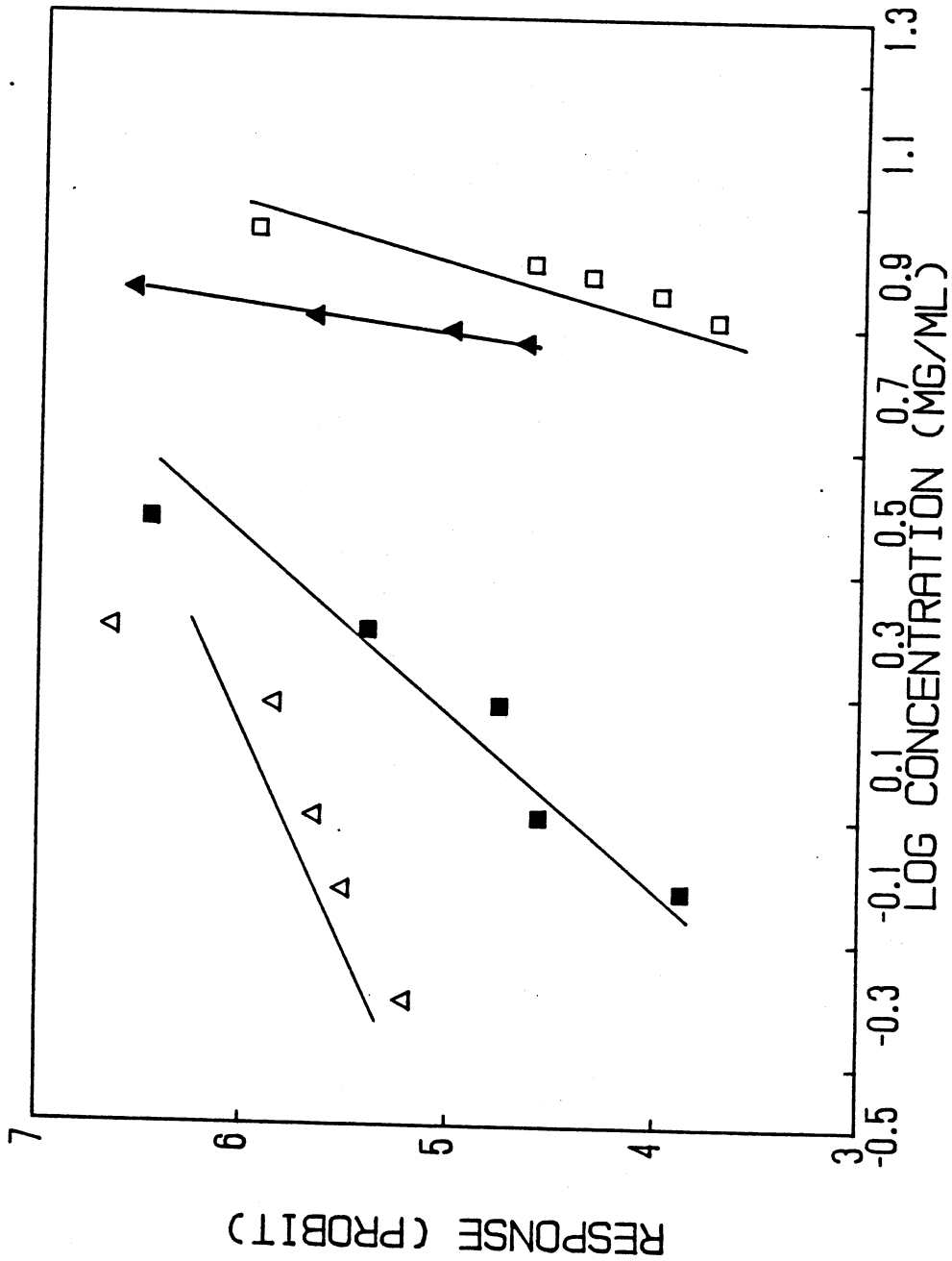
RESPONSE (PROBIT)
7
6
5
4
3
-0.5
-0.3
-0.1
0.1
0.3
0.5
0.7
0.9
1.1
1.3
LOG CONCENTRATION (MG/ML)

