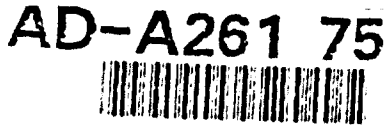


Phase I Progress Report

Contract No.: DAAD17-91-C-1048

AD_____

CONTRACT NO: DAMD17-91-C-1048

TITLE: FURTHER DEVELOPMENT AND VALIDATION OF THE FROG EMBRYO TERATOGENESIS ASSAY-XENOPUS (FETAX)

PRINCIPAL INVESTIGATOR: John A. Bantle

CONTRACTING ORGANIZATION: Oklahoma State University
Department of Zoology
Life Sciences West 430
Stillwater, Oklahoma 74078

REPORT DATE: November 23, 1992

TYPE OF REPORT: Phase I Progress Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	23 November 1992	Phase I Progress Report

4. TITLE AND SUBTITLE
Further Development and Validation of the Frog Embryo Teratogenesis Assay-Xenopus (Fetax)

5. FUNDING NUMBERS
Contract No.
DAMD17-91-C-1048

62720A
3E16270A835.PD.012
WUDA335831

6. AUTHOR(S)
John A. Bantle

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Oklahoma State University
Department of Zoology
Life Sciences West 430
Stillwater, Oklahoma 74078

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Prepared with the cooperation of Greg Linder, James Dumont, Douglas Fort, Robert Finch and Dennis Burton

12a DISTRIBUTION AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The Frog Embryo Teratogenesis Assay Xenopus (FETAX) is a 96 hour whole embryo developmental toxicity screening assay. It was designed to detect xenobiotics that selectively impair embryo development, growth and survival (developmental toxicants)at concentrations far less than those required to impair adults. Not all xenobiotics act in this manner. Consequently, it is necessary to use tests that include developmental endpoints or risk missing toxic effects on a weak link in the life cycle of vertebrates. The purpose of the contract was to establish the repeatability and reliability of FETAX by conducting an inter-laboratory study in three phases. The results of Phase I employing Hydroxyurea. Isoniazid and 6-Aminonicotinamide are presented here. The same lot numbers were used throughout the study so that variation in chemical purity did not affect results. Identity of the compounds was known. Three concentration-response tests were performed for each compound in each laboratory. There was good correlation between historical data and new test results especially for isoniazid and 6-aminonicotinamide. Results suggest that Phase II should be

14 SUBJECT TERMS	15. NUMBER OF PAGES
Frog Embryo Teratogenesis Assay - Xenopus; FETAX; ILS; Validation; developmental; toxicology; teratology; Lab animals; RAD III; Phase I	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18 SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20 LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

performed. The contract called for participation in an annual workshop and for technology transfer. The results from the workshop and the book chapter for "Fundamentals of aquatic Toxicology" are provided.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N.A. Where copyrighted material is quoted, permission has been obtained to use such material.

N.A. Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI Signature John A. Bantle Date 11/22/92

FETAX -
A DEVELOPMENTAL TOXITY ASSAY USING FROG EMBRYOS

By:
John A. Bantle

INTRODUCTION

The Need for Developmental Toxicity Testing

Developmental toxicity tests are designed to detect xenobiotic agents that affect embryonic development. Embryonic development can be considered a "weak link" in the life cycle of an organism. During this period unique cellular and molecular processes operate to generate a complex multicellular organism from a zygote. These processes are sensitive and easily perturbed by many chemicals. Developmental toxicants are chemicals that can exert their effects at concentrations lower than that required to affect adults or cause general cellular toxicity. For example, semicarbazide causes malformation in frog embryos at 1/3000th the concentration required to kill embryos and affects embryonic growth at even lower concentrations (Schultz et al., 1988). Chronic full life cycle tests account for all significant life stages and usually take longer to run for vertebrates than the four day developmental toxicity test presented here. Short-term developmental toxicity tests can then be considered sub-chronic tests that may predict chronic effects in far less time and cost.

FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) is a four day whole embryo developmental toxicity tests that utilizes the embryos of the South African Clawed frog, *Xenopus laevis*. FETAX was initially designed as an indicator of potential human developmental health hazards. The assay is well suited for complex mixtures (e.g., industrial effluents) testing and has been validated using single chemicals of known mammalian developmental toxicity (See: Validation Study Results). The assay also is applicable to aquatic toxicology assessments. This chapter will emphasize this latter use.

Uses of FETAX

FETAX data can be extrapolated to other species, because an evoluntionarily conserved genetic program controls embryonic development. This program may be thought of as a series consecutively expressed genes that guide the formation of basic embryonic structures. If differences such as metabolic activation and placentation are taken into account, it is even possible to extrapolate the data to mammals. However, there are some features of the amphibian egg that make it unique. These features allow the use of this assay to find developmental toxicants that affect amphibians. This amphibian developmental toxicity test may help in studies designed to discover the reasons for the reported world-wide disappearance of amphibians even in pristine locations (Wake and Morowitz, 1990; Wake, 1991). This decline may be due in part to normal population fluctuations caused by climatological factors or by anthropogenic factors (Pechmann et al., 1991). In at least one case, frog eggs failed to develop in pond water but developed normally when moved to the laboratory (Science Briefings, 1991). It is, therefore, possible that some decline may be due to chemical pollution and FETAX may be used to investigate the extent and causes of the decline.

When FETAX is used for aquatic toxicology evaluation, it must be remembered that stunted and malformed embryos would be swiftly removed from the normal population through the inability to feed or by predation. This means that species survival can be compromised by developmental toxicants. For humans, developmental abnormalities persist in live offspring with attendant social and health costs.

Developmental Toxicity Assay Design

In designing a developmental toxicity assay, it is imperative to account for the normal molecular and cellular mechanisms that guide embryonic development. As mentioned earlier, a genetic program guides development and it entails the sequential expression and repression of genes. Many of these genes are expressed for a short period only during a specific stage of embryonic development. Therefore, genotoxic agents are often developmentally toxic as well. Five cellular mechanisms operate in concert during development and each is critical in embryogenesis. These mechanisms are cell division, interaction (induction), migration, differentiation and selective cell death. Division is an obvious mechanism as a single-celled zygote cleaves to form a hollow ball of cells followed by normal mitosis during embryogenesis. This continues until a complex multicellular organism forms. Cell interaction is an important mechanism by which cells signal one another via short and long range mechanisms. This signalling is informational and target cells respond by changing developmental pathways. The induction of the lens of the eye by underlying neural ectoderm is a classic example of this process. Cells also migrate in the embryo either as individual cells, as tissues, or as entire organs. Thus, primordial germ cells (future spermatozoa and ova) wander from their point of origin throughout the body until they find the presumptive gonad. The kidneys of mammals form in one location and move posteriorly as development progresses. After cells arrive in their final locations they differentiate and acquire their cell-specific functions. Neurons form by cell

enlargement and then elaborate complex cytoplasmic processes that differentiate to become axons and dendrites. Perhaps the most difficult of cell processes for the student to comprehend is cell death. Programmed cell death is actually an important feature in the generation of normal embryonic structure. Whole embryonic systems form only to be modified or removed during development. The eradication of the pronephros and mesonephros in mammals are typical examples. The interruption of any of these mechanisms may cause abnormal development or even death. This means that endpoints of any developmental toxicity assay must consider all these mechanisms. FETAX endpoints are the 96-hr LC50 (embryo death), the 96-hr EC50 (malformation) and the Minimum Concentration to Inhibit Growth (MCIG). These endpoints account for all important cell and molecular mechanisms since the assay is based on the whole embryo and not on embryo parts or cultured cells.

Some developmental toxicants only affect certain stages of embryonic development. The drug thalidomide only exerts its devastating effect of abnormal limb development and stunting during a very short period. Treatment with thalidomide before or after this period results in little or no effect. Therefore, exposure conditions in any assay must be designed to ensure that exposure occurs during all the sensitive developmental stages. Exposure is continuous in FETAX throughout the four day period of primary organogenesis thereby ensuring that all sensitive stages are affected. During the four day exposure period, the embryo proceeds from a hollow blastula stage of a few hundred cells (Figure 1) to a free-swimming larva (Figure 2) that is ready to feed. All primary organogenesis is complete although limbs have not yet formed.

Lastly, it is important to note two other fundamental concepts in developmental toxicology. Karnofsky's law (Karnofsky, 1965) states that any material can be teratogenic when administered at concentrations approaching general cell toxicity. This feature will be seen later in the description of the Teratogenic Index (TI) concept. The second concept is that insult to early stages is far more deleterious than damage to later stages of development. Early injury to a primordium (a group of undifferentiated cells destined to produce an organ) of cells can result in damage to whole organ systems while damage later may affect only a single organ. Damage to an early group of cells is transmitted to succeeding generations of daughter cells and the initial fault is magnified throughout the embryo. Therefore, a rule is that the earlier damage occurs, the worse and more widespread the damage.

FETAX LITERATURE REVIEW

Amphibian embryos and larvae have been exposed experimentally to a wide variety of toxic chemicals. Herbicides (Anderson and Prahlad, 1976), fungicides (Bancroft and Prahlad, 1973), insecticides (Cabejsezed and Wojcik, 1968), metals (Abbasi and Soni, 1984; Chang et al., 1974) and many other chemicals and mixtures have been tested in a variety of species (Cooke, 1972; Ghate and Mulherkar, 1980; Ghate, 1983; Ghate, 1985a; Ghate, 1985b; Green, 1954). The results of this work convinced early researchers that amphibian embryos were sensitive indicators of water quality. However, factors such as species used, exposure time, diluent water differences, temperature, number of embryos/dish varied greatly from one experiment to another. Comparison of results became impossible and none of the experiments set good criteria for determining whether a chemical was a developmental toxicant or simply causing malformations at or near the concentration of general cellular toxicity. Birge's group did extensive work in comparing the relative sensitivities of several different anuran species although assay conditions often varied (Birge and Black, 1979; Birge et al., 1979). In the mid-seventies Dial (1976) and Greenhouse (1976a; 1976b; 1977), began the process of standardization by simply publishing the results of several studies that used the same basic methodology. It was then possible to begin comparing the developmental toxicity of several chemicals. Greenhouse's use of *Xenopus laevis* paved the way for FETAX because he recognized the many advantages of using this imported frog (See: Test Organism).

It was Dumont and his coworkers who named the assay, defined the basic protocol, fixed the endpoints and proposed the concept of the Teratogenic Index (TI) that helped to differentiate developmental toxicants from other chemicals (Dumont et al., 1979; Dumont et al., 1983a). FETAX was first used in studying the developmental toxicity of selenium (Browne and Dumont, 1979; Browne and Dumont, 1980), aromatic amines (Davis et al., 1981) and complex shale oil mixtures (Dumont and Schultz, 1980). Work soon turned to solidifying the protocol and initial efforts were made to validate the assay for use in screening for human developmental toxicants (Dumont et al., 1983a). The laboratories of Sabourin and Bantle followed Dumont's work by first comparing FETAX to other developmental toxicity assays (Sabourin et al., 1985) and then continuing the validation process (Sabourin and Faulk, 1987; Bantle and Courchesne, 1985; Bantle and Dawson, 1988; Bantle et al., 1989a; Bantle et al. 1989b; Bantle et al., 1990; Courchesne and Bantle, 1985; Dawson and Bantle, 1987a; Dawson et al., 1988a; Dawson et al., 1989; DeYoung et al., 1991; Fort et al., 1989; Rayburn et al., 1991a; Rayburn et al., 1991b). As the assay began to prove itself to be both sensitive and specific, the validation process demonstrated certain weaknesses in the protocol. An American Society for Testing and Materials E.47 Aquatic Toxicology Taskforce was formed to define the best protocol and write a New Standard Guide which has just been accepted for publication (Bantle and Sabourin, 1991). This guide recommends a basic FETAX protocol to be used for all testing. An "Atlas of Abnormalities-A Guide for the Performance of FETAX" (Bantle et al., 1991) was written as a companion to the guide (Available free from the author). This atlas facilitates learning the assay and helps in staging the embryos and identifying malformations. Many investigators wish to add endpoints or alter

exposure conditions. Dumpert and coworkers have extended exposure periods and have waited extended time periods before recording results (Dumpert and Zeitz, 1984; Dumpert, 1986; Dumpert, 1987). This practice is not discouraged so long as the investigator indicates a deviation from the standard FETAX has been made so comparisons are not made erroneously. FETAX serves as a base from which other work can proceed so it is recommended that standard FETAX be performed and modifications made later.

Bantle and coworkers realized early that a metabolic activation system (MAS) was required if the predictive accuracy of FETAX was to be high enough to be useful as a developmental toxicity screen for mammals. Early experimentation showed that *Xenopus* embryos lacked a competent metabolic activation system. Cultured hepatocytes were initially tried but the system was costly and awkward (Bantle unpublished). S9 supernatant proved to be too toxic for the frog embryos (Bantle unpublished). S9 supernatant is a post-mitochondrial supernatant produced by the high speed centrifugation of rat liver homogenate. Residual Aroclor 1254 was likely the toxic component. The best system proved to be a mixture microsomes derived from Aroclor 1254- and Isoniazid-induced rat livers (Bantle and Dawson, 1988; Bantle et al., 1989a; Bantle et al., 1990b; Dawson et al., 1988a; Fort et al., 1988; Fort et al., 1989; Fort and Bantle, 1990a; Fort and Bantle, 1990b; Fort et al., 1991).

Work during this time period also proved that FETAX could be used to assess the developmental toxicity of surface water (Dawson et al., 1984), ground water (Bantle et al., 1989b) and sediment extracts (Dawson et al., 1988b).

Schultz and coworkers and Dawson have showed that FETAX can be used in structure activity studies (Schultz et al., 1980; Schultz et al., 1988; Schultz and Ranney, 1988; Dawson et al., 1990a; Dawson et al., 1990b; Dawson et al., 1991a; Dawson et al., 1991b). These studies have also progressed to complex mixture analysis which is currently ongoing (Dawson and Bantle, 1987b; Dawson and Wilke, 1991a; Dawson and Wilke, 1991b). Finch (unpublished) has worked with FETAX using flow-through exposure and is developing methods for the use of the assay in a biomonitoring trailer. Linder et al. (1990) also has shown that the assay is flexible enough to be used *in situ* for on site biomonitoring. Linder has devised a simple inexpensive plastic mesh cage that confines the embryos securely. This is staked to a stainless steel pole and marked with a bobber.

Currently, an interlaboratory study is being conducted by laboratories from academia, private industry and government to assess the repeatability and reliability of FETAX. Attempts are now being made to discover useful biomarkers in *Xenopus* and to couple the effects of toxicants at the molecular level to whole embryo effects. This will allow FETAX to become an important model system with which to study molecular mechanisms of developmental toxicity.

TERMINOLOGY

For general terminology the reader is referred to the glossary in this book. A developmental toxicant is a chemical material that affects any developmental process. Developmental toxicants exert their effects (embryo mortality, malformation, growth inhibition etc.) at concentrations lower than that required to cause general cellular toxicity or adult effects. There are a variety of criteria for effects or endpoints of developmental toxicity that could be used to compare chemically-exposed organisms with unexposed organisms. Embryo death, malformation and growth are typically effect criteria that account for most of the potentially adverse effects that may occur in developmental processes. These effects are used in FETAX endpoints to define the 96-hr LC50 (embryo death), 96-hr EC50 (malformation) and the MCIG. Rarely used endpoints such as pigmentation and swimming ability can be used to account for neural damage (Courchesne and Bantle, 1985). Other endpoints are possible but entail added labor. It is important to differentiate between a developmental toxicant and a teratogen. Teratogens cause malformation at concentrations lower than those required to cause general cellular toxicity or adult effects. Thus, teratogens are a subset of developmental toxicants. The Teratogenic Index or TI is a measure of developmental hazard (Dumont et al, 1983a; Dawson and Bantle, 1987a). The TI is defined as the 96-hr LC50 divided by the 96-hr EC50 (malformation). TI values higher than 1.5 signify a larger separation of the concentration ranges that produce mortality and malformation and, therefore, a greater potential for all embryos to be malformed in the absence of significant embryo mortality. TI values have ranged from 1 to 3,000 depending upon the nature of the test material. The Minimum Concentration to Inhibit Growth (MCIG) is the lowest concentration of test material that significantly inhibits growth as measured by head-tail length. A significant difference in growth may be determined by the T-test for group observations at the $P=0.05$ level.

An *in vitro* developmental toxicity assay is defined here as any assay that does not use whole mammalian embryos *in vivo*. FETAX is classified as an *in vitro* test even though whole embryos are used. An exogenous metabolic activation system (MAS) is added to an *in vitro* test system to provide conditions for the metabolism of the test material as if it were in a whole animal system. FETAX uses a 1:1 combination of Aroclor 1254- and Isoniazid-induced rat liver microsomes plus generator system (Bantle et al., 1990b). A MAS is incorporated when FETAX is used for predicting developmental toxicity in mammals.

FETAX REFERENCE SOURCES

The ASTM new standard guide for the conduct of FETAX gives the basic protocol for FETAX without the MAS (Bantle and Sabourin, 1991). Different options for exposure are given as well as the possible utilization of alternative anuran species when it is necessary to use endemic

species. The "Atlas of Abnormalities" (Bantle et al., 1991) is a companion manual to the ASTM guide. It covers topics that could not be included in the guide such as adult care, identification of stages and malformations and the preparation of the MAS. It also contains standard data forms for use in conducting the assay.

Two other reference sources are available which aid in animal husbandry and breeding. The first is by Dawson et al., (1992) which provides information on continuous flow tanks which simplify care and alternative methods of inducing mating. The second source is edited by Kay and Peng (1992). Besides care and breeding information, other uses of *Xenopus* are given which show the tremendous flexibility of the *Xenopus* model and further proof that FETAX is a high connectivity system.

TEST ORGANISM

The selection of a test organism is critical to the success of an assay. It plays a critical factor in the specificity, sensitivity, repeatability and reliability of the assay. It also plays a role in determining assay costs. *Xenopus laevis* is the South African Clawed Frog and was selected over North American species for the following reasons: they can be raised from birth to death in the laboratory, they eat dead food as opposed to the live food required by most North American species, because the adults are totally aquatic, they can be kept in aquaria like fish, the adults are resistant to most diseases, and they can be bred a number of times before replacement.

Breeding is accomplished anytime of the year by a simple injection of commercially-available human chorionic gonadotropin into the dorsal lymph sacs of the male and female (Figures 3-5). Amplexus ensues (the male clasps the female and both release gametes) (Figure 6) and several thousand eggs are available the next day. Dejellied normally-developing eggs must be selected from abnormal or over-ripe eggs but this is not time consuming. This is important because selection of abnormal/over-ripe eggs would lead to high mortality and malformation in controls.

In contrast, most North American frogs require the injection of pituitary gland extracts to induce ovulation and the eggs must be stripped from the female by pressing firmly on her flanks. Fertilization must be accomplished by artificial semination because the frogs will not go into amplexus. Artificial semination involves mincing testes in buffer and applying the sperm suspension over the freshly stripped eggs. When donor frogs are taken into account, several frogs are killed for every breeding. In addition to this costly breeding scheme, it is only possible to carry out breeding during the Spring. Since native species are in decline in most parts of the globe, it makes little sense to use the remaining frogs as test organisms when a readily available substitute is available.

Another important reason for the selection of *Xenopus* is that it is a high connectivity model (NRC, 1985). The reason for this high connectivity is that *Xenopus* have been used for many years in biology. A great deal is already known about its normal development (Nieuwkoop and Faber, 1975; Deuchar, 1972), biology and biochemistry (Deuchar, 1975). Consequently, test results from FETAX may be more readily explainable in light of this knowledge. This makes it much easier to understand and interpret the results of mechanistic studies and can help in the development of biomarkers in amphibians.

It is important to note that the larvae of *Xenopus* are transparent (Figure 2). This makes it easy to observe internal malformations as a number of internal organs are easily visible from the outside (Figure 7). This obviates the need for dissection or histological sectioning when assessing abnormal development.

In cases where endemic species must be tested, the ASTM guide lists alternative species that Birge and coworkers (Birge and Black, 1979; Birge et al., 1979) found to give suitable numbers of eggs per year. When native species are used, the FETAX protocol can be employed but the results cannot be compared with typical FETAX results.

Although *Xenopus* generally exhibit good breeding characteristics, breeding is generally less successful in the Fall and it may be necessary to simultaneously breed as many as three pair of frogs in order to ensure that at least one breeding pair will produce acceptable eggs. Even if eggs are produced, there can be problems with the fertilization rate. Problems with egg quality can be ameliorated by breeding the adults every three months and conditioning the frogs prior to breeding. Conditioning starts by selecting healthy, sexually mature adults and feeding them every day for three weeks prior to mating. Daily water changes (or use of a flow-through system) will help the conditioning of the frogs. As with all toxicity tests, consistent success is directly dependent upon the quality of the animal husbandry. It is better to maintain a small colony of well cared for frogs than a large colony that is maintained indifferently.

FETAX ENDPOINTS AND EXPOSURE REGIMENS

FETAX Endpoints and Assay Data

FETAX has three standard endpoints and a TI ratio calculated from two of the endpoints. Embryo death is measured by the 96-hr LC50 and embryo malformations by the 96-hr EC50 (malformation). Only malformations in live embryos are recorded. Standard concentration-response experiments are performed and mortality and malformation curves constructed using probit analysis. The probit analysis results in the appropriate LC50 or EC50 values with 95% confidence limits. Both concentration-response curves are usually placed on the same plot to demonstrate the separation between the two curves. The TI is found by dividing the 96-hr

During the technician training period, it is advisable to have a second dissection microscope present with control larvae for direct comparison with abnormal larvae. Larvae should be moved and rolled over during inspection and the scope light can be moved to place light on different parts of the embryo. After several tests, data collection becomes routine as most malformations are severe enough to be easily observable. It takes time to observe minor malformations such as inproper gut coiling. Despite the subjective nature of the malformation endpoint, the ongoing interlaboratory validation study of FETAX indicates that the data is no more variable than the objective mortality endpoint.

Figure 11 shows a ventral view of a control larva with a tightly coiled gut. This figure illustrates the transparency of the larvae at stage 46 which makes it unnecessary to section the embryo to observe internal malformations. Gills and aortic arches are easily seen. Figure 14 shows a severely malformed larva. All organs are malformed in this larvae and the malformations are easily observed. Most technicians have trouble detecting facial malformations and Figure 15 shows a control larva on top and a larva with obvious facial malformation on the bottom. Once again staining helps in identification during the training phase until competency is attained. In this Figure, the tails of the larvae are inserted into fiberglass insect screen which is suspended in a Petri dish.

Some developmental toxicants cause very specific malformations. For example, one groundwater sample caused all larvae to be completely devoid of pigmentation and have incomplete gut coiling (Bantle et al., 1989b). Another study with plant glycoalkaloids caused anencephaly (headless larvae) in most embryos at high concentrations of toxicant (Friedman et al., 1990). These abnormalities give important information as to the target organ/s of the toxicant.

Growth inhibition data is collected by placing each Petri dish between the condenser and lens in the negative carrier compartment of a standard darkroom enlarger. A glass plate can substitute for the negative carrier to hold the dishes. Prior to purchasing the enlarger, the opening of the negative carrier should be measured to make sure that the Petri dish will fit and that the image can be enlarged and focused on the digitizing pad. Enlargement of 2.5-3 diameters greatly increase the resolution of each measurement. The enlargement should be great enough to accurately measure length but not exceed being able to image all of the larvae on the pad at once.

The "Sigmascan" software is loaded and the length program calibrated by magnifying a transparent ruler onto the pad and measuring a 1 cm length to serve as a standard reference. This is the approximate length of a stage 46 larva. Once calibrated, each Petri dish in ascending concetration series order is enlarged and the each larvae's head-tail length measured. Sigmascan allows the user to follow the contours of those larvae that have been misshappen by the toxicant. The T-test for grouped observations is part of the "Sigmascan" software package so that data analysis can proceed immediately following collection. Each concentration is compared with the diluent control. In cases where a cosolvent is required, the concentrations must be compared with the solvent control. Summary data can be sent to "Sigmaplot" software for graphic analysis along with concentration-response data for mortality and malformation. There is teratogenic hazard when growth is significantly affected ($P=0.05$) at concentrations below 30% of the LC50 (See: Data Analysis and Interpretation).

Frequently Encountered Problems. With care, FETAX can be routinely performed with excellent results. When problems do occur they are likely to be in the following areas: breeding, selection of good embryos, identification of malformations and controls not falling within acceptable limits. Breeding problems have already been discussed but many problems surface when the investigator attempts to stretch an experiment by selecting embryos that are marginal in quality. It is better to scale back the experiment and reduce high and low concentrations rather than choose poorly pigmented or abnormally cleaving blastulae. It is important to remember that the stage 8 embryos are very fragile and must be handled with great care. If the double selection process is performed carefully and the glassware to be used in the assay is properly cleaned, then the solvent or FETAX solution controls will fall within acceptable limits. When they are not within acceptable limits, each phase of the experiment needs to be carefully analyzed starting with embryo selection and proceeding to glassware washing, bacterial contamination and pH changes.

Another area of difficulty is in identifying malformations in the stage 46 larvae. This problem usual disappears with experience but it is important to remember that developmental toxicants may delay development in a manner similar to cold temperatures. Ordinarily, this is obvious as the embryo appears normal but simply at an earlier stage of development. Sometimes there is confusion as to whether the gut is abnormally coiled or simply delayed in its development. If there are doubts, the surviving embryos should be placed in clean FETAX solution and cultured past the 96-hr time period. If the gut finally coils and the embryo is normal in appearance, then developmental delay is indicated. This should not be recorded as a malformation but it should be noted as developmental delay in the report. Pericardial or abdominal edema may be observed during the test. Many times, the edema will disappear so that all embryos appear normal at the end of 96 hr. This should be recorded but not as a malformation.

DATA ANALYSIS AND INTERPRETATION

FETAX gives concentration-response data for mortality, malformation and growth. These data can be compared with similar data on a molar basis using other pure chemicals to yield relative

developmental toxicity. For example, saccharin has an LC50 of 18.4 mg/ml while hydroxyurea has an LC50 of 1.8 mg/ml. Once corrected for molarity, hydroxyurea will be at least 10 times more toxic.

For assessing the developmental toxicity of complex mixtures, it must be remembered that any significant difference between the 100% concentration and controls represents some developmental toxicity and associated hazard. It is more difficult to assign the teratogenic hazard to a single test material or pure compound. Recall that a teratogen is any agent that causes malformation in living offspring. In order to rank compounds according to their teratogenic hazard, Dumont and coworkers (Dumont et al., 1983a) developed the Teratogenic Index or TI. This value is found by dividing the 96-hr LC50 by the 96-hr EC50 (malformation). Values <1.5 indicate little or no teratogenic hazard while values greater than 1.5 indicate increasing hazard. The TI is similar to the commonly used Therapeutic Index in pharmacology in that a specific effect (malformation) is compared to general toxicity (mortality). The LC50 and EC50 are used in the determination of TI because they are automatically calculated by probit analysis programs and there is the least variation in confidence intervals at the 50% response level. There is some danger the TI will not correctly identify the teratogenic hazard if the slopes of the mortality and the malformation curves are different. However, this has not occurred very often. Most of the concentration curves obtained during the validation phase of FETAX demonstrated similar slopes for mortality and malformation (Figure 16). There is no reason to assume that the TI in FETAX will be indicative of the TI generated by another species. However, it is likely that these values will be fairly close, given that the predictive accuracy of FETAX is nearly 90% when compared to chemicals of known mammalian and human developmental toxicity (See: Validation Study Results).

Figure 16 shows several concentration-response curves that will illustrate how decisions are made in assessing developmental toxicity. It is important to remember that most probit analysis programs do not run when 0 and 100% response data points are entered. Generally, only partial response concentrations are used in establishing the curve. Therefore, there are usually many other data points collected than what appears in Figure 16. α-chaconine (Figure 16, top panel) is a plant glycoalkaloid that is quite toxic to embryos (Friedman et al., 1990). Normally a TI of only 1.03 would allow a determination of low teratogenic hazard. However, this compound causes extremely severe head abnormalities in most surviving embryos. Nonteratogens such as cycloheximide and puromycin (Courchesne and Bantle, 1985) have low TIs like α-chaconine but only cause slight abnormalities even at high concentrations. Figure 17 shows the growth inhibition curves for α-chaconine, hydroxyurea and isoniazid. This data is graphed as the percent of mean control head-tail length (ordinate) vs. the percent of the LC50 concentration (abscissa). Note that stronger teratogens such as hydroxyurea and isoniazid cause significant effects on growth at much lower concentrations than α-chaconine and have steeper slopes. If the MCIG is less than 30% of the 96-hr LC50, then the teratogenic hazard of a compound is considered significant. α-chaconine only shows a significant effect on growth at much higher concentrations. Because of these differences, α-chaconine can be considered a low hazard teratogen qualifying as a teratogen only on the basis of the severity of the malformations caused.

Note that in Figure 16 (middle panel) the mortality and malformation concentration-response curves for hydroxyurea are farther apart than α-chaconine resulting in a TI of 2.78. The curves are parallel and the correspondence of the data points to the lines are good. The growth-inhibition curve shows a significant effect near the 30% of the LC50 level indicating some teratogenic hazard (Figure 17). Note that at the highest concentrations embryos are only 40% of controls lengths. Hydroxyurea, a DNA synthesis inhibitor, also causes severe malformations. Thus, hydroxyurea poses significant teratogenic hazard by all three criteria.

Isoniazid, an antibiotic, is a false positive in FETAX (Figure 16, bottom panel). In mammals it is normally metabolized before it can affect development but it is a severe teratogen in FETAX unless isoniazid-induced microsomes are added. Note that the mortality and malformation concentration-response curves are widely separated resulting in a TI of 44.0. Between concentrations of 0.1 mg/ml and 6.0 mg/ml, all embryos survive but are all malformed. This is indicative of a highly hazardous teratogen. This hazard is also demonstrated in the growth-inhibition curve presented in Figure 17 as a significant effect is observed within 10% of the 96-hr LC50. Once again, the embryos are reduced in size to as low as 40% of controls at 65% of the LC50. Isoniazid causes severe malformations in surviving embryos.

In the foregoing sections it is important to realize that all three criteria (i.e., mortality, malformation and growth inhibition) play a role in assessing relative teratogenic hazard. Each criterion should be considered on its own merits and reported. Only then can teratogenicity be adequately assessed.

VALIDATION STUDY RESULTS

Table 1 shows the results of validation studies carried out in the author's laboratory using 65 compounds (See also: Dumont et al, 1983b). Attempts were made to select compounds from several different chemical classes and to choose nearly as many nonteratogens as teratogens. Blind testing was used for many of the compounds. In cases where a metabolic activation system (MAS) was known to play a role in developmental toxicity in mammals, the MAS was added to FETAX. For the purposes of routine testing for hazard to humans, MAS must be routinely added to all samples. Although the validation study was of more use for human developmental toxicity

as soon as they come off the bottom and continue dejellying in the flask.

Dejellying Embryos. Embryos are dejellied in a fresh 2% w/v cysteine solution at pH 8.1. This efficiently strips the jelly coat from the embryo. Treatment should not last more than 3 minutes or the embryos could be irreversibly damaged, resulting in death or malformations. This can occur even though the embryos appear normal after treatment. In practice, dejellying is not a difficult process when properly performed. A stopwatch should be started upon addition of the 2% cysteine. The solution is gently swirled and watched. As the jelly is stripped, the solution progressively turns cloudy and gray. The embryos then begin to roll freely on the bottom. At this point the cysteine solution should be poured off and the embryos allowed to pile up on the bottom of the flask. Two to three additions of fresh FETAX solution are immediately added with gentle swirling to wash away all of the cysteine solution. Underdejellied embryos stick to glassware while overdejellied embryos die or are malformed. If high mortality or malformation rates are observed in both controls in glass and plastic, the possibility of overdejellying should be considered. Large bore blood bank Pasteur pipets or regular pipets that have had the ends snapped off and have been fire polished are used to transfer the 1.5 to 2 mm embryos to large 100 mm Petri dishes. The large dishes prevent overcrowding and allow large amounts of FETAX solution to bath the embryos. If some embryos and unfertilized eggs disintegrate and release degradative enzymes into the media, the stress on the normal embryos will be minimized by the large volume.

Selection of Normal Embryos. With the exception of judging malformation, no other procedure is more important in FETAX than selecting normally developing stage 8 to 11 embryos (Nieuwkoop and Faber, 1975). The pace of embryonic development is dependent upon a number of factors the most important of which is temperature. Staging is a process of accounting for the speed of embryonic development by noting the appearance of certain morphological markers. Stage 8 embryos are blastulae (Figure 8). A blastula is an embryo composed of a hollow ball of cells just approaching primary organogenesis. Selecting embryos prior to this stage is risky because apparently normal cleavage stage embryos can quickly become abnormal. By waiting until stage 8, the chance of selecting embryos that will grow and develop normally is greatly enhanced. It must be remembered that the female uses egg deposition to shed normal, abnormal and overripe eggs. This is a normal process. Because FETAX measures what goes wrong during development, it is imperative that only normal embryos be used at the start of the test. Stage 11 embryos are at the gastrula stage (Figure 9). At this stage, the formation of the embryonic gut has commenced and events are leading to the formation of the neural tube. Waiting past this stage risks missing a sensitive stage of development. This is important since damage done during early developmental stages causes extensive damage to a number of organ systems. A double selection process materially helps the proper selection of normal embryos between stages 8 and 11. After the embryos are placed in large Petri dishes, an attempt should be made to quickly eliminate large white necrotic eggs before they begin to disintegrate. The number of these varies with the mating but they are usually more prevalent in females which have not been bred for long periods of time. Most technicians put too many embryos in a single dish prior to sorting. It is better to provide ample volume for the embryos. If the FETAX solution becomes clouded, the solution should be quickly changed and the selection process accelerate. After the necrotic eggs and bad embryos are removed, normally pigmented embryos are selected and the "Atlas of Abnormalities" should be consulted for examples of normal stage 8-11 embryos. In order to select only normally cleaving embryos, a double selection process is used. Double selection means that embryos are sorted quickly a first time and then a second, more careful, selection is then performed.

During the training period for new technicians, they should breed frogs, select normal stage 8-11 embryos and culture them in FETAX solution for 96 hr. If mortality is < 10% and malformation < 7%, then actual experiments may be conducted.

Adding Test Material. The best method of preparing test material dilutions is to pipet the stock solution into volumetric flasks (TC). Volumetric flasks are sold as either those that deliver a prescribed volume (marked TD: to deliver) or contain a prescribed volume (marked TC:to contain). FETAX solution is then added to the line. Once the solutions are prepared, add the embryos to the empty dish, quickly remove the solution with a Pasteur pipet and then immediately add the test material from the volumetric flask. The concentration of the stock solution should be adjusted so that reasonable amounts of stock are added to the flasks in the dilution series, since it is fairly difficult to add small volumes.

Another approach to making test solutions is to calculate the number of ml of stock solution and the number of ml of FETAX solution required to prepare a specific concentration of the test material. Embryos are added to the dish and all excess FETAX solution removed using a Pasteur pipet. The calculated amount of FETAX solution is added first and then the test material is added with gentle swirling. This avoids the need for volumetric flasks and subsequent washing problems. It allows for the use of disposable pipets. The technique does yield adequate concentration-response curves assuming the proper size pipet is used and the pipeting is done very carefully. Great care should be taken not to apply the concentrated stock solution directly on the embryos. This latter method is not as accurate as using volumetric flasks. The standard FETAX test follows the renewal procedure which entails fresh replacement of test material every 24 hrs during the test. Renewal is accomplished by removing test solution with a Pasteur pipet then adding 10 ml of fresh solution to each dish.

Assignment to the Incubator (or constant temperature chamber). When all test and control dishes have been prepared, the dishes are placed on an ordinary cafeteria tray lined with absorbent paper. It is possible to fit all of the dishes on a single tray and thus use only a single shelf of the incubator. This allows several tests to be performed concurrently. In addition, locating the dishes on a single shelf reduces the chance for temperature variation. If the dishes are randomized, a simple random number generator can be used to assign the dishes to tray positions. Incubator temperature must be between 23 and 24 ° C and it is best to continuously monitor the temperature throughout the test using a recording thermometer. When a recording thermometer is not available temperature measurments should be made at least twice daily. Many electronics stores sell digital thermometers that report the temperature and keep track of the high and low event. These are ideal for FETAX as long as they are calibrated before use. If the concentration of test material is to be analyzed to ensure the dilutions were properly made, then additional dilution dishes free of embryos should be prepared and analyzed. Any analysis samples taken should be placed in vials with minimum head space and tightly sealed. If the analysis cannot be performed immediately, the samples should be frozen. Some head space is warranted so that the vials will not crack if frozen. If shipment is required, they should be shipped on dry ice to prevent test material degradation.

Conducting the Test

Temperature and pH Measurements. Incubator temperature plays a primary role in determining whether the control embryos reach stage 46 by 96 hr. Over 90% of the controls should be at stage 46 at the end of the test if the temperature averages 23.5° C. Too low of a temperature retards development and too high of a temperature leads to high rates of malformation in the controls. This is why temperature must be carefully controlled and monitored. When mortality is low but rates of malformation exceed 7%, the temperature record of the incubator should be reviewed.

The ASTM guide (Bantle and Sabourin, 1991) requires the measurement of pH in the diluent control and the highest test concentration every 24 hr of the test. This measurement should be made just before changing the test solutions and at the end of the test (see below). A semimicro pH probe can be used by tilting the dish so that the electrode bulb and reference wick is fully immersed. The pH should be recorded on the standard forms. If the pH in the highest test concentration is low it may be a sign of bacterial growth. If the solution appears cloudy, this is another sign that bacterial growth has occurred. All previous attempts to perform FETAX gnotobiotically have failed. Some test materials even serve as a substrate for bacterial growth. In these cases it might prove necessary to conduct FETAX in the presence of 100 U/ml Penicillin and 100 U/ml Streptomycin and report it as a deviation from standard FETAX procedure. It should be noted that antibiotics may interact, however, with the test material and alter toxicity.

Changing Test Solutions. A large bore fire-polished Pasteur pipet is used to remove test solutions from dishes. By starting with the lowest concentrations and proceeding to higher concentrations, it is possible to use the same pipet to remove the test material. The procedure should be accomplished quickly to avoid drying the embryos and care should be taken to count and remove dead embryos from the dish. If the embryo has disintegrated, all remaining yolk material and tissue should be removed or it will serve as a substrate for bacterial growth. New test material solution is quickly renewed as is any MAS and generator system. The embryos have usually hatched from the fertilization membrane and are quite fragile. Great care must be taken to avoid damaging them in the pipet. The tops of the Pasteur pipet can be broken off and fire polished to produce larger bores to accomodate larger embryos without damage in case they are accidently picked up. Depending on the toxicity of test material, this operation is performed in a safety hood or while using a respirator mask.