Figure 2

Figure 3

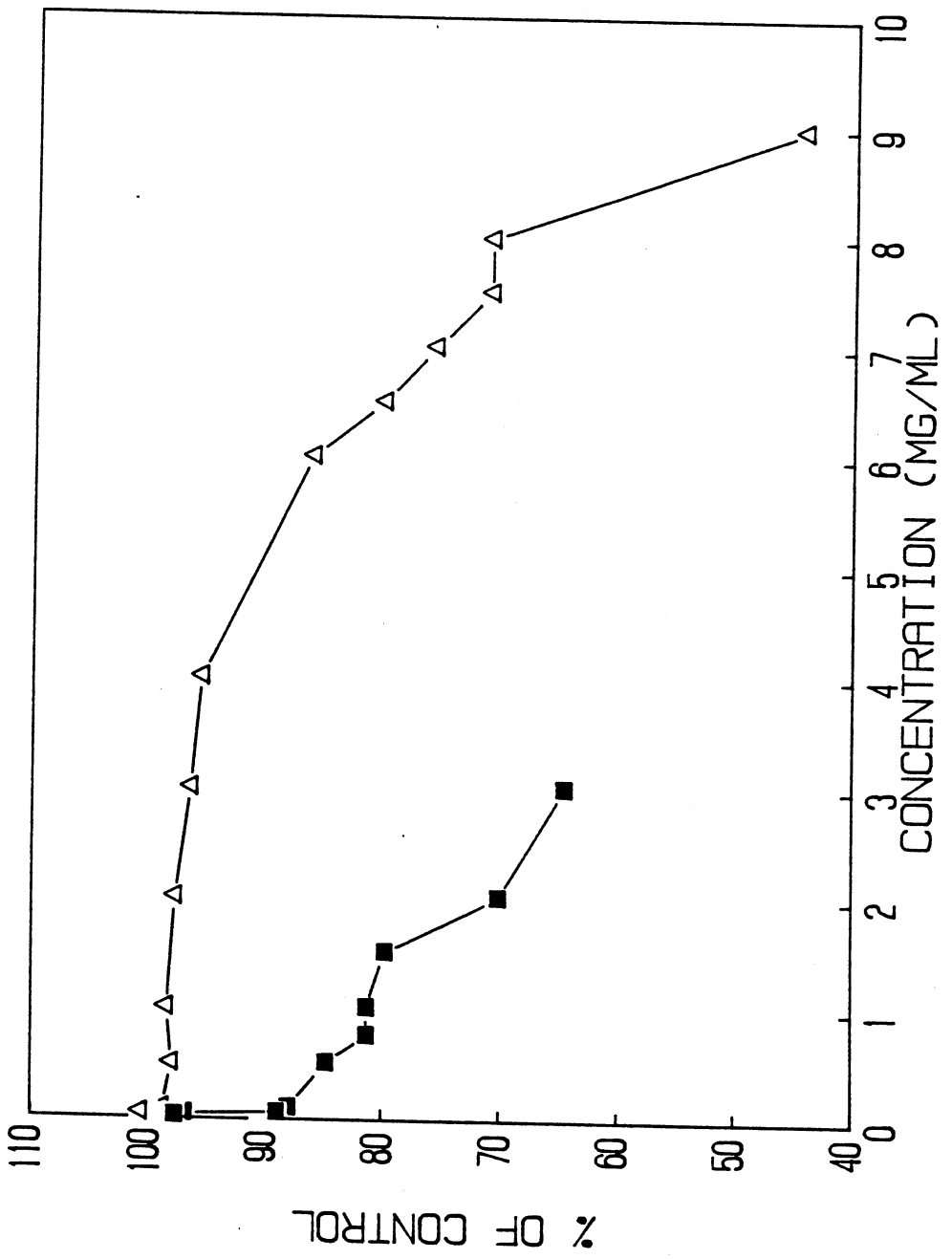
% OF CONTROL
110
100
90
80
70
60
50
40
0
1
2
3
4
5
6
7
8
9
10
CONCENTRATION (MG/ML)

Figure 4

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