Ending the Test and Biological Data

General Procedure. If the temperature has been held constant at 24 ± 2° C, 90% of the controls should attain stage 46 by the end of 96 hr. It may be acceptable to delay ending the test for an hour or two until the controls reach stage 46 (Figure 2). The "Atlas of Abnormalities" shows the morphological markers indicative of stage 46 (Figures 2 and 7). The presence of hindlimb buds are the easiest of the markers for the novice to recognize (See arrow on top larvae in Figure 10). Staining procedures covered in the "Atlas" make it easy to recognize the hindlimb bud during training.

After determining that a sufficient number of embryos have reached stage 46 by 96 hr, the next step is to remove the dead larvae. The absence of heartbeat is an unambiguous sign of death that can be used at 48 (stage 35), 72 (stage 42) and 96 hr (stage 46)(See heart labeled "H" in Figures 7 and 11). At 24 hr, live embryos will move if gently irritated. Necrosis is easily observable in dead embryos. Death at 24 hrs is ascertained by skin pigmentation, structural integrity and irritability. Once the dead larvae are removed, the total number dead at 96 hr is recorded on the standard form (Figure 12) and MS-222 anesthetic is added followed by 3% formalin to fix the remaining live embryos. Some laboratories obeserve the anethestized embryos for cardiac malformations prior to fixation. It is much easier to see heart structure while the heart is slowly pumping red blood. Gross congenital malformations are now recorded with particular reference to severity, type and number in each category (Figure 13). Some test materials cause only very slight malformations while others may cause extremely severe malformations. Increasing concentrations of test materials usually increase the severity of the malformations (Figure 10). It is important to refer to the "Atlas of Abnormalities" in order to identify and categorize the various malformations.

Glassware. Because FETAX is a sensitive developmental toxicity test, any residual contaminant or detergent left on glassware following the wash procedure given in the ASTM guide will cause increased mortality, malformation and growth inhibition in controls. Only soap that is intended for cell culture glassware should be used for cleaning and only minimal amounts of this detergent should be used. It is wise to separate FETAX glassware from other laboratory glassware and have each investigator wash their own glassware to assure cleanliness. The controls employing plastic Petri dishes run concurrently with glass Petri dishes help identify glassware washing problems. When the problem of cleaning glassware proves intractable, glassware may be taken to a glassblower who is equipped with a high temperature bake oven in which glassware can be heated to 500° F. Most organics are removed by high temperature treatment such as this and the glassware becomes nontoxic.

The ASTM guide gives the criteria for selection of glass or plastic dishes for FETAX. Glass dishes should be chosen when the nature of the test material is unknown. Generally, the decision is made when it is known that the test material will bind to either plastic or glass. Dishes made of the non-binding material are then selected. Covered 60 mm Petri dishes should be used as test chambers. However, limited capacity is the only problem when the 60 mm plastic Petri dishes are used. Generally, they are only able to accommodate 20 embryos in 8 ml of test solution instead of the standard 25 embryos in 10 ml of test solution in glass dishes. However, plastic dishes are the best choice for experiments employing a metabolic activation system because they can be purchased sterile and can be disposed of following the test.

Apparatus and Equipment. FETAX was designed to be compatible with common fish tests in terms of equipment and glassware. For handling test materials, safety equipment such as hoods, respirator masks, gloves, acid buckets and bench matting is required along with safety guides and material safety data sheets. Analytical and pan balances are required for weighing test materials and weighing salts for balanced salt solutions. Standard weights should be used prior to each weighing. A pH meter is required for the test. Disposable pipets and adjustable pipettors are also required for precisely handling liquids. Adjustable pipettors should be calibrated prior to the start of each test. This is easily done by repeatedly filling disposable micropipets of appropriate volumes available from science supply houses. Glassware should include volumetric flasks, graduated cylinders and a variety of beakers and Erlenmeyer flasks. 60 mm glass and plastic Petri dishes can be purchased by the case. Dishes must remain covered throughout the test.

A high quality binocular dissection microscope and scope light are essential for staging embryos and recording death and malformations. Fine camel's hair artist brushes and watchmakers forceps are handy for manipulating the embryos and larvae in the dish.

Animal culture equipment is covered in the ASTM guide, but considerable flexibility is allowed. Fiberglass raceways, fiberglass bathtubs, vegetable crispers (restaurant sizes) and standard fish aquaria may be used to house adult frogs. Sidewalls must be at least one foot high to prevent adults from escaping and hardware cloth must never be used as covers or mating platforms because of the potential for metal ion contamination. Cost seems to be the determining factor in the selection of animal husbandry equipment. Heaters may be required for the animal room and, if other than well water is used to culture the adults, carboys and carbon filters are required for water treatment. Some investigators have successfully used reverse osmosis water to culture the adults with no apparent adverse effects on results. Only non-toxic tubing is to be used to pipe water to the tanks. Standard Tygon tubing is toxic. A variety of commercially available valves may be attached to frog holding tanks to allow drainage from tanks. Some investigators use simple siphoning to empty tanks. Small water pumps are available from hardware stores to help drain water from the tank.

One of the best systems for culturing adults is to use a continuous flow system. Those investigators with high quality well water, can frequently afford to keep slowly flowing water going through the tanks. A simple 5 cm standpipe with a plastic mesh covering over the drain converts a tank to a flow-through system. In flow-through systems, it is still necessary to brush sidewalls. Since *Xenopus* are wholly aquatic there is no need to provide "dry land" for the adults.

Perhaps the single most important piece of equipment for FETAX is the incubator or environmental chamber that holds the embryos throughout the four day test. Incubators are now available that can regulate ± 1/4° C and these are the most desirable type. A recording thermometer is also important to ensure that temperature maxima and minima are not exceeded. The ASTM guide for randomization of dishes is probably unnecessary for modern circulating air incubators. If there is any question as to temperature variation within the incubator, dish randomization must be carried out.

A personal computer is essential for FETAX data analysis. An inexpensive dark room enlarger is used to enlarge the images of the embryos two-three times on a digitizing pad for length measurements. When selecting a darkroom enlarger, a transparent ruler initially should be projected in several different orientations and measured the length of the projected image. It should be the same regardless of orientation. Head-tail length measurements that once took many hours by hand using a map measurer now take only two hours per test by computer digitizing. Software for data analysis includes "Toxstat", available from the University of Montana, and EPA probit analysis. The "Manual of Pharmacologic Calculations" by Tallarida and Murray (1987) is commercially available in software compatible with IBM compatible

computers. "Sigmascan" (Jandell Scientific, Corte Madera, CA) software is used in analyzing head-tail length and for performing the T-test for grouped observations. "Sigmaplot" (Jandell Scientific, Corte Madera, CA) is used in graphing results from concentration-response curves for mortality, malformation and growth inhibition.

Water. FETAX solution was developed as a standard balanced salt solution and its formulation is listed in the ASTM guide (Bantle et al., 1991). Only a standard solution would allow FETAX to yield data that was repeatable, reliable and comparable. In designing FETAX solution, Dawson and Bantle (1987a) found that relatively high Mg^{++} concentrations were required for normal growth and development of frog embryos. This may affect results in metal toxicity studies. It is difficult to dissolve lead salts in FETAX solution (unpublished) and even if it gets into solution the Mg^{++} concentration may affect toxicity. This must be taken into account when toxicity tests with metals are performed and it may be desirable to alter FETAX solution in certain cases. This and any other alteration must be reported as a variation from standard procedure and additional controls may need to be performed. Another problem area with FETAX solution is buffering capacity. A number of zwitterionic buffers such as HEPES and MOPS were initially tried (unpublished). All caused unacceptably high levels of malformation and it was decided that no buffer should be included. Consequently, the buffering capacity of FETAX solution is small and the test material may change the pH. FETAX should be performed between pH 6.5 and 9 and it is best if the pH is between 7.0 and 7.8. However, there are situations when it is inadvisable to adjust the pH after the addition of test material beyond pH 6.5 or 9.0. In some cases toxicity is altered and in others the test material will not stay in solution. This shows the importance of the requirement in the FETAX guide for daily measuring of pH in the control and the highest test concentration. These measurements are made in the dishes after each 24-hr of exposure. Bacteria can also change the pH of the test solution and this situation must be monitored. The ASTM guide allows for the use of other diluent water in continuous flow experiments but pH, metals content and other chemical factors must be monitored as specified in the guide. The best indicator of diluent water quality is the embryo. If mortality is less than 10%, malformation less than 7%, and embryos grow to at least 0.9 cm by stage 46 (See: selection of normal embryos for staging), then the water is usable as diluent so long as the pH is between 6.5 and 9.0.

Beginning the Test

Breeding. One of the advantages of using *Xenopus laevis* is that breeding may be induced by injection of commercially available human chorionic gonadotropin (HCG) into the dorsal lymph sac of the male and female (Figure 4). Advice has already been given on preparing the adults for mating by conditioning and adjusting the number of breeding pairs. Additional modifications can be made to ensure an acceptable clutch of embryos for FETAX. The standard scheme of injecting males with 400 international units (IU) of HCG into the dorsal lymph sac and females with 1,000 units is given in the ASTM guide and the "Atlas of Abnormalities" shows pictures of the injection procedure. The animals are then placed in a breeding tank together and left to breed overnight. The next day eggs which have been laid and fertilized have fallen through the mesh suspended 3 cm off the bottom of the aquarium. The eggs and embryos are on then deposited on the bottom of the aquarium and away from the adults.

In the injection of the HCG, it is important to use a 26 gauge needle because the wound in the frog's skin does not close as readily as that in mammalian skin. A larger needle would allow the release of HCG from the lymph sac. If several breedings are unsuccessful, a two day injection scheme may be initiated to allow the HCG more time to act. In this scheme, the male and female are injected with 200 and 400 units, respectively, of HCG 48 hr prior to mating. On the night before mating, inject the male with 400 units and the female with 800 units and then place them into the breeding tank as usual. It might be necessary to monitor breeding success by observing egg deposition around 11:30 PM on the night of mating. If the female has begun to deposit eggs, then the test should start very early the next morning. When breeding *Xenopus*, try to minimize disturbances that may interfere with amplexus. If the sidewalls of the breeding tank are glass, they should be painted in order not to disturb the frogs. If a large colony is established and individual frogs marked by liquid N_2 branding, then consistently unsuccessful breeders can be eliminated from the colony.

Collection and Staging. The embryos that are found on the tank bottom following mating are covered in a viscous, sticky jelly coat. It was decided that this jelly coat should be removed (dejellying) for FETAX because it slowed production and several experiments suggested its presence did not alter toxicity. Two methods can be employed to remove the embryos from the tank. The first method is to simply invert a Petri dish and place it on the bottom of the tank. Move the dish slowly into the eggs with an even motion. The embryos will pile up on the leading edge of the dish. When a sufficient number accumulate, slowly invert the dish raising the edge with the embryos first. With practice, the embryos will fall into the open dish after it is inverted. A spatula can then be used to scrap the embryos into a 125 ml bottle or Erlenmeyer flask for dejellying. The second method is easier for the novice but requires more dejellying solution. If a small breeding aquarium is used and the distance from the support mesh to the bottom is kept about 2.5 cm, then it is possible to dejelly the embryos in the breeding aquarium. This also helps recover embryos stuck to the support mesh and the embryos can simply be poured out into a large beaker. The greatest danger of this technique is to continue the dejellying process too long. If this occurs, the embryos may be irreversibly damaged and will not be usable in FETAX. Perhaps the best approach is to pour the embryos into the flask

LC50 by the 96-hr EC50 (malformation). The ASTM taskforce agreed that values less than 1.5 indicate little developmental hazard while values greater than 1.5 indicate increasing developmental hazard. It is very rare that TI values exceed 1,000 and developmental toxicants more commonly have TIs in 10-200 range.

The MCIG is the Minimum Concentration to Inhibit Growth and is calculated by measuring the head-tail length of each embryo following the contour of the embryo. The embryos are fixed in 3.0% formalin prior to this procedure and fixation does not seem to alter embryo length. Length data from each concentration set are compared to control length data using the T-test for grouped observations. The lowest concentration set that inhibits growth at the $p=0.05$ level of significance is the MCIG. The data is usually plotted as the percentage of control versus the percentage of the 96-hr LC50. This makes it possible to compare results from different test materials.

Many other endpoints are possible in FETAX as long as the required endpoints are performed. Additional endpoints such as pigmentation, locomotion and hatchability are described in the ASTM New Standard Guide (Bantle and Sabourin, 1991). An EC50 can usually be obtained for all three of the latter endpoints. However, pigmentation and locomotion are very subjective and much work needs to be done to make data collection objective.

Exposure Regimens

Dumont initially used a four day static exposure in all the early work performed in his laboratory (Dumont et al., 1979; Dumont and Schultz, 1980). The advantage of this exposure regimen is that it is very gentle on the embryos. Test material is only added at the beginning of the test. Dead embryos were removed every 24 hrs to prevent bacteria from multiplying and killing other embryos. However, decomposition of the test material is a real possibility and the ASTM task force decided that the static-renewal (also called "renewal") procedure was the best compromise for standard FETAX. Under this exposure regimen, test material is renewed every 24 hrs. Dead embryos are removed at these times. This also helps reduce the number of bacteria and fungi in the dish. When rat liver microsomes for metabolic activation are used in the test, they also are renewed at this time along with the generator system.

It is possible to use continuous exposure with FETAX (Bantle and Sabourin, 1991). One simple method of exposing the embryos is to cut the bottom off of 25 ml beakers and glue 50 micron monofilament mesh to the open bottom using a small amount of silicon sealant. The embryos are placed inside and the beaker clipped to the sidewall of an exposure tank. In this manner FETAX can be conducted simultaneously with other exposure assays such as acute fish toxicity tests. Because of the limited availability of diluters for most labs and their attendant costs, the 24-hr renewal procedure was chosen as the best exposure compromise.

Recently, Greg Linder (Linder et al., 1990) has developed an exposure chamber for *in situ* exposure of *Xenopus* or other amphibian embryos. This chamber is constructed of a 2.5 cm wide and 7.0 cm diameter circlet of large plastic polyethylene mesh (1 mm mesh size). This material can be purchased from hobby shops. The circlet is held together by means of a small nylon bolt and nut. Two large 7.5 cm disks of the same mesh are pressed to each side of the circlet and held by a centrally-located nylon bolt and wing nut. The inside of the large mesh is lined with teflon 50-100 micron mesh to keep the embryos from passing through the larger mesh. The assembled chamber with embryos is lowered into the site just below the surface of the water and anchored in place using a stainless steel stake. A common fishing bobber is used to locate the chamber and each chamber is checked daily.

FETAX POSITIVE AND NEGATIVE CONTROLS

It is necessary to use appropriate controls in order for FETAX data to be valid. The standard negative control is 4 dishes of 25 embryos each in either a standard buffer solution (FETAX solution) or diluent water. With 100 embryos in the control group, a single death or malformation is only a 1% response. Mortality must be $< 10\%$ and malformation $< 7\%$ for the test to be valid. Generally, mortality and malformation rates can be held to 5% or less. When carrier solvents are to be used to solubilize water-insoluble materials, appropriate solvent controls must be performed to ensure the solvent is not causing mortality or malformations. Even with these controls it is still difficult to discount the possibility that the carrier solvent is not interacting with the test material to cause synergism or antagonism.

When a positive control is required, 6-aminonicotinamide is used to cause a predicted level of mortality or malformation (Bantle and Sabourin, 1991). Concentrations of 5.5 and 2,500 mg/l are used. At 5.5 mg/l, malformation should be between 40 and 60%, while at 2,500 mg/l mortality should be between 40 and 60%. 6-aminonicotinamide was selected as a reference toxicant because it has a TI of 455 representing good separation between mortality and malformation concentration-response curves. It is relatively stable and solution concentrations can be checked spectrophotometrically at A_{257}. The compound has an extinction coefficient of 13.9 mM at a pH of 1.8. Growth inhibition is not considered with the 6-aminonicotinamide reference toxicant.

Cyclophosphamide is used as a reference toxicant for the metabolic activation system (MAS). At 4.0 mg/ml cyclophosphamide and no MAS, there should be no mortality. However, if MAS and generator system are included, then 100% mortality can be expected. If there is less than this level of mortality at 96 hr, then the MAS

did not bioactivate properly and the test must be repeated.

As expected, the controls are critical in establishing the reliability of the data. Every effort needs to be made to set up controls accurately. If there are enough embryos during test set up, it is advisable to set up at least two plastic dishes as a control for proper dish washing techniques of the glass Petri dishes. If mortality, malformation and growth inhibition are greater in the glass dishes than the plastic dishes, then it is likely that either the glassware was not cleaned properly or that residual detergent was present in the dishes. If mortality and malformation in the controls remain high and the solution becomes slightly turbid with time, then it is possible that bacterial contamination is causing these effects. In subsequent experiments, at least two dishes of 25 embryos each should be made up with 100 U/ml Penicillin G and 100 U/ml Streptomycin sulfate. If this improves the mortality and/or malformation rates relative to standard controls, then bacterial contamination is the cause of the problem. The experiments can be repeated, using penicillin and streptomycin in all the dishes although a caution will have to be added when presenting the data. Although there are no data indicating antibiotic-test material interactions, the possibility of interactions cannot be eliminated.

FETAX METHODOLOGY

Introduction

Both the ASTM New Standard Guide (Bantle and Sabourin, 1991) and the "Atlas of Abnormalities" (Bantle et al., 1991) adequately cover basic FETAX methodology. This section will emphasize rationale and methods not included previously that will make it easier to perform the assay and understand the data. The strict format of ASTM does not allow an explanation of why procedures are carried out and the "Atlas of Abnormalities" provides photographic information not allowed in the ASTM guide.

Preparation

Animals. Animal husbandry is the greatest single determinant in the success of FETAX. *Xenopus* can be purchased from a number of commercial sources but many supply very small adults that will breed but not provide the 700 plus embryos required for most tests. Seven hundred are usually required for the controls and a concentration series. Proven breeders should be purchased and animals should not be "bred out" or bred within the preceding 60 days. Animals that have been bred too often and not conditioned prior to breeding generally degrade over time and are termed bred out. About 60 days between breedings are required for the frogs to produce and adequate number of gametes. Males should be 7.5 to 10 cm in crown-rump length and females 10-12.5 cm. In nature, it is likely that the female will continue to increase clutch size as she ages. Some females can produce 4,000 plus eggs per mating. However, for a variety of reasons, the literature suggests that animals kept in laboratory situations progressively decline in their reproductive capabilities. Every attempt should be made to provide quality care for the adults. This process is known as conditioning and entails feeding the frogs daily and changing their water each day (if the adults are kept in static tanks) at least three weeks prior to breeding. Because their food is typically beef liver and/or lung, considerable accumulation of debris may build up on the side of the tank. An inexpensive toilet cleaning brush may accompany each aquarium and be used to clean the sidewalls prior to water changes. Having a brush for each aquarium helps to keep down the spread of disease.

Although very hardy and able to spend long periods in foul water without contracting disease, *Xenopus* can still become diseased, most notably with redleg and nematode infections. The "Atlas" covers the recognition and treatment for both diseases. However, proper cleaning and care may be more effective than any treatment regimen in reducing mortality in the colony. Sick frogs stop eating even before they manifest any outward sign of disease. As frogs sicken, their skin color darkens and subsequently sloughs off. Care must be exercised here as *Xenopus* have the ability to change skin color with their surroundings. In the advanced stages of any disease, the frogs waste away; a process that takes several days. Diseased frogs should be removed from the colony as soon as possible in order to reduce the probability that healthy frogs will be subsequently infected.

Another problem that must be overcome is cyclical breeding. Despite housing the adults in a lightproof room and placing them on a constant 12 hr day/night light cycle and rigidly controlling the water temperature ($23 \pm 3°C$), *Xenopus* still seem to display seasonality in their breeding. When breeding success seems to decline in the Fall despite the best animal husbandry, it is time to increase the number of breeding pairs in order to increase the chances of success. In Spring, two pair should suffice while in Fall, it may be necessary to breed three pairs. However, the embryos from separate breeding pairs must never be mixed. This caution stems from the observation that some animals produce abnormal offspring even though the early embryos are initially acceptable. By mixing embryos, it may invalidate a test which would otherwise be successful. If embryo numbers are few, the test should be scaled back or converted to a small range-finder experiment.

Another caution is to make sure that the temperature of the animal room housing the adults does not fall below 21° C. Older literature suggests that reducing the temperature below this level prevents the females from shedding their eggs. Temperatures above 26° C can be harmful to embryonic development. This means that the animal room temperature should be precisely controlled even if it becomes necessary to install electric heaters or air conditioning. While precise data is unavailable, it seems that the animals can sense impending weather fronts and that this may affect mating and egg deposition. Good animal husbandry remains as much an art as it is a science and experience and patience play important roles in the success of any test employing live animals.

testing purposes, it showed that FETAX gives predictable results with a wide variety of known developmental toxicants. The confidence intervals are narrow in most cases and the results are repeatable. In this list only actinomycin D (false negative- [FN]), isoniazid (false positive- [FP]), coumarin (false positive- [FP]) and zinc (false positive- [FP]) (Table 1) gave incorrect results. It must be remembered that considerable controversy surrounds the classification of many of these compounds as mammalian and human teratogens. This makes it difficult to carry out this type of study.

SUMMARY AND CONCLUSIONS

FETAX is a useful developmental toxicity test that has already been used in assaying environmental mixtures both in a laboratory setting, a biomonitoring trailer and *in situ*. FETAX can be used for ecotoxicological risk and for human health. The validation study and the *in vitro* metabolic activation system support the latter use of FETAX. An ongoing interlaboratory validation study suggests that FETAX will prove to be repeatable and reliable. Once the basic assay is performed, FETAX is flexible enough to allow a number of variations in terms of exposure, endpoints and species used. This can provide additional valuable data. The greatest drawback to the assay is occasional breeding problems. Once good embryos are available, however, FETAX is straightforward and provides excellent data.

Multispecies testing is needed to adequately safeguard the environment. Additionally, acute toxicity tests alone may not suffice. It is likely that reproductive and developmental toxicity tests will be needed along other testing to adequately evaluate the hazards posed by environmental toxicants. An effort is already being made to replace FETAX with even simpler molecular-based tests. These tests involve special dyes which quantitatively fluoresce when excited by light. The dyes can give a specific indication as to cell pH, membrane function, DNA content etc. (unpublished). FETAX will serve to indicate the predictive value of these new tests since results will be compared between the new cellular tests and FETAX. Because a consistent protocol was followed in FETAX it will be possible to compare results. Additional efforts must be expended to ensure that developmentally relevant, rapid and inexpensive tests are available to the scientific community.

ACKNOWLEDGEMENT

This work support by U.S. Army Medical Research and Development Contract #DAMD17-91-C-1048. The opinions, interpretations, conclusions and recommendations are those of the author and not necessarily endorsed by the U.S. Army. In conducting research using animals, the investigator adhered to the "Guide for Care and Use of Laboratory Animals", prepared by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research council (NIH Publication No. 86-23, Revised 1985). Special thanks goes to Jim Rayburn and Mendi Hull for their assistance in the laboratory and in preparation of this manuscript.

REFERENCES

Abbasi SA, Soni R: Teratogenic effects of chromium(VI) in the environment as evidenced by the impact of larvae of amphibian *Rana tigrina*: Implications in the environmental management of chromium. Int J Environ Stud 23(2):131-138, 1984.

Anderson RJ, Prahlad KV: The deleterious effects of fungicides and herbicides on *Xenopus laevis* embryos. Arch Environ Contam Toxicol 4:312-323, 1976.

Bancroft R, Prahlad KV: Effect of ethylenebis [dithiocarbamic acid] disodium salt (nabam) and ethylenebis [dithio carbamato] manganese (maneb) on *Xenopus laevis* development. Terat 7:143-150, 1973.

Bantle JA, Courchesne CL: The combined use of genotoxicity and whole embryo teratogenicity screening assays in predicting teratogenic risks. Proc 2nd Int Conf Ground-Water Qual Res, Okla Stillwater, pp. 175-176, 1985.

Bantle JA, Dawson DA: Uninduced rat liver microsomes as an *in vitro* metabolic activation system for the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). In: Aquatic Toxicology and Hazard Assessment: Tenth volume, edited by WG Landis, WH van der Schalie, ASTM STP 971, pp. 316-326. Philadelphia, Pa: ASTM, 1988.

Bantle JA, Fort DJ, Dawson DA: Bridging the gap from short-term teratogenesis assays to human health hazard assessment by understanding common modes of teratogenic action. In: Aquatic Toxicology and Hazard Assesment: Twelth volume, edited by WG Landis, WH van der Schalie, ASTM STP 1096, pp. 46-58, Philadelphia, Pa: ASTM, 1989a.

Bantle JA, Fort DJ, James BL: Identification of developmental toxicants using the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). Hydrobiol 188/189: 577-585, 1989b.

Bantle JA, Fort DJ, Rayburn JR, DeYoung DJ, Bush SJ: Further validation of FETAX: Evaluation of the developmental toxicity of five known mammalian teratogens and non-teratogens. Drug Chem Toxicol 13(4): 267-282, 1990a.

Bantle JA, Dumont JN, Finch R, Linder G: Atlas of abnormalities: A guide for the performance of FETAX. Stillwater: Oklahoma State Publications Department, 1990b.

Bantle JA, Sabourin TD: New standard guide for conducting the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). ASTM, In Press, 1991.

Birge WJ, Black JA: Copper in the Environment, pt II. Health effects. New York, Wiley, 1979.

Birge WJ, Black JA, Westerman AG, Hudson JE: The Biogeochemistry of Mercury in the Environment. Elsevier/North-Holland Biomedical Press, 1979.

Browne CL, Dumont JN: Toxicity of selenium to developing *Xenopus laevis* embryos. J Toxicol Environ Health 5:699-709, 1979.

Browne CL, Dumont JN: Cytotoxic effects of sodium selenite on *Xenopus laevis* tadpoles. Arch Environ Contam Toxicol 9:181-191, 1980.

Cabejszed I, Wojcik J: Trial application of tadpoles of *Xenopus laevis* for estimation of harmfulness of pesticides in water. Rocz Panstq Zakl Hig 19:499-506, 1968.

Chang LW, Reuhl KR, Dudley AW: Effects of methylmercury chloride on *Rana pipiens* tadpoles. Environ Res 8:82-91, 1974.

Cooke AS: The effects of DDT, Dieldrin, and 2,4-D on amphibian spawn and tadpoles. Environ Pollut 3:51-68, 1972.

Courchesne CL, Bantle JA: Analysis of the activity of DNA, RNA, and protein synthesis inhibitors on *Xenopus* embryo development. Teratogenesis Carcinog Mutagen 5:177-193, 1985.

Davis KR, Schultz TW, Dumont JN: Toxic and teratogenic effects of selected aromatic amines on embryos of the amphibian *Xenopus laevis*. Arch Environm Contam Toxicol 10:371-391, 1981.

Dawson DA, McCormick CA, Bantle JA: Detection of teratogenic substances in acidic mine water samples using the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). J Appl Toxicol 5(4):234-244, 1984.

Dawson DA, Bantle JA: Development of a reconstituted water medium and initial validation of FETAX. J Appl Toxicol 7:237-244, 1987a.

Dawson DA, Bantle JA: Co-administration of methylxanthines and inhibitor compounds potentiates teratogenicity in *Xenopus* embryos. Terat 35:221-227, 1987b.

Dawson DA, Fort DJ, Smith GL, Newell DL, Bantle JA: Comparative evaluation of the developmental toxicity of nicotine and cotinine with FETAX. Teratogenesis Carcinog Mutagen 8:329-338, 1988a.

Dawson DA, Stebler E, Burks SA, Bantle JA: Teratogenicity of metal contaminated sediment extracts to frog (*Xenopus laevis*) and fathead minnow (*Pimephales promelas*) embryos. Environ Toxicol Chem 7(1):27-34, 1988b.

Dawson DA, Fort DJ, Newell DL, Bantle JA: Developmental toxicity testing with FETAX: Evaluation of five validation compounds. Drug Chem Toxicol 12(1):67-75, 1989.

Dawson DA, Schultz TW, Baker LL, Wilke TS: Comparative developmental toxicity of acetylenic alcohols on embryos and larvae of *Xenopus laevis*. In: Aquatic Toxicology and Risk Assesment: Thirteenth Volume, edited WG Landis, WH van der Schalie. pp.267-277. ASTM STP 1096. Philadelphia, PA: ASTM, 1990a.

Dawson DA, Schultz TW, Baker LL, Mannar A: Structure-activity relationships for oesteolathyrism. III. Substituted thiosemicarbazides. J Appl Toxicol 10(1):59-64, 1990b.

Dawson DA, Wilke TS: Initial evalution of developmental malformation as an endpoint in mixture toxicity hazard assessment for aquatic vertebrates. Ecotox Environ Safety 21:215-226, 1991a.

Dawson DA, Wilke TS: Evaluation of the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) as a model system for mixture toxicity hazard assessment. Environ Toxicol Chem 10:941-948, 1991b.

Dawson DA, Schultz TW, Baker LL: Structure-activity relationships for osteolathyrism: IV. *Para*-substituted benzoic acid hydrazides and alkyl carbazates. Environ Toxicol Chem 10(4):455-461, 1991a.

Dawson DA, Wilke TS, Schultz TW: Joint action of binary mixtures of osteolathyrogens at malformation-inducing concentrations for *Xenopus* embryos. J Appl Toxicol 11(6):415-421, 1991b.

Dawson DA, Schultz TW, Shroeder EC: Laboratory Care and Breeding of the African Clawed Frog. Lab Animal 21: 3136, 1992.

Deuchar EM: *Xenopus laevis* and developmental Biology. Biol Rev 47:37-112, 1972.

Deuchar EM: *Xenopus: The South African Clawed Frog*. New York: Wiley, 1975.

DeYoung DJ, Bantle JA, Fort DJ: Assessment of the developmental toxicity of ascorbic acid, sodium selenate, coumarin, serotonin, and 13-cis retinoic acid using FETAX. Drug Chem Toxicol 14(1&2):127-141, 1991.

Dial N: Methylmercury: Teratogenic and lethal effects in frog embryos. Terat 13:327-334, 1976.

Dumont JN, Schultz TW, Jones RD: Toxicity and teratogenicity of aromatic amines to *Xenopus laevis*. Bull Environ Contam Toxicol 22:159-166, 1979.

Dumont JN, Schultz TW: Effects of coal-gasification sour water on *Xenopus laevis* embryos. J Environ Sci Health A15:127-138, 1980.

Dumont JN, Schultz TW, Buchanan MV, Kao GL: Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) - a short-term assay applicable to complex environmental mixtures, In: Symposium on the Application of Short-Term Bioassays in the Analysis of Complex Environmental Mixtures, edited by M. Waters et al., pp. 393-405, New York: Plenum Press, 1983a.

Dumont JN, Schultz TW, Epler RG: The response of the FETAX to mammalian teratogens. Terat 27(2):A39-A40, 1983b.

Dumpert K, Zietz E: Platanna (*Xenopus laevis*) as a test organism for determining the embryotoxic effects of environmental chemicals. Ecotoxicol Environ Safety 8:55-74, 1984.

Dumpert K: Tests with the South African clawed toad (*Xenopus laevis*) for detecting chemical causes of the decrease of amphibians. Chemosphere 15(6):807-811, 1986.

Dumpert K: Embryotoxic effects of environmental chemicals: Tests with the South African clawed toad (*Xenopus laevis*). Ecotoxicol Environ Saf 13(3):324-338, 1987.

Fort DJ, Dawson DA, Bantle JA: Development of a metabolic activation system for the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX). Teratogenesis Carcinog Mutagen 8:251-263, 1988.

Fort DJ, James BL, Bantle JA: Evaluation of the developmental toxicity of five compounds with the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX). J Appl Toxicol 9(6):377-388, 1989.

Fort DJ, Bantle JA: Use of Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) and an exogenous metabolic activation system to evaluate the developmental toxicity of diphenylhydantoin. J Fund Appl Toxicol 14:720-733, 1990a.

Fort DJ, Bantle JA: Analysis of the developmental toxicity of isoniazid using exogenous metabolic activation systems with Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). Teratogenesis Carcinog Mutagen 10:463-476, 1990b.

Fort DJ, Rayburn JR, DeYoung DJ, Bantle JA: Developmental toxicity testing with Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX): Efficacy of Aroclor 1254-induced exogenous metabolic activation system. Drug Chem Toxicol 14(1&2):143-160, 1991.

Friedman M, Rayburn JR, Bantle JA: Developmental toxicology of potato alkaloids in the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX). Food Chem Toxicol 29:537-547, 1990.

Ghate HV, Mulherkar L: Effect of sodium diethyldithiocarbamate on developing embryos of the frog *Microhyla ornata*. Indian J Exp Biol 18(9):1040-1042, 1980.

Ghate HV: Notochordal anomaly in frog embryos exposed to tetraethythiuram monosulphide and tetraethylthiuram disulphide. Toxicol Lett 19:253, 1983.

Ghate HV: Toxicity and teratogenic effects of carbon disulphide in frog embryo. Riv Biol 78(1):129-132, 1985a.

Ghate HV: Dithiocarbamate induced teratogenesis in frog embryo. Riv Biol 78(2):288-291, 1985b.

Green EV: Effects of hydrocarbon-protein conjugates on frog embryos. I. Arrest of development by conjugates of 9,10-dimethly-1,2-benzanthracene. Cancer Res 14:591-598, 1954.

Greenhouse GA: Evaluation of the teratogenic effects of hydrazine , methylhydrazine, and dimethylhydrazine on embryos of *Xenopus laevis*, the South African clawed toad. Teratol 13:167-178, 1976a.

Greenhouse GA: The evaluation of toxic effects of chemcials in freshwater by using frog embryos and larvae. Environ Pollut 11:303-315, 1976b.

Greenhouse GA: Toxicity of N-phenyl-a-naphthylamine and hydrazine to *Xenopus laevis* embryos and larvae. Bull Environ Contam Toxicol 18:503-511, 1977.

Karnofsky DA: In: Teratology: Principles and Techniques, edited by J.G Wilson, J. Warkany, p. 185, 1965.

Kay, BK, Peng, HB: *Xenopus laevis:* Practical uses in cell and molecular biology. In: Methods in Cell Biol. 36:, 1992.

Linder G, Barbitta J, Kwaiser T: Short-term amphibian toxicity tests and paraquat toxicity assessment. Aquatic Toxicology and Risk Assessment: Thirteenth volume, edited by WG Landis, WH van der Schalie. ASTM STP 1096, pp. 189-198. Philadelphia, Pa: ASTM, 1990.

NRC. Models for Biomedical Research: a new perspective. Committee on Models for Biomedical Research, Board on Basic Biology, Commission of Life sciences, National Research Council. National

Academy Press, Washington, DC, 1985, 180 pp.

Nieuwkoop PD, Faber J: Normal table of *Xenopus laevis* (Daudin), 2d ed. Amsterdam: North Holland, 1975.

Pechman JHK, Scott DE, Semlitsch RD, Caldwell JP, Vitt LJ, Gibbons JW: Declining amphibian populations: The problem of separating human impacts from natural fluctuations. Science 253:892-895, 1991.

Rayburn JR, Fort DJ, McNew R, Bantle JA: Synergism and antagonism induced by three carrier solvents with t-retinoic acid and 6-aminonicotinamide. Bull Environ Contam Toxicol 46:625-632, 1991a.

Rayburn JR, Fort DJ, DeYoung DJ, McNew R, Bantle JA: Altered developmental toxicity caused by three carrier solvents. J Appl Toxicol 11(4):253-260, 1991b.

Sabourin TD, Faulk RT, Goss LB: The efficacy of three non-mammalian test systems in the identification of chemical teratogens. J Appl Toxicol 5:227-233, 1985.

Sabourin TD, Faulk RT: Comparative evaluation of a short-term test for developmental effects using frog embryos. In: Banbury Report 26: Developmental Toxicology: Mechanisms and Risk. pp. 203-223, Cold Spring Harbor Laboratory, 1987.

Schultz TW, Cajina-Quezada M, Dumont JN: Structure-toxicity relationships of selected nitrogenous heterocyclic compounds. Arch Environ Contam Toxicol 9:591-598, 1980.

Schultz TW, Ranney TS, Riggin GW, Cajina-Quesada M: Structure-activity relationships for osteolathyrism. I. Effects of Altering the Semicarbazide Structure. Trans Am Microsc Soc 107(2):113-126, 1988.

Schultz TW, Ranney TS: Structure-activity relationships for oesteolathyrism: II. Effects of alkyl-substituted acid hydrazides. Toxicol 53:147-159, 1988.

Science Briefings: New Task Force on Declining Amphibians. Science 253:509, 1991.

Tallarida RJ, Murray RB: Manual of Pharmacologic Calculations with Computer Programs. New York: Springer-Verlag, 2nd ed. 1987.

Wake DB: Declining Amphibian Populations. Science 253: 860, 1991.

Wake DB, Morowitz HJ: Declining Amphibian Populations-a global phenomenon, report of a workshop sponsored by the Board on Biology, National Research Council, held at Irvine, CA, February 19-20, 1990.

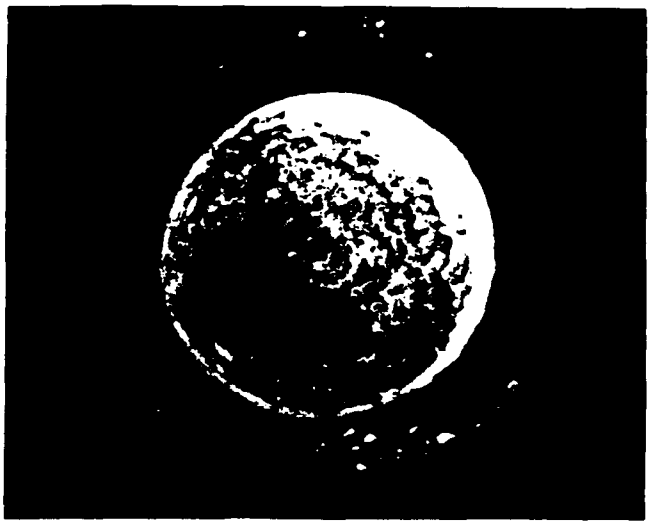


Figure 1. A Typical Blastula.

This figure shows a dorsal view of a typical *Xenopus* Stage 8 blastula as it might be collected from the bottom of the breeding aquarium. The jelly coat has not been removed. It is quite sticky and attempts to manipulate the embryo with a spatula will prove time consuming (photo by M.A. Hull).

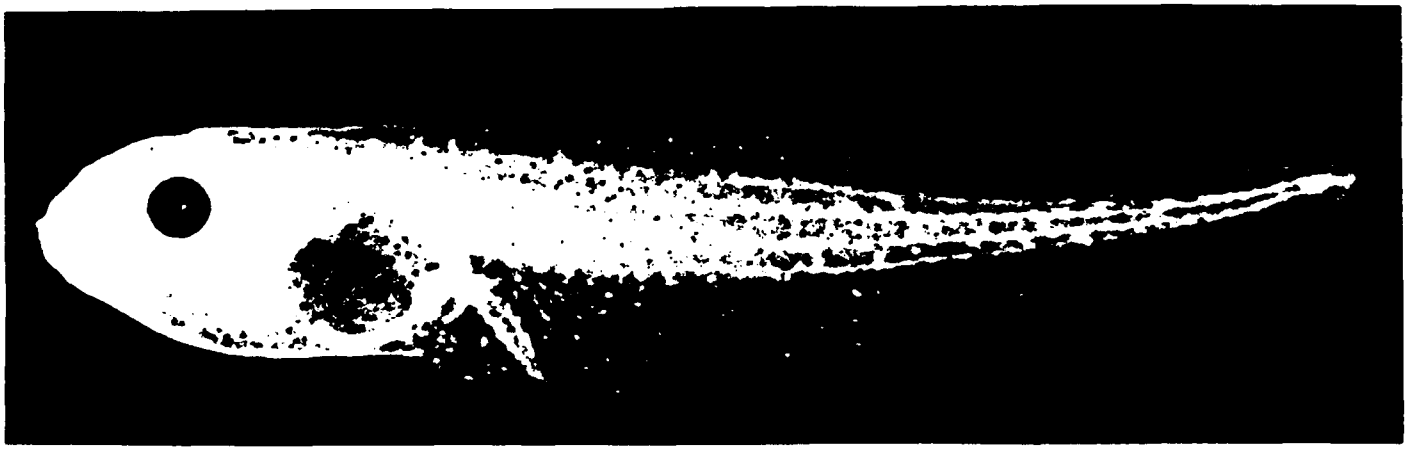


Figure 2. The Normal Stage 46 (96-hr) Larva.

A full length view. Refer to Figure ". for structures (photo by M.A. Hull).

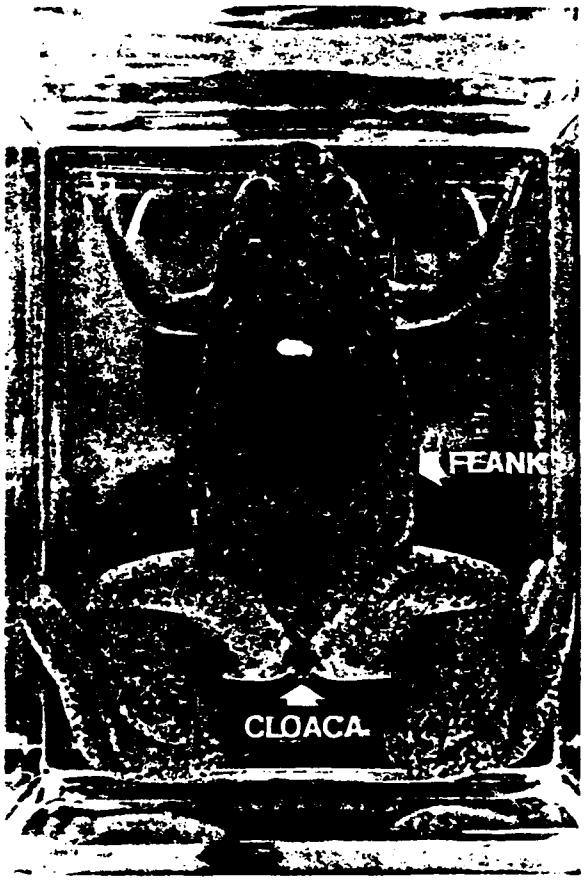


Figure 3. Normal Adult *Xenopus* Female.

Females are identified by their fleshy cloacal lips (arrow) which increase in size after injection with HCG. Gravid females show bulging flanks. This female had been bred recently so it does not show the enlarged flanks (photo by M.A. Hull).

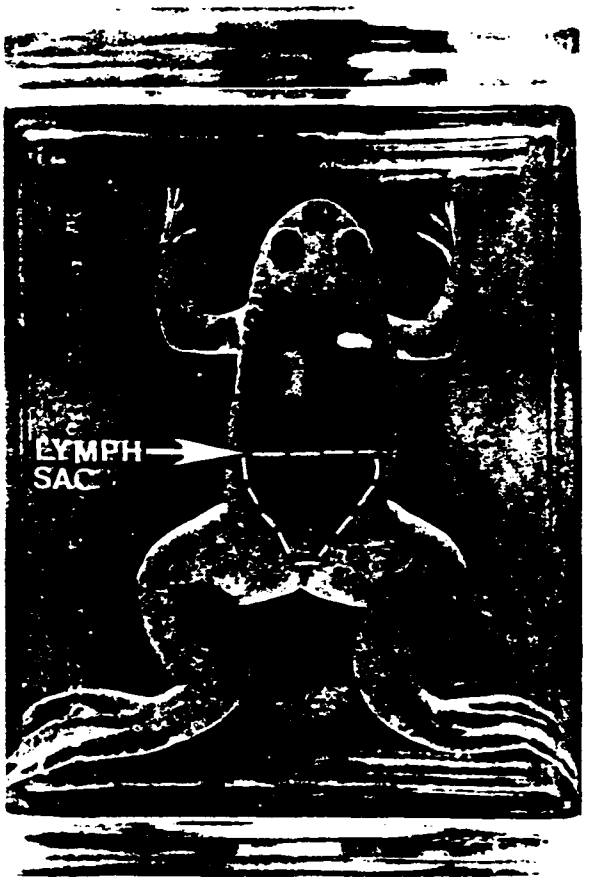


Figure 4. Normal Adult *Xenopus* Male.

Males are generally smaller than females and do not possess cloacal lips. They have dark forearm pads (nuptial pads) on the ventral surface of the hand and forearm which darken even further after HCG injection. The pale marks on the back of this male are the result of liquid nitrogen branding for identification purposes (photo by M.A. Hull).

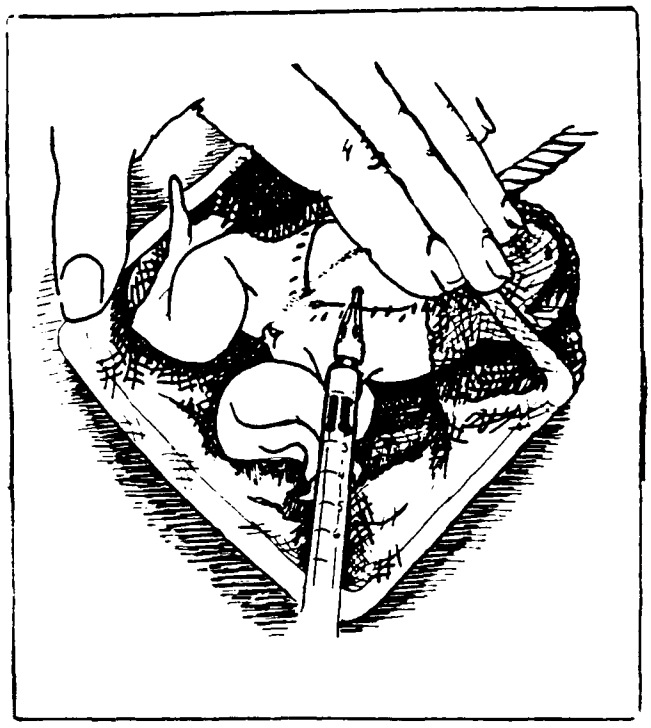


Figure 5. Method of Injecting Human Chorionic Gonadotropin (HCG).

It is easy to inject *Xenopus* by carefully immobilizing the adult in an ordinary aquarium net. Use a tuberculin syringe with a 1/2" long, 26 gauge needle to inject the HCG into the dorsal lymph sac. The lymph sac is bounded by the lateral line which runs along the side of the frog and appears as "stitching" on the skin. The dorsal lymph sac has been surrounded by a dashed white line in Figure 4. Injections should be inside (centrad) the lateral line. Note that one fourth of the needle tip enters the skin at a shallow (10-15 degree) angle. When injecting the frog, wrinkle the skin so that the injection can be administered subcutaneously. Keep the point of the needle well away from the spinal cord (sketch by D.J. DeYoung).

Figure 6. Amplexus in the Mating Tank.

A male and female frog are shown here in amplexus. A ten gallon glass aquarium has been used a mating chamber. A plastic screen made from a fluorescent light diffuser grate (available from most hardware stores) has been used as a grate to support the adults. Mating is carried out in the dark at a temperature of 23°C. FETAX solution (ASTM Standard Guide) is used as the medium and there should 2.5 inches of solution above the grate in the mating chamber. The eggs fall to the bottom where they can be scraped off into plastic Petri dishes. Another useful grate material is a 1 cm plastic mesh (Cat #XV-0350) manufactured by InterNet Inc. [2730 Nevada Ave. North, Minneapolis, MN 55427; phone (612) 541-9690]. However, this must be purchased in large quantities. Heavy duty aluminum foil is used as a top and a bubbler is used for aeration. If a tank with shorter sides is used, a weighted lid may be necessary to preclude escape (photo by M.A. Hull).

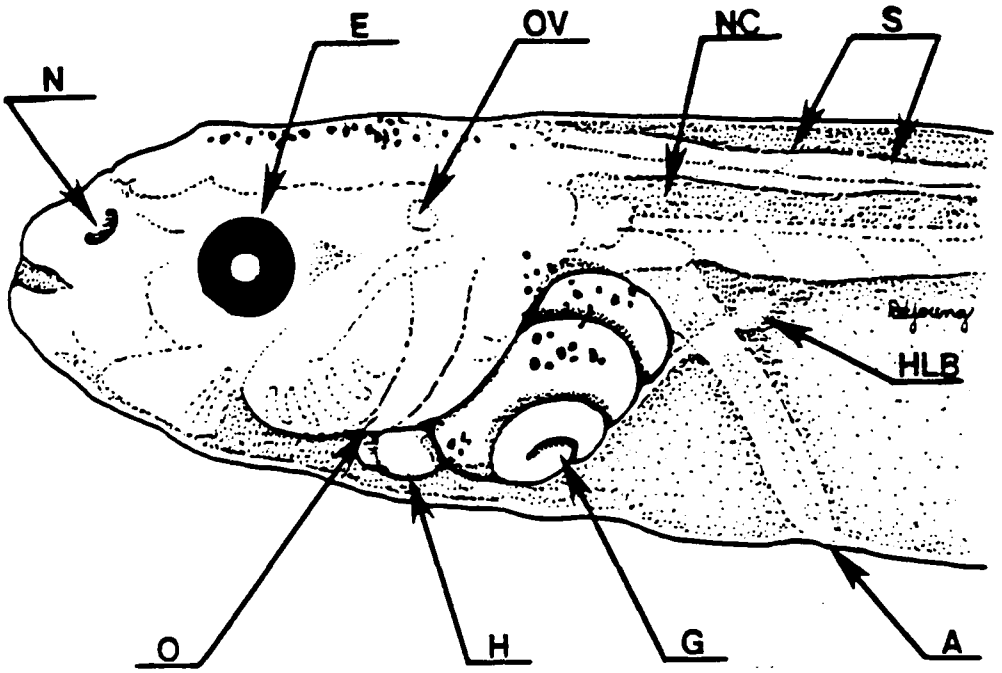


Figure 7. Diagram of the Head Region of the Stage 46 (96-hr) Larva.

A Stage 46 larva is recognized by the appearance of the hind limb bud, the coiling of the gut, and the shape of the operculum covering the gills. The best indicator that the larva has attained Stage 46 is the appearance of the hind limb bud. Gut coiling is also easily observed. N=nares, E=eye, OV=otic vesicle, S=somite, O=operculum, H=heart, G=gut, HLB=hind limb bud, A=anus (diagram by D.J. DeYoung).

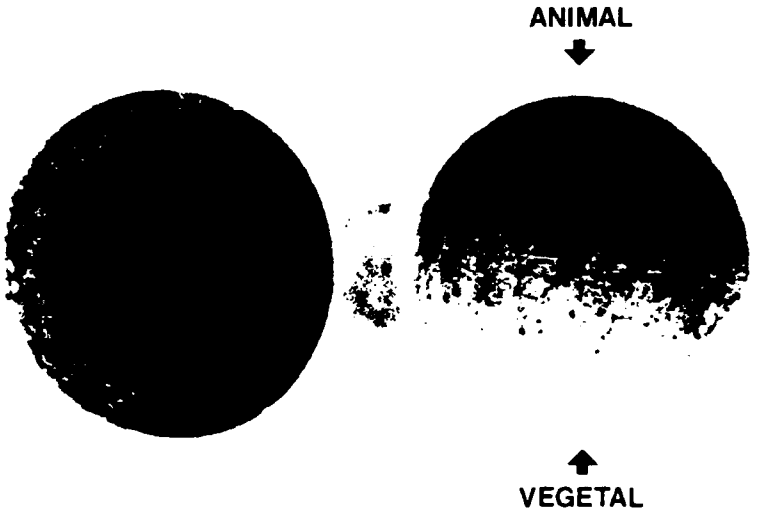


Figure 8. Normal Stage 8 Blastulae.

These are medium cell blastulae. The figure on the left is a dorsal view and the figure on the right is a lateral view. Notice that there is a gradual reduction in size of the cells in the animal hemisphere area compared to the size of the cells in the vegetal hemisphere. The lateral view of the blastula shows the gradual reduction in cell size and the progressive movement of the pigmented animal hemisphere cells down over the larger white vegetal hemisphere cells (photo by M.A. Hull).

Figure 9. Normal Stage 11 Gastrulae.

The figure on the left is a dorsal view while the figure on the right is a lateral view. The blastopore (arrow) now encircles the lower part of the embryo and the white circle of yolk cells are now referred to as a yolk plug. The lateral view shows the extent which the animal hemisphere cells have now enveloped the embryo (photo by M.A. Hull).

Figure 10. Increasing Concentrations of a Developmental Toxicant.

The effects of increasing concentration of the teratogen hydroxyurea. Note that as the concentration increases, the malformations become more severe. Concentrations from top to bottom are Control, 0.2, 0.4, and 0.7 mg/ml (photo by M.A. Hull).

Figure 11. Ventral View of a Stained Stage 46 Control Embryo.

A ventral view of the heart of a normal 96-hr *Xenopus laevis* embryo. The transparency of the embryos allows a technician to easily identify malformations. Since the embryos are transparent, this specimen was stained for clarity in the photo. H=heart, AA=aortic arches, E=eye, GR=gill rakers, G=gut, A=anus (photo by M.A. Hull).

FETAX MORTALITY DATA

Test Material:								Investigator		
Test No.		Date: / /						*Stock Concentration:		
Concentration	Stock (ml)*	FETAX (ml)	24-hr	48-hr	72-hr	96-hr	Total No.	% / dish	T M
Controls (1&2)	0 00	10 00							X
Controls (3&4)	0 00	10 00							

*T M stands for total mortality at that concentration

Figure 12. FETAX Standard Mortality Form

This form is used to record the number of dead embryos in each dish at each 24 hr period. This procedure is performed at the same time as the solution renewal for each dish.

FETAX MALFORMATION DATA

Test Material: Test No. Date: / / Investigator:

CONCENTRATION: Controls

MALFORMATION	1	2	3	4	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	TOTAL
Severe																											
Gut																											
Edema - optic																											
- cardiac																											
- abdominal																											
- facial																											
- cephalic																											
- blisters																											
Tail																											
Notochord																											
Fin																											
Face																											
Eye																											
Brain																											
Hemorrhage																											
Cardiac																											
Other-specify*																											
No. Malformed																											
Total No.																											

*Comments:

Figure 13. FETAX Standard Malformation Form.

This form is used to record the amount of different types of malformations seen in each individual dish. The number of malformed embryos are recorded at the end of the test. The judging of malformaitons entails comparing the exposed embryos in the differing concentrations to the control embryos. The control embryos are staged by comparing them to pictures of normal stage 46 embryos in the "Atlas of Abnormalities".

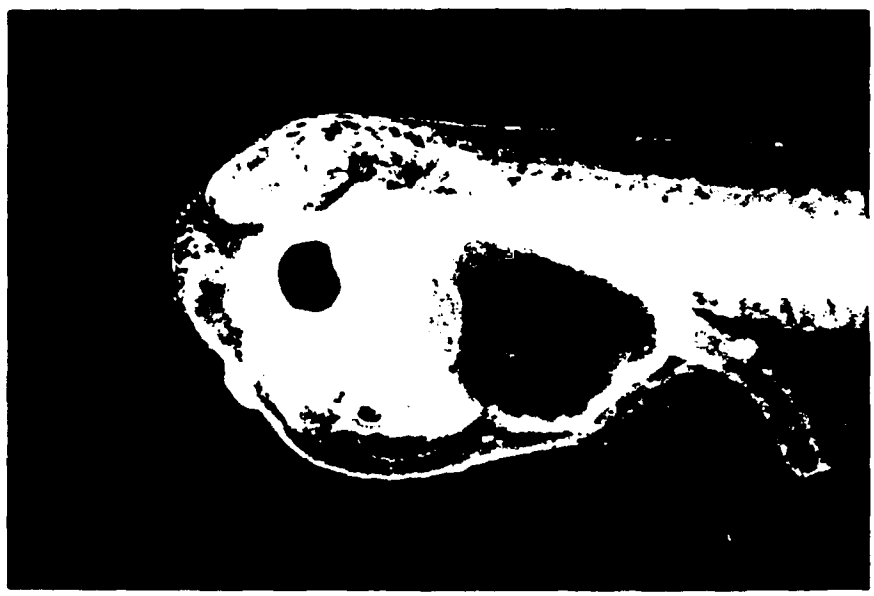


Figure 14. Embryo Exposed to a Severe Teratogen.

This embryo displays severe abnormalities. Its face, brain, eye and gills are damaged. It was exposed to 5 ng/ml of 13-cis retinoic acid (photo by M.A. Hull).

Figure 15. Contrasting the Normal 96 hr *Xenopus laevis* Embryo with One that has Facial Malformations.

Facial abnormalities are also easy to distinguish in the *Xenopus* embryo. At the tip, a 96-hr control, and below lies a severely malformed embryo. By using the control as a comparison, the malformations in the lower embryo are easily detected. The lower embryo was exposed to 0.07 mg/ml of the teratogen coumarin (photo by M.A. Hull).

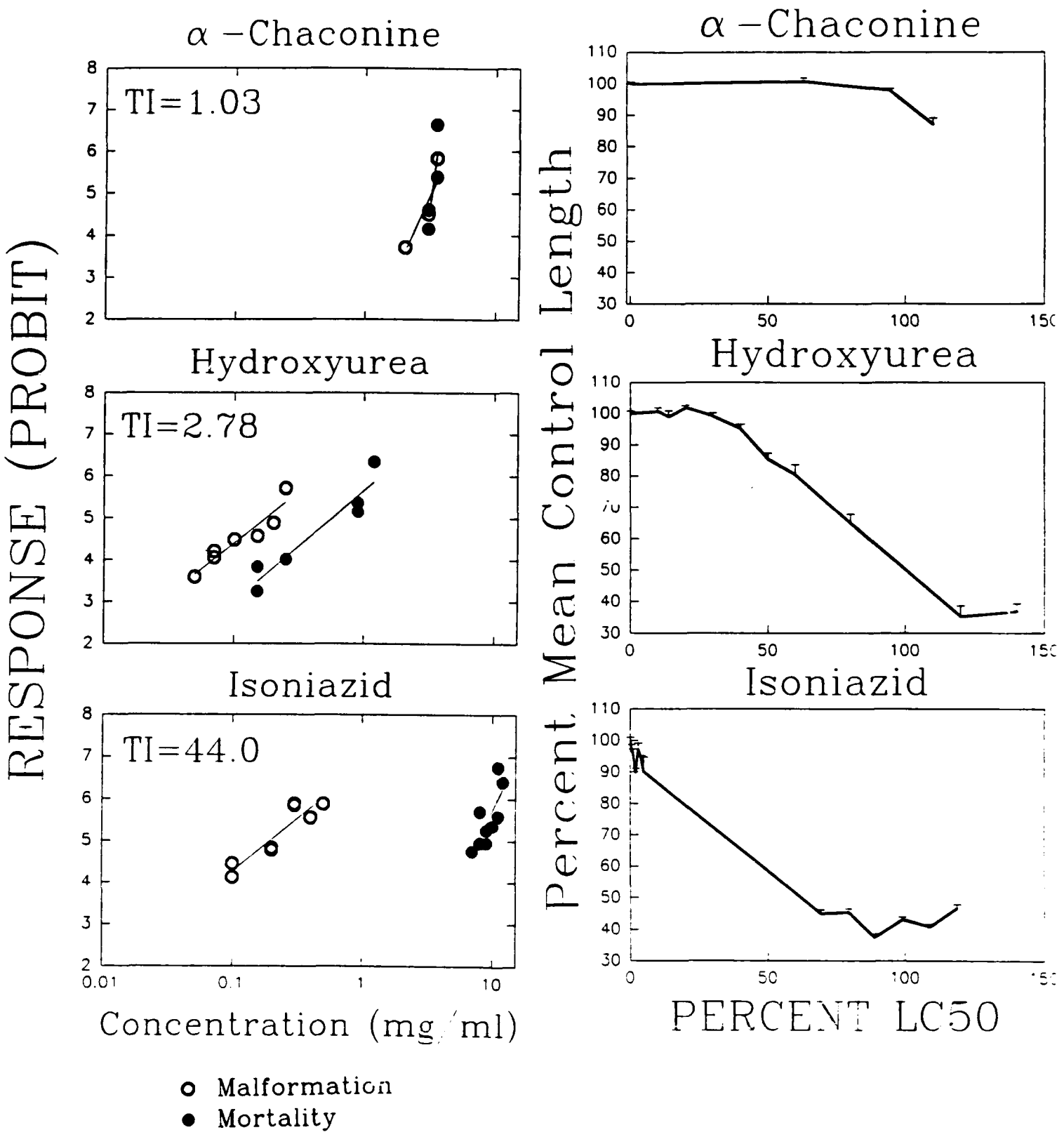


Figure 16. Probit Analysis Graphs of Chemicals Tested with FETAX.

Representative concentration-response curves and respective Teratogenic Index values for three compounds tested with FETAX.

Figure 17. Growth Graphs for Chemicals Tested with FETAX.

Representative embryo growth curves for the three compounds. Concentrations are expressed as percent of the respective compound LC50. Growth is expressed as a percent of mean FETAX solution control length.

STATUS AND FUTURE DEVELOPMENT OF FETAX

John A. Bantle
Department of Zoology
430 LSW
Oklahoma State University
Stillwater, OK
74078

ABSTRACT

Embryonic development is a weak link in the life cycle of most organisms. Many chemicals affect embryonic development at far lower concentrations than required for adult toxicity making it necessary to screen for developmental toxicants. FETAX is a 96-h developmental toxicity test that utilizes the embryos of the South African Clawed Frog *Xenopus laevis*. The assay has both human health and ecotoxicology applications depending on whether an *in vitro* rat liver microsomal metabolic activation system (MAS) is used to simulate mammalian metabolism. The endpoints of the assay are the 96-h LC50, the 96-h EC50 (malformation) and the minimum concentration that inhibits growth (MCIG). The teratogenicity of a compound is assessed by the Teratogenic Index (TI=96-h LC50/96-h EC50 (malformation)), the severity of malformations and an inhibition of growth at concentrations <30% of the 96-h LC50. The state of development and future research directions of FETAX as a screen are addressed.

INTRODUCTION

The Need for Developmental Toxicity Testing

Developmental toxicity tests are designed to detect xenobiotic agents that affect embryonic development.22. Embryonic development can be considered a "weak link" in the life cycle of an organism. During this period unique cellular and molecular processes operate to generate a complex multicellular organism from a zygote. These processes are sensitive and easily perturbed by many chemicals. Developmental toxicants are chemicals that can exert their effects on embryos at concentrations lower than that required to affect adults or cause general cellular toxicity. For example, semicarbazide causes malformation in frog embryos at 1/3000th the concentration required to kill embryos and affects embryonic growth at even lower concentrations (Schultz and Ranney, 1988). Chronic tests account for all phases of the life cycle but usually take longer to run for vertebrates than the four day test presented here. Developmental toxicity tests can then be considered sub-chronic tests that come close to predicting chronic effects in far less time and cost. While a developmental toxicant causes <u>any</u> deficit in an embryo, a teratogen has a more narrow definition in that it causes

malformation. Therefore, teratogens are a subset of developmental toxicants.

FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*) is a four day whole embryo developmental toxicity tests that utilizes the embryos of the South African Clawed frog, *Xenopus laevis*. FETAX was initially designed as an indicator of potential human developmental health hazards. The assay is well suited for complex mixtures testing and has been validated using chemicals of known mammalian developmental toxicity. The assay also is applicable to ecotoxicology.

Uses of FETAX

FETAX data can be extrapolated to other species, because a conserved genetic program controls embryonic development. If differences such as metabolic activation and placentation are taken into account, it is even possible to extrapolate the data to mammals. However, there are some features of the amphibian egg that make it unique. These features allow the use of this assay to find developmental toxicants that affect amphibians. This will help in studies designed to discover the reason for the reported world-wide disappearance of amphibians even in pristine locations (Wake and Morowitz, 1990; Wake, 1991). This decline may be due in part to normal population fluctuations caused by

climatological factors or by anthropogenic factors (Pechmann et al., 1991). In at least one case, frog eggs failed to develop in pond water but developed normally when moved to the laboratory (Science Briefings, 1991). It is, therefore, possible that some decline may be due to pollution and FETAX can be used to investigate the extent the decline is caused by environmental degradation.

When FETAX is used for ecotoxicological purposes, it must be remembered that stunted and malformed embryos would be swiftly removed from the population through the inability to feed or by predation. This means that species survival can be compromised by developmental toxicants. For humans, developmental abnormalities persist in live offspring with attendant social and health costs.

The purpose of this paper is to review the state of development of FETAX for both human health and ecotoxicology purposes and indicate the direction of current research.

MATERIALS AND METHODS

The procedure for FETAX has been standardized through the American Society for Testing and Materials (ASTM) committee process in the form of a New Standard Guide (Bantle and Sabourin, 1991). Additionally, an

atlas ("Atlas of Abnormalities- A Guide for the Conduct of FETAX") has been published that complements the ASTM guide. Both publications are available free from the author and provide a detailed description of FETAX.

Briefly, adult *Xenopus* are purchased from *Xenopus* I (Ann Arbor, MI). Male and female *Xenopus* are induced to breed by injecting human chorionic gonadotropin into the dorsal lymph sac. The animals then mate normally and eggs are collected from the aquarium the following day. The jelly coat of the eggs are removed by treatment with L-cysteine and normally cleaving blastulae are selected. Typically, a concentration series is constructed consisting of several concentrations plus controls. Two glass 60 x 15 mm Petri dishes each containing 25 embryos are used for each concentration. Therefore, each dead or malformed embryo represents a 2% response. The assay is a 4-d renewal ending with free swimming larvae that have undergone all major phases of organogenesis. Exposure to the test material is continuous and dead embryos are removed on a daily basis to reduce microbial contamination. When used for screening mammalian developmental toxicants, Aroclor 1254-induced and isoniazid-induced rat liver microsomes must also be used (Bantle et al., 1991). A balanced salt solution (FETAX solution) is used from mating onwards in order to minimize variation caused by different diluent waters

(Dawson and Bantle, 1987). Controls include a FETAX solution-only control, a positive control employing 6-aminonicotinamide, a positive metabolic activation system control employing cyclophosphamide, and a solvent control (when a cosolvent is needed to solubilize the test material). At the conclusion of the 96-h assay, mortality and gross congenital malformations are recorded and the embryos are fixed in 3% formalin for length measurements. Head-tail length is measured using a common darkroom enlarger to magnify the embryos onto a digitizing pad. Sigma Scan software (Jandel Scientific, Corta Madera, CA) is then used to measure head-tail length which is an indicator of growth. FETAX takes a single work week to perform and approximately 20 h of technician labor.

CONCLUSIONS

Developmental Toxicity Assay Design

In designing a developmental toxicity assay, it is imperative to account for the normal molecular and cellular mechanisms that guide embryonic development. As mentioned earlier, a genetic program guides development and it entails the sequential expression and repression of genes. Many of these genes are expressed for a short period only during a specific stage of embryonic development. Therefore,

genotoxic agents are often developmentally toxic as well. Five cellular mechanisms operate in concert during development and each is critical in embryogenesis. These mechanisms are cell division, interaction (induction), migration, differentiation and selective cell death. The interruption of any of these mechanisms may cause abnormal development or even embryo death. This means that endpoints of any developmental toxicity assay must consider all of these mechanisms. FETAX endpoints are the 96-h LC50, the 96-h EC50 (malformation) and the minimum concentration that inhibits growth (MCIG). These endpoints account for all important cell and molecular mechanisms since the assay is based on the whole embryo and not on embryo parts or cultured cells.

Some developmental toxicants only affect certain stages of embryonic development. The drug thalidomide only exerts its devastating effect on normal limb development and stunting during a very short period. Treatment with thalidomide before or after this period results in little or no effect. Therefore, exposure conditions in any assay must be designed to ensure that exposure occurs during all the sensitive stages. Exposure is continuous in FETAX throughout the four day period of primary organogenesis thereby ensuring that all sensitive developmental stages are affected. During the four day exposure period, the embryo proceeds from a hollow blastula stage of a few hundred cells

to a free-swimming larva that is ready to feed. All primary organogenesis is complete although limbs have not yet formed.

Lastly, it is important to note two other fundamental concepts in developmental toxicology. Karnofsky's law (Karnofsky, 1965) states that any material can be teratogenic when administered at concentrations approaching general cell toxicity. This feature will be seen later in the description of the Teratogenic Index (TI) concept. The second concept is that insult to early stages is far more deleterious than damage to later stages of development. Early injury to a primordium of cells can result in damage to whole organ systems while damage later may affect only a single organ. Damage to an early group of cells is transmitted to succeeding generations of daughter cells and the initial fault is magnified throughout the embryo. Therefore, the earlier damage occurs, the more severe and widespread the damage.

FETAX Endpoints and Assay Data

FETAX has three standard endpoints and a TI ratio calculated from two of the endpoints. Embryo death is measured by the 96-h LC50 and embryo malformations by the 96-h EC50 (malformation). Only malformations in live embryos are recorded. Standard concentration-response

experiments are performed and curves constructed using probit analysis. The probit analysis results in the appropriate LC50 or EC50 values with 95% confidence limits. Both concentration-response curves are usually placed on the same plot to demonstrate the separation between the two curves. The TI is found by dividing the 96-h LC50 by the 96-h EC50 (malformation). Values less than 1.5 indicate little developmental hazard while values greater than 1.5 indicate increasing developmental hazard. It is very rare that TI values exceed 1,000 and developmental toxicants more commonly have TIs in 10-200 range.

The MCIG is calculated by measuring the head-tail length of each embryo following the contour of the embryo. The embryos are fixed in 3.0% formalin prior to this procedure. Fixation does not seem to alter embryo length. Length data from each concentration set are compared to control length data using the tTest for grouped observations. The lowest concentration set that inhibits growth at the p=0.05 level of significance is the MCIG. The data is usually plotted as the % of control versus the % of the 96-h LC50. This makes it possible to compare results from different compounds.

The use of other endpoints are possible in FETAX as long as the required endpoints are performed. Additional endpoints such as pigmentation, locomotion and hatchability

are described in the ASTM New Standard Guide (Bantle and Sabourin, 1991). An EC50 can usually be obtained for all three. However, pigmentation and locomotion are very subjective and much work needs to be done to make data collection objective.

Data Interpretation

Although additional endpoints exist, FETAX gives concentration-response data for mortality, malformation and growth. These data can be compared with similar data on a molar basis using other pure chemicals to yield relative developmental toxicity. For example, saccharin has an LC50 of 18.4 mg/ml while hydroxyurea has an LC50 of 1.8 mg/ml. Once corrected for molarity, hydroxyurea will be at least 10 times more toxic.

For assessing the developmental toxicity of complex mixtures, it must be remembered that any significant difference between the 100% concentration and controls represents some developmental toxicity and associated hazard. It is more difficult to assign the teratogenic hazard to a test material or pure compound. Recall that a teratogen is any agent that causes malformation in living offspring. In order to rank compounds according to their teratogenic hazard, Dumont and coworkers (Dumont et al., 1983) developed the Teratogenic Index or TI. This value is

found by dividing the 96-h LC50 by the 96-h EC50 (malformation). Values <1.5 indicate little or no teratogenic hazard while values greater than 1.5 indicate increasing hazard. The TI is similar to the commonly used Therapeutic Index in Pharmacology in that a specific effect (malformation) is compared to general toxicity (mortality). The LC50 and EC50 are used in the determination of TI because they are automatically calculated by probit analysis programs and there is the least variation in confidence intervals at the 50% response level. There is some danger the TI will not correctly identify the teratogenic hazard if the slopes of the mortality and the malformation curves are different. However, this has not occurred very often. Most of the concentration curves obtained during the validation phase of FETAX demonstrated similar slopes for mortality and malformation (Fig. 1, Table 1). There is no reason to assume that the TI in FETAX will be indicative of the TI generated by another species. However, it is likely that these values will be fairly close, given that the predictive accuracy of FETAX is about 90% when compared to chemicals of known mammalian and human developmental toxicity.

Fig. 1A shows a typical FETAX concentration-response curve that will illustrate how decisions are made in assessing developmental toxicity. It is important to remember that most probit analysis programs do not run when 0 and 100% response data points are entered. Generally,

only partial response concentrations are used in establishing the curve. Therefore, there are usually many other data points collected than what appears in Fig. 1A. Each data point represents 50 embryos and it should be noted that there is a close fit of the data to the line. The 96-h LC50 for this experiment was 2.54 mg/ml while the EC50 (malformation) was 0.0045 mg/ml. The TI was quite high at 550.6 indicating considerable developmental hazard for malformation. It is very important to note the very wide concentration range that exists where all exposed embryos would be malformed but would not die. The separation of the two curves is indicative of relative developmental hazard and is represented by the TI.

Treatment with 6-aminonicotinamide causes severe malformations to all organ systems even at concentrations <30% of the 96-h LC50 (Dawson et al., 1989). This is a second criterion for predicting developmental hazard. Some compounds, such as plant glycoalkaloids, have low TIs but cause very severe malformations (Friedman et al., 1990). Saccharin and sodium cyclamate are compounds that have little developmental toxicity because they have low TIs and cause only moderate malformations even at very high concentrations (Dawson and Bantle, 1987; Dawson et al., 1989).

Growth inhibition is a third criterion of developmental hazard. Fig. 1B shows the concentration-response curve for growth inhibition. Since this particular experiment was designed to delineate the mortality and malformation curves, Fig. 1B shows some discontinuity. Experiments have previously been performed that investigated the effect of 6-aminonicotinamide on growth in the concentration range of 0.01-1.5 mg/ml (Dawson et al. 1989). This early data clearly showed significant growth inhibition (MCIG=0.01 mg/ml or about 3% of 96 h-LC50) below 30% of the 96-h LC50. Most chemicals that have high developmental toxicity cause inhibition at <30% of the 96 h-LC50 and cause a reduction >20% of control length.

In the foregoing sections it is important to realize that all three criteria play a role in assessing relative teratogenic hazard. Each criterion should be considered on its own merits and reported. Only then can teratogenicity be adequately assessed.

Past and Future Developments of FETAX that Affect All Uses of the Assay

Use of Cosolvents to Solubilize Hydrophobic Compounds

Since a number of developmental toxicants are hydrophobic, some work has been done on the use of

cosolvents in FETAX. Rayburn et al., 1991a, Rayburn et al., 1991b and Dresser et al., 1992 have used a number of cosolvents in an attempt to solubilize hydrophobic compounds. Some of the chemicals are solubilized by the cosolvents but in most cases interactions occurred between cosolvent and test chemical. This currently makes it necessary to use the lowest possible concentration of solvent. This often allows the test material to come out of solution. Better methods of solubilizing test chemicals are needed. One approach might be to continuously suspend insoluble materials by high speed mixing or sonication and deliver the suspension to the embryo.

Use of FETAX On Site

Slight modifications are being made to the assay to make it possible to conduct FETAX onsite in a biomonitoring trailer. There is a considerable advantage in performing FETAX onsite since biomonitoring wells can deliver groundwater directly to the test setup. Other bioassays can be run concurrently with FETAX using the same sample water.

Interlaboratory Study

A seven member interlaboratory study is ongoing to determine the repeatability and reliability of FETAX data. The study is divided into three phases. In the first phase,

technicians are trained and then test three compounds previously assayed in FETAX. Each lab was given the concentrations to be tested and the identity of the chemical was known. The second phase was the same as Phase I except that the study was performed using a blind testing format. In Phase III, each lab had to perform range-finding tests and then perform three full or definitive tests on each chemical using a blind testing format. Phase III closely duplicated real world testing. Between phases, FETAX protocol was to be reevaluated. Phase I was completed using the test compounds hydroxyurea, isoniazid and 6-aminonicotinamide. The coefficients of variation (CVs) for the 96-h LC50 and EC50 (malformation) were consistently below 75 except for the 6-aminonicotinamide 96-h EC50 (malformation). When the results from a single lab were eliminated, the EC50 (malformation) CV fell well below 75. The variation was deemed acceptable and the developmental toxicity of each compound was adequately assessed. The only change in protocol that was necessitated by the Phase I study was the 6-aminonicotinamide positive control. The ASTM task force had suggested that 2 dishes of 25 embryo each be set up at the anticipated 96-h LC50 and two dishes at the EC50 (malformation). The number dead or malformed had to fall within a 10-90% response range at the end of the test. This occurred in only 32 of 42 measurements. Analysis of the full concentration-response data showed excellent overall results for 6-aminonicotinamide suggesting

that the committee set too tight a range for the positive control. Contributing to the problem was the use of different lot numbers between the ASTM guide study and the interlaboratory study. This problem is now being addressed by the ASTM task force. The repeatability and reliability of FETAX data is acceptable as judged by Phase I results. The main source of variation in the study appeared to be the concentration of test chemical and not in judging malformations as expected. By the end of Phase III, the FETAX protocol should be proven.

Past and Future Developments of FETAX for use as a Screen for Developmental Toxicants that Affect Humans

Research Areas

Past research has concentrated primarily on validation and the development of a metabolic activation system (MAS) using rat liver microsomes. Validation is the process whereby known mammalian and human developmental toxicants are tested in FETAX in order to determine the predictive accuracy of the assay. The MAS substitutes for the maternal liver and placenta for metabolizing xenobiotic compounds.

Validation Study Results

Table 1 shows the results of validation studies carried out in the author's laboratory using 65 compounds. Attempts were made to select compounds from several different chemical classes and to choose nearly as many nonteratogens as teratogens. Blind testing was used for many of the compounds. In cases where metabolic activation was known to play a role in developmental toxicity in mammals, the MAS was added to FETAX. For the purposes of routine testing for hazard to humans, MAS must be routinely added to all samples. Although the validation study was of more use for human developmental toxicity testing purposes, it showed that FETAX gives predictable results with a wide variety of known developmental toxicants. The confidence intervals are narrow in most cases and the results are repeatable. In this list only actinomycin D (false negative- [FN]), isoniazid (false positive- [FP]), coumarin (false positive- [FP]) and zinc (false positive- [FP]) (Table 1) gave incorrect results according to our review of the mammalian literature. It must be remembered that considerable controversy surrounds the classification of many of these compounds as mammalian and human teratogens. Most validation schemes are based on result from testing on rodents. This makes validating an *in vitro* teratogenesis screening assay an additional step removed from humans. Additional uncertainty is introduced as a result because

rodent systems also have an error rate (Marks, 1991). This makes it very difficult to carry out this type of study. Additional validation studies need to be performed using chemicals where the human developmental toxicity has already been established. These studies should be carried out in a blind testing format and always with and without a MAS. Clearly, it will not be possible to predict human developmental toxicity in all cases but FETAX should still be useful in the preliminary screening of compounds for developmental toxicity.

Metabolic Activation System

Xenopus embryos have a limited capability to metabolize xenobiotic compounds. In order to simulate mammalian metabolism and thereby increase the predictive accuracy of FETAX, an *in vitro* MAS using rat liver microsomes was developed (Bantle and Dawson, 1988a; Dawson et al., 1988; Fort et al., 1988; Bantle et al., 1989a; Fort et al., 1989; Fort and Bantle 1990a; Fort and Bantle1990b; Bantle et al., 1991; Fort et al., 1991). Initial experimentation was performed using cultured hepatocytes and whole rat liver. However, it quickly became clear that S9 supernatant or microsomes were the best choice. Unfortunately, Aroclor 1254-induced S9 supernatant proved toxic to *Xenopus* embryos. Uninduced microsomes were then tried with better results. However, it subsequently proved possible to use Aroclor

1254-induced microsomes to effectively activate proteratogens and inactivate compounds that are normally detoxified by the mammalian liver. Aroclor 1254 is a broad spectrum inducer which helped improved the predictive accuracy to nearly 90%. Perhaps the best microsomal preparation is a 50:50 mix of Aroclor 1254-induced and Isoniazid-induced rat liver microsomes which covers a very broad spectrum of cytochrome P-450.

Even though the MAS is added daily along with fresh test compound, it must be remembered that the microsomes only work for five h after addition. Any parent test compound remaining is then free to affect the test. This makes it necessary to qualify interpretation of test results. A two fold change in TI and a significant change in the severity of malformations (Fig. 2) is sufficient to lead to a conclusion that a compound is either a proteratogen or it poses little developmental toxicity because of detoxification. When a compound is evaluated for potential human or mammalian developmental toxicity, it is necessary to test with and without microsomes. Preliminary tests with limited concentrations can be performed that will indicate whether there is significant metabolism.

Future research must performed to replace Aroclor 1254 induction with Phenobarbitol and B-napthoflavone induction because of the difficulty of disposing of treated rats and

the variability in lots of Aroclor 1254. It is possible that a continuous flow apparatus can deliver toxicant and metabolic activation system continuously so that the embryos are exposed to parent compound, intermediate metabolites and metabolites. This will help in data interpretation. Finally, work must be done on modeling human placentation if FETAX is to attain its maximum predictive accuracy. No matter how well developed and validated FETAX becomes, it will always have a failure rate.

Past and Future Developments of FETAX for use in Ecotoxicology

Research Areas

FETAX has already been used to detect the developmental toxicity
of surface and groundwaters. Sediment extracts have also been tested. When used for ecotoxicology it is not necessary to use MAS but it is important to state the goals of the test to avoid confusion with human health objectives (However, both can be performed simultaneously). Modifications of the assay are allowing *in situ* applications and it will soon be necessary to validate FETAX in the field.

Surface water, Groundwater and Sediment Extract Testing

Amphibian embryos and larvae have been previously exposed to a wide variety of toxic chemicals. Herbicides (Anderson and Prahlad, 1976), fungicides (Bancroft and Prahlad, 1973), insecticides (Cabejsezed and Wojcik, 1968), metals (Abbasi and Soni, 1984; Chang et al., 1974) and many other chemicals and mixtures have been tested in a variety of species (Cooke, 1972; Ghate and Mulherkar, 1980; Ghate, 1983; Ghate, 1985a; Ghate, 1985b; Green, 1954). The results of this work convinced early researchers that amphibian embryos were sensitive indicators of water quality. Birge's group did extensive work in comparing the relative sensitivities of several different anuran species although assay conditions often varied (Birge and Black, 1979; Birge et al., 1979). Although there are a number of more sensitive anurans, there are compelling reasons for employing *Xenopus*. *Xenopus* can be raised from birth to death in the lab and are not an endangered species. Contrary to most anurans, *Xenopus* can be induced to breed year round by injections of commercially available human chorionic gonadotropin. Lastly, the numerous offspring are transparent making identification of abnormalities easy within a short 4 d time span. These advantages far outweigh other disadvantages.

FETAX has been tested with metal contaminated surface waters and sediments and well as with groundwater contaminated with volatile organics (Dawson et al., 1984; Dawson et al., 1988b; Bantle et al., 1989b). FETAX also detected chemicals causing high malformation rates in 8 of 12 Yellowstone National Park water samples (Bantle and Peterson, 1991, unpublished). Because FETAX is a 4 d test, it is fast enough to be used in TI/TRE studies. The department of Ecology of the State of Washington is currently evaluating FETAX as a test for soil and sediment toxicity as a part of their biomonitoring program. A field validation program is required in order to prove that data collected with FETAX can be used to explain developmental toxicity in wild amphibian populations. Additional work needs to be performed with sample preparation and evaluation of the role of FETAX in multispecies testing formats.

In Situ Applications

Linder and Co-workers have been developing methods for *in situ* applications of FETAX at contaminated waste sites (Linder et al., 1990). They have developed plastic mesh exposure cages to allow *in situ* exposure of developing *Xenopus* embryos. The exposure cage is placed into the test matrix (e.g., sediment and water column) at on-site locations and then secured with stainless steel stakes. This *in situ* exposure method recently has been used at an

abandoned mine site in Montana. Care must be taken in performing these experiments to prevent the release of organisms in areas where the frogs can over winter.

SUMMARY

FETAX is a useful developmental toxicity test that has already been used in assaying environmental mixtures both in a lab setting, a biomonitoring trailer and *in situ*. FETAX can be used for ecotoxicological purposes and for human health. The validation study and the *in vitro* metabolic activation system allow the latter use of FETAX. An ongoing interlaboratory validation study suggests that FETAX will prove to be repeatable and reliable. Once the basic assay is performed, FETAX is flexible enough to allow a number of variations in terms of exposure, endpoints and species used. This can provide additional valuable data. The greatest drawback to the assay is occasional breeding problems. Once good embryos are available, however, FETAX is straightforward and provides excellent data.

It is likely that multispecies testing is needed to adequately safeguard the environment. Additionally, acute toxicity tests alone may not suffice. It is likely that reproductive and developmental toxicity tests will be needed along with mutagenesis, carcinogenesis and other testing to adequately evaluate the hazards posed by environmenta.

toxicants. An effort is already being made to replace FETAX with even simpler cellular based tests. FETAX will serve to indicate the predictive value of these new tests. Results will be compared between the new cellular tests and FETAX. Because a consistent protocol was followed in FETAX it will be possible to compare results. Additional efforts must be expended to ensure that developmentally relevant, rapid and inexpensive tests are available to the scientific community.

ACKNOWLEDGEMENT

This work support by U.S. Army Medical Research and Development Contract #DAMD17-91-C-1048. The opinions, interpretations, conclusions and recommendations are those of the author and not necessarily endorsed by the U.S. Army. In conducting research using animals, the investigator adhered to the "Guide for Care and Use of Laboratory Animals", prepared by the committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research council (NIH Publication No. 86-23, Revised 1985). I thank Jim Rayburn and Mendi Hull for their assistance in the laboratory and in preparation of this manuscript.

REFERENCES

Abbasi, S.A., and Soni, R. 1984. Teratogenic effects of chromium(VI) in the environment as evidenced by the impact of larvae of amphibian *Rana tigrina*: Implications in the environmental management of chromium. *Int. J. Environ. Stud.* 23(2):131-138.

Anderson, R.J., and Prahlad, K.V. 1976. The deleterious effects of fungicides and herbicides on *Xenopus laevis* embryos. Arch. Environ. Contam. Toxicol. 4:312-323.

Bancroft, R., and Prahlad, K.V. 1973. Effect of ethylenebis [dithiocarbamic acid] disodium salt (nabam) and ethylenebis [dithio carbamato] manganese (maneb) on *Xenopus laevis* development. Terat 7:143-150.

Bantle, J.A., and Dawson, D.A. 1988. Uninduced rat liver microsomes as an *in vitro* metabolic activation system for the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). In: *Aquatic Toxicology and Hazard Assessment: Tenth volume*, W.G. Landis, W.H. van der Schalie, eds. ASTM STP 971, pp. 316-326. Philadelphia, Pa.

Bantle, J. A., Fort, D.J., and Dawson, D.A. 1989a. Bridging the gap from short-term teratogenesis assays to human health hazard assessment by understanding common modes of teratogenic action. In: *Aquatic Toxicology and Hazard Assesment: Twelth volume*, W.G. Landis and W.H. van der Schalie eds. ASTM STP 1096, pp. 46-58, Philadelphia, Pa.

Bantle, J.A., Fort, D.J., and James, B.L. 1989b. *Identification of developmental toxicants using the Frog Embryo Teratogenesis Assay-Xenopus* (FETAX). Hydrobiol 188/189: 577-585.

Bantle, J.A., Dumont, J.N., Finch, R., and Linder G. 1991. *Atlas of abnormalities: A guide for the performance of FETAX.* Stillwater: Oklahoma State Publications Department.

Bantle, J.A., and Sabourin, T.D., 1991. *New standard guide for conducting the Frog Embryo Teratogenesis Assay-Xenopus* (FETAX). American Society for Testing and Materials., Special Publication E1439-91, pp 1-11.

Birge, W.J., and Black, J.A. 1979. *Copper in the Environment*, pt II. Health effects. New York, Wiley.

Birge, W.J., Black, J.A., Westerman, A.G., and Hudson, J.E. 1979. *The Biogeochemistry of Mercury in the Environment*. Elsevier/North-Holland Biomedical Press.

Cabejszed, I., and Wojcik, J. 1968. Trial application of tadpoles of *Xenopus laevis* for estimation of harmfulness of pesticides in water. Rocz. Panstq. Zakl. Hig. 19:499-506.

Chang, L.W., Reuhl, K.R., and Dudley, A.W. 1974. Effects of methylmercury chloride on *Rana pipiens* tadpoles. Environ. Res. 8:82-91.

Cooke, A.S., 1972. The effects of DDT, Dieldrin, and 2,4-D on amphibian spawn and tadpoles. Environ. Pollut. 3:51-68.

Dawson, D.A., McCormick, C.A., and Bantle, J.A. 1984. Detection of teratogenic substances in acidic mine water samples using the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). J. Appl. Toxicol. 5(4):234-244.

Dawson, D.A., and Bantle, J.A. 1987. Development of a reconstituted water medium and initial validation of FETAX. J Appl Toxicol 7:237-244.

Dawson, D.A., Fort, D.J., Smith, G.L., Newell, D.L., and Bantle, J.A. 1988a. Comparative evaluation of the developmental toxicity of nicotine and cotinine with FETAX. Teratogen. Carcinog. Mutagen. 8:329-338.

Dawson, D.A., Stebler, E., Burks, S.A., and Bantle, J.A. 1988b. Teratogenicity of metal contaminated sediment extracts to frog (*Xenopus laevis*) and fathead minnow (*Pimephales promelas*) embryos. Environ. Toxicol. Chem. 7(1):27-34.

Dawson, D.A., Fort, D.J., Newell, D.L., and Bantle, J.A. 1989. Developmental toxicity testing with FETAX: Evaluation of five validation compounds. Drug Chem Toxicol 12(1):67-75.

Dresser, T., Rivera, E., Hoffman, F. and Finch, R. 1992. Teratogenic assessment of four solvents using the Frog Embryo Teratogenesis Assay. *Xenopus* (FETAX). J. Appl. Toxicol. 12:49-56.

Dumont, J.N., Schultz, T.W., Buchanan, M.V., and Kao, G.L. 1983. Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) - a short-term assay applicable to complex environmental mixtures, In: *Symposium on the Application of Short-Term Bioassays in the Analysis of Complex Environmental Mixtures*. M. Waters ed. pp. 393-405, New York: Plenum Press.

Fort, D.J., Dawson, D.A., and Bantle, J.A. 1988. Development of a metabolic activation system for the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX). Teratog. Carcinog. Mutagen. 8:251-263.

Fort, D.J., James, B.L., and Bantle, J.A. 1989. Evaluation of the developmental toxicity of five compounds with the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX). J. Appl. Toxicol. 9(6):377-388.

Fort, D.J., and Bantle, J.A. 1990a. Use of Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) and an exogenous metabolic activation system to evaluate the developmental toxicity of diphenylhydantoin. J. Fund. Appl. Toxicol. 14:720-733.

Fort, D.J., and Bantle, J.A. 1990b. Analysis of the developmental toxicity of isoniazid using

exogenous metabolic activation systems with Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). Teratog. Carcinog. Mutagen. 10:463-476.

Fort, D.J., Rayburn, J.R., DeYoung, D.J., Bantle, J.A. 1991. Developmental toxicity testing with Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX): Efficacy of Aroclor 1254-induced exogenous metabolic activation system. Drug. Chem. Toxicol. 14(1&2):143-160.

Friedman, M., Rayburn, J.R., and Bantle, J.A. 1990. Developmental toxicology of potato alkaloids in the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). Food Chem. Toxicol. 29:537-547.

Ghate, H.V., and Mulherkar, L, 1980. Effect of sodium diethyldithiocarbamate on developing embryos of the frog *Microhyla ornata*. Indian J. Exp. Biol. 18(9):1040-1042.

Ghate, H.V., 1983. Notochordal anomaly in frog embryos exposed to tetraethythiuram monosulphide and tetraethylthiuram disulphide. Toxicol. Lett. 19:253.

Ghate, H.V. 1985a. Toxicity and teratogenic effects of carbon disulphide in frog embryo. Riv. Biol. 78(1):129-132.

Ghate, H.V. 1985b. Dithiocarbamate induced teratogenesis in frog embryo. Riv. Biol. 78(2):288-291.

Green, E.V. 1954. Effects of hydrocarbon-protein conjugates on frog embryos. I. Arrest of development by conjugates of 9,10-dimethly-1,2-benzanthracene. Cancer Res. 14:591-598.

Karnofsky, D.A. 1965. In: *Teratology: Principles and Techniques*, J.G. Wilson ed., J. Warkany, p. 185.

Linder, G., Barbitta, J., and Kwaiser, T. 1990. Short-term amphibian toxicity tests and paraquat toxicity assessment. *Aquatic Toxicology and Risk Assessment: Thirteenth volume*, edited by W.G. Landis, W.H. van der Schalie. ASTM STP 1096, pp. 189-198. Philadelphia, Pa: ASTM.

Marks, T.A. 1991. A retrospective appraisal of the ability of animal tests to predict reproductive and developmental toxicity in humans. J. Amer. Coll. of Toxicol.

Pechman, J.H.K., Scott, D.E., Semlitsch, R.D., Caldwell, J.P., Vitt, L.J., and Gibbons, J.W. 1991. Declining amphibian populations: The problem of sepparating human impacts from natural fluctuations. Science 253:892-895.

Rayburn, J.R., Fort, D.J., McNew R., Bantle, J.A. 1991a. Synergism and antagonism induced by three carrier solvents with t-retinoic acid and 6-aminonicotinamide. Bull. Environ. Contam. Toxicol. 46:625-632.

Rayburn, J.R., Fort, D.J., DeYoung, D.J., McNew, R., and Bantle, J.A. 1991b. Altered developmental toxicity caused by three carrier solvents. J. Appl. Toxicol. 11(4):253-260.

Schultz, T.W., and Ranney, T.S. 1988. Structure-activity relationships for oesteolathyrism: II. Effects of alkyl-substituted acid hydrazides. Toxicol. 53:147-159.

Science Briefings. 1991. *New Task Force on Declining Amphibians*. Science 253:509.

Wake, D.B., 1991. Declining Amphibian Populations. Science 253: 860.

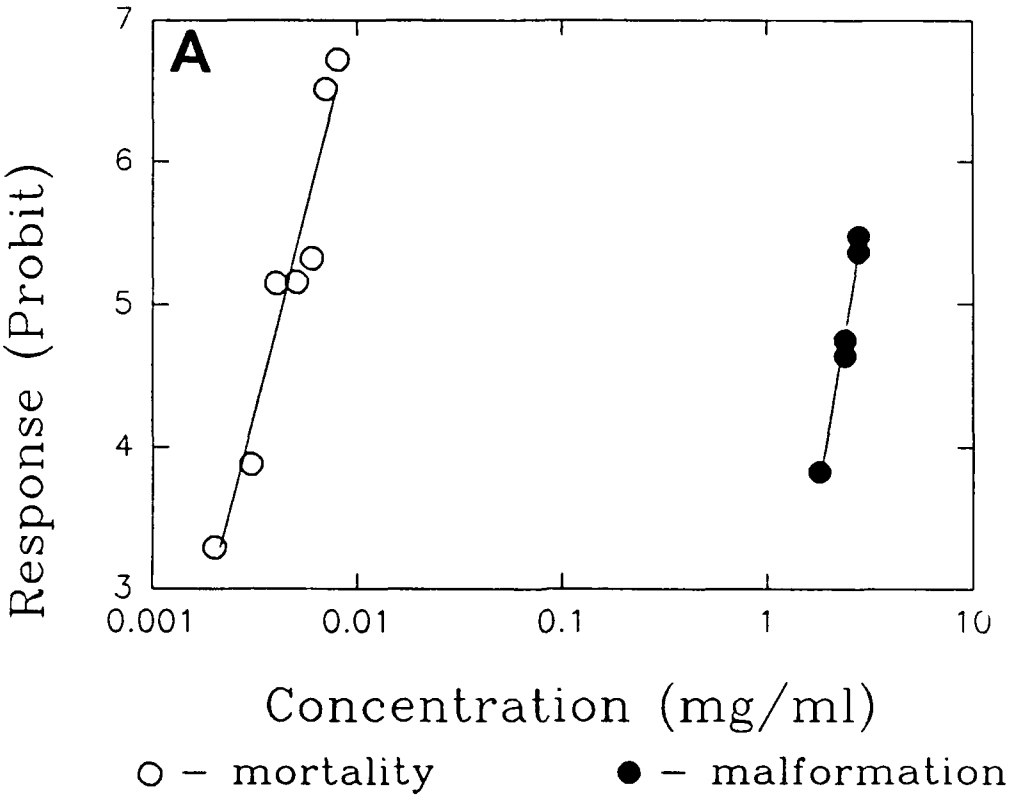
Wake, D.B., and Morowitz, H.J. 1990. *Declining Amphibian Populations-a global phenomenon, report of a workshop sponsored by the Board on Biology,* National Research Council, held at Irvine, CA, February 19-20.

FIGURE LEGENDS

Figure 1. Representative concentration-response curves for mortality, malformation and growth. A) Mortality and malformation curves for 6-aminonicotinamide, B) Growth inhibition. Note that the concentrations selected bracket the mortality and malformation ranges but not growth. This particular case is unusual because growth is usually the most sensitive indicator of toxicity.

Figure 2. The effect of solanidine on *Xenopus* embryos in the presence or absence of Aroclor 1254-induced rat liver microsomes plus generator system. Solanidine is a plant glycoalkaloid that exhibits considerable developmental toxicity even though it has a low TI. In this experiment embryos were treated with 7 mg/L solanidine for 96 h with and without Aroclor 1254-induced rat liver microsomes. Fresh solanidine, metabolic activation system and NADPH generator system were added to each dish daily. The top embryo is an untreated stage 46 control embryo while the middle embryo was treated with solanidine without microsomes and the bottom embryo was exposed to solanidine and microsomes. Note the significant increase in the severity of malformations in the presence of the microsomes suggesting the conversion of solanidine to a more teratogenic metabolite.

FIG 1
A&B


A
Response (Probit)
7
6
5
4
3
0.001
0.01
0.1
1
10
Concentration (mg/ml)
○ – mortality
● – malformation

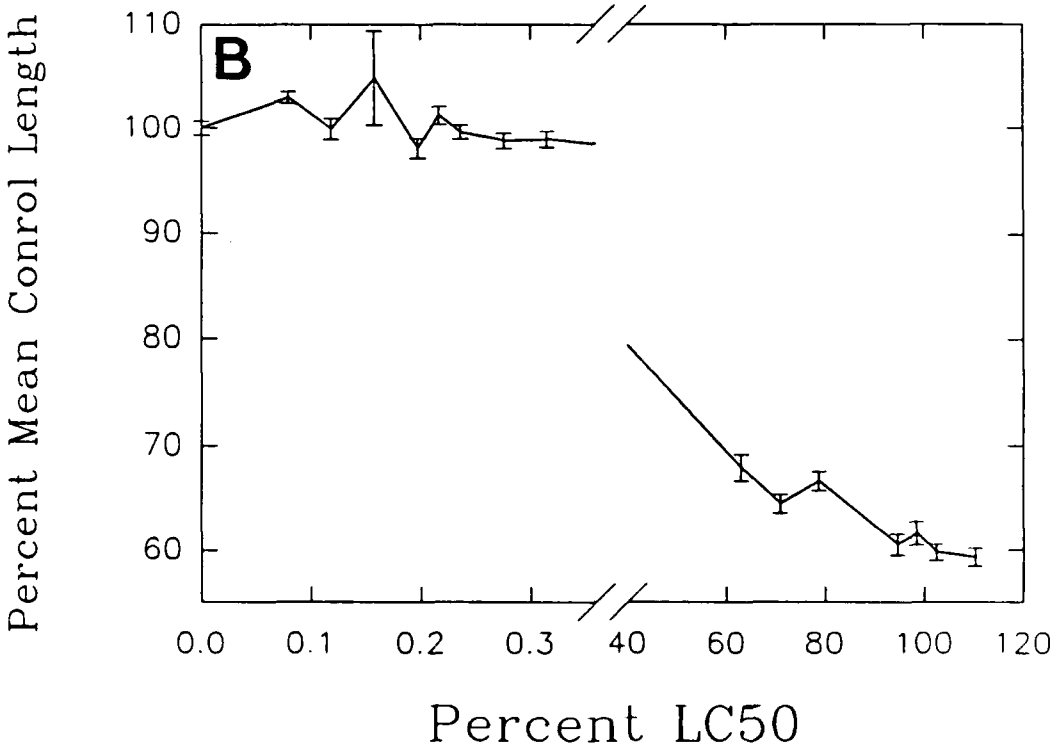

B
Percent Mean Conrol Length
110
100
90
80
70
60
0.0
0.1
0.2
0.3
40
60
80
100
120
Percent LC50

Figure 2.

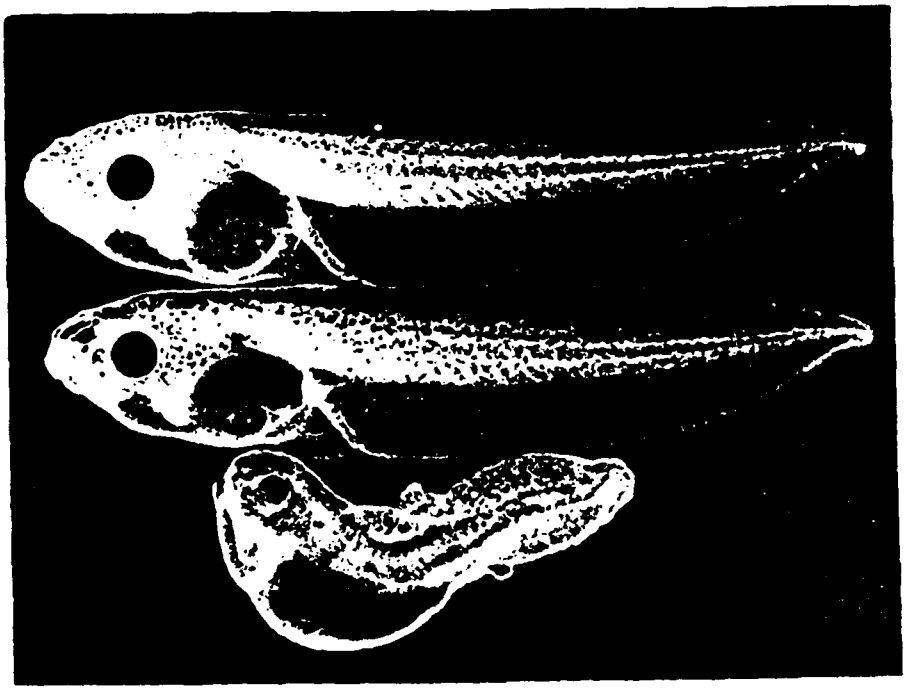


Table 1 Results of FETAX Validation Studies.

SUBSTANCE	UNITS	96-hr LC50	96-hr EC50	TI	MCIG	MAS LC50	MAS EC50	TI	M M
acetaminophen	(mg/ml)	0.153	0.140	1.1	0.105				
acetone	(%v/v)	2.190	1.287	1.7	1.430				
acetylaminofluorine(2-)[a]	(mg/l)	88.50	7.15	12.4		42.50	2.60	16.35	
acetylhydrazide	(mg/ml)	12.42	0.050	248.4	0.050	11.14	0.06	185.6	0.
actinomycin D [FN][c]	(mg/ml)	0.019	0.217	0.1	0.016	1.80	0.12	15.00	0.
amaranth	(mg/ml)	3.386	3.500	1.0	>4.0				
aminonicotinamide(6-)	(mg/ml)	3.070	0.006	511.7	0.100				
ascorbic acid	(mg/ml)	19.700	12.130	1.6	<10.00				
aspartame	(mg/ml)	13.920	13.140	1.1	7.000				
azacytidine(5-)	(mg/ml)	0.516	0.044	11.7	0.090				
benzo(a)pyrene[a]	(mg/l)	>10.0	11.0	0.9		>10.0	1.65	6.11	n
busulfan	(mg/ml)	>0.20	0.160	>1.25					
caffeine	(mg/l)	0.257	0.128	2.0	0.100				
chaconine(α)	(mg/l)	1.880	1.730	1.1	<4.00				
chaconine(ß1)	(mg/l)	2.520	1.750	1.4	4.00				
chaconine(ß2)	(mg/l)	>10.0	6.500	>1.5	6.000				
chaconine(gamma)	(mg/l)	>10.0	7.820	>1.28	4.000				
copper sulfate	(mg/l)	1.190	0.130	9.2	0.050				
cotinine	(mg/ml)	4.340	0.720	6.0	0.325				
coumarin[a] [FP][d]	(mg/ml)	0.129	0.040	3.2					
cycloheximide	(mg/ml)	0.159	0.119	1.3	0.056				
cyclophosphamide	(mg/ml)	1.370	0.390	3.5		>10.0	1.65	>6.06	
cytochalasin D	(mg/l)	461.5	121.0	3.8		805.5	546.0	1.48	
cytosine arabinoside	(mg/ml)	5.410	0.760	7.1	0.7				
diazapam	(mg/ml)	0.032	0.020	1.6	0.013				
dimethylsulfoxide	(%v/v)	1.183	1.310	0.9	1.500				
diphenhydramine HCl	(mg/ml)	0.031	0.003	10.7	0.003				

Table 1 cont.

SUBSTANCE	UNITS	96-hr LC50	96-hr EC50	TI	MCIG	MAS LC50	MAS EC50	TI	MAS MCIC
diphenylhydantoin[a]	(mg/l)	74.450	32.000	2.3		126.35	69.15	1.83	
doxlyamine succinate	(mg/ml)	0.22	0.04	4.8	0.035				
ethanol (L)	(%v/v)	1.440	1.010	1.4	1.000				
ethidium bromide	(mg/ml)	0.050	0.035	1.4	0.050				
ethyl(N-)-N-nitrosourea	(mg/ml)	0.258	0.051	5.0	4.870	3.70	7.30	5.07	
flourouracil(5-)	(mg/ml)	1.620	0.137	11.8	0.120				
furazolidone	(mg/ml)	0.014	0.007	2.0	0.008				
hydroxydilantin(M-)[a]	(mg/l)	>150.0	>150.0	1.0		>150	>150		
hydroxydilantin(P-)[a]	(mg/l)	>150.0	>150.0	1.0		>150	>150		
hydroxyurea	(mg/ml)	1.820	0.430	4.2	0.3				
isoniazid [FP][d]	(mg/ml)	9.893	0.270	36.6	0.170	8.90 6.34	0.29 0.41	30.17 15.28	0.25 0.13 [b]
nicotinic acid	(mg/ml)	3.180	1.260	2.5	1.250	3.29 3.29	1.51 153.0	2.18 0.02	1.13 1.27 [b]
methotrexate	(mg/ml)	0.504	0.026	19.4	0.019				
methylmercury chloride	(mg/l)	0.08	0.02	3.6	0.038				
naphthalene	(mg/l)	6.540	5.750	1.1					
nicotine	(mg/l)	136.50	0.41	332.9	0.463	20.35	5.85	3.48	
nitriloacetic acid	(mg/ml)	0.570	0.52	1.1	0.375				
nitrosodimethylamine(N-)	(mg/ml)	3.38	2.295	1.5	1.250				
procarbazine	(mg/ml)	3.170	1.310	2.4	1.000				
propylene glycol	(%v/v)	2.697	1.740	1.5	1.375				
pseudoephedrine [FP][d]	(mg/ml)	0.414	0.237	1.7	0.200				
retinoic acid(-cis)	(ng/ml)	30.42	2.49	12.2	9.000				
retinoic acid(-trans)	(mg/l)	0.375	0.034	11.0	0.070				
rifampicin[a]	(mg/ml)	>2.00	>2.000	1.0	1.38	0.50	2.75		
saccharin	(mg/ml)	18.370	19.340	0.9	17.000				
serotonin	(mg/ml)	3.005	0.370	8.1	0.425				

Table 1 cont.

SUBSTANCE	UNITS	96-hr LC50	96-hr EC50	TI	MCIG	MAS LC50	MAS EC50	TI	MAS MCIG
dium acetate	(mg/ml)	4.200	3.300	1.3	2.500				
sodium cyclamate	(mg/ml)	16.135	14.845	1.1	12.000				
sodium salicylate	(mg/ml)	2.320	1.450	1.6	1.250				
sodium selenate	(mg/ml)	0.021	0.007	3.0	0.009				
solanine (a)	(mg/l)	10.900	8.800	1.2	12.000				
solasonine	(mg/l)	5.600	5.110	1.1					
trichloroethylene	(%v/v)	0.030	0.008	3.9	0.020				
triethylene glycol	(% v/v)	2.450	2.150	1.1	1.430				
urethane	(mg/ml)	5.645	1.730	3.3	1.250				
zinc sulfate [FP][d]	(mg/l)	34.40	2.69	12.8		36.70	2.88	12.72	

[a] These chemcials were used with DMSO as the solvent.
[b] These values were obtained using isoniazid-induced microsomes. All other MAS data were generated using aroclor-induced microsomes.
[c] This chemical gave false negative results.
[d] These chemicals gave false positive results.

INITIAL INTERLABORATORY VALIDATION STUDY OF FETAX.

John A. Bantle[1Ψ], Dennis R. Burton[2], Douglas A. Dawson[3], James N. Dumont[4], Robert A. Finch[5], Douglas J. Fort [6], Greg Linder[7] and James R. Rayburn[1].

[1]Department of Zoology, Oklahoma State University, Stillwater, OK, 74878
[2]WYE Research & Educational Center, PO Box 169, Queenstown, MD, 21658
[3]College of Veterinary Medicine, University of Tennessee, PO Box 1071, Knoxville, TN, 37901
[4]Quality Department, Bldg. 2024, MS 6052, Oak Ridge National Labs, OakRidge, TN, 37830
[5]US Army Biomedical, R&D Laboratory, Bldg. 568, Fort Detrick, Frederick, MD, 21702
[6]Stover & Associates Inc., PO Box 2056, Stillwater, OK, 74076
[7]US EPA, Environmental Research Laboratory, 200 SW 35th St, Corvallis, OR, 97333

RUNNING HEAD: ILS of FETAX

KEYWORDS: FETAX, *Xenopus*, teratogen, hydroxyurea, isoniazid, 6-aminonicotinamide, interlaboratory validation,

Address correspondence to:

ΨDr. J. A. Bantle
Department of Zoology
Oklahoma State University
Stillwater, OK, 74078
Phone: (405)-744-8708
Fax: (405)-744-7074

ABSTRACT

FETAX is a 96-hr whole embryo developmental toxicity screening assay that can be used in ecotoxicology and in detecting mammalian developmental toxicants when an *in vitro* metabolic activation system is employed. A standardized ASTM New Standard Guide has been published along with a companion Atlas that helps in staging and identifying malformations. As part of the ASTM process, an interlaboratory validation study was undertaken to evaluate the repeatability and reliability of FETAX. A three phase experimental plan with seven participants was carried out. Phase I was a training and protocol evaluation phase. The identity of the three test materials was known and, because they had been previously tested in FETAX , the same concentrations needed to establish the 96-hr LC50 and EC50 (malformation) were used by all laboratories. Phase II was similar to Phase I except that the identity of the test materials was not known. Phase III was designed to test FETAX in a blind testing format with each lab responsible for determining the concentrations to be tested. The results of Phase I are reported here. FETAX proved to be as repeatable and reliable as many other bioassays. In Phase I, some excess variation was observed in individual labs. Some of this variation may have been due to training difficulties. One error in protocol design proved to be the use of the reference toxicant 6-aminonicotinamide. While 6-aminonicotinamide provided excellent dose-response data in most laboratories, the protocol was written too strictly based on historical FETAX data. Phase II provided less variable data than Phase I and Phase III is currently in progress.

INTRODUCTION

FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*) is a 4 day whole embryo developmental toxicity test that utilizes the embryos of the South African Clawed frog, *Xenopus laevis*. FETAX was initially designed as an indicator of potential human developmental health hazards (Dumont et al., 1983). This use was aided by the development of an *in vitro* metabolic activation system using Aroclor 1254 and Isoniazid-induced rat liver microsomes (Bantle and Dawson, 1988; Bantle et al., 1989; Dawson et al. ,1988; Fort et al., 1988; Fort et al., 1990; Fort and Bantle, 1990a, Fort and Bantle, 1990b, Fort et al., 1991; Fort et al., 1992). FETAX is well suited for testing complex mixtures (e.g., industrial effluents) and has undergone extensive validation using single chemicals of known mammalian developmental toxicity (Bantle, 1992).

FETAX is also applicable to aquatic toxicity assessments (Dumont and Schultz, 1980; Dawson et al. 1985; Dawson et al., 1988; Bantle et al., 1989). This amphibian developmental toxicity test may help in studies designed to discover the reasons for the reported world-wide disappearance of amphibians even in pristine locations (Wake and Morowitz, 1990; Wake, 1991). This decline may be due in part to normal population fluctuations caused by climatological factors or by anthropogenic factors (Pechmann et al., 1991). However, in at least one case, frog eggs failed to develop in pond water but developed normally when moved to the laboratory (Science Briefings, 1991). It is, therefore, possible that some decline may be due to chemical pollution and FETAX may be used to investigate the extent and causes of the decline.

A standardized ASTM New Standard Guide for the conduct of FETAX (Bantle and Sabourin, 1991) has been published along with a companion "Atlas of Abnormalities" (Bantle et al., 1991) that helps in staging and identifying malformations. As part of the ASTM process, an interlaboratory validation study (ILS) was undertaken to determine the repeatability and reliability of FETAX. A three phase experimental plan with seven participants was carried out. Phase I was a training and protocol evaluation phase. The identity of the three test materials was known and, because they had been previously tested in FETAX , the same concentrations needed to establish the 96-hr LC50 and EC50 (malformation) were used by all laboratories. The *in vitro* metabolic activation system was not employed. The results of this phase are reported here. Phase II was similar to Phase I except that the identity of the test materials was not known. Phase III was designed to test FETAX in a blind testing format with each lab responsible for determining the concentrations to be tested. The repeatability and reliability of the metabolic activation system were also to be evaluated.

EXPERIMENTAL

Participants

Each laboratory of this interlaboratory study included a supervisor and a primary technician. The technicians performed FETAX while the supervisors compiled and reported the data to a central coordinator. The data was screened by the coordinator to see that it complied with the standard protocol established in the New Standard Guide (Bantle and Sabourin, 1991). The supervisors and their technicians were as follows: Dr. J.A. Bantle, J.R. Rayburn, M.A. Hull; Dr. D. Burton, S. Turley; Dr. D.A. Dawson; Dr. R.A. Finch, M. Maurice; Dr. D.J. Fort, M. Gray; Dr. G. Linder, D. Buchwalter. The coordinator was Dr. J.N. Dumont.

ILS Phase I Procedure

Chemicals were chosen that had been previously tested using FETAX. Abundant historical data were used to give a concentration range that would allow the determination of the LC50 and EC50 (malformation). All technicians used the same concentration ranges and knew which chemical they were testing.

Tests were performed as specified in the ASTM New Standard Guide (Bantle and Sabourin, 1991). After completing the Hydroxyurea and Isoniazid experiments,

adjustments were made to the ASTM procedure for 6-aminonicotinamide. Originally, the stock and FETAX solutions were added individually to the dishes using pipettes and pipetors. For 6-aminonicotinamide, 50 ml Erlenmeyer flasks (Fisher) were used to make 25 ml of each concentration of 6-aminonicotinamide, 10 ml samples were then added to each of the two dishes.

Solutions were renewed every 24 h of the four d tests and any dead animals removed. At 96 h, surviving embryos were fixed in 3.0% (w/v) formalin (Sigma). The number of dead, the number of malformed survivors, as well as the developmental stage were determined using a dissecting microscope.

Samples were kept from selected concentrations in specially supplied borosilicate glass tubes with teflon-lined lids (Fisher). At the end of the experiment the samples were shipped on dry ice to Dr. Bantle's lab for concentration analysis. Embryos were preserved in formalin (Sigma) and stored in scintillation vials (RPI) and were sent to Dr. Bantle's laboratory for verification of results when necessary.

Assay Procedure

Xenopus culture, breeding procedures, and egg sorting were described previously in the ASTM New Standard Guide (Bantle and Sabourin, 1991) and the *Atlas of*

Abnormalities (Bantle et al., 1991). Adult *Xenopus laevis* frogs were obtained from Xenopus I (Ann Arbor, Michigan).

The supervisors and their technicians attended a FETAX workshop before the study began. At the workshop, participants were familiarized with the FETAX protocol outlined in the ASTM New Standard Guide. The interlaboratory study was also explained in detail. Standard data entry forms obtained from J.N. Dumont were used throughout the study (Bantle et al., 1991).

Test Materials

Chemicals tested in Phase I were Hydroxyurea (CAS # 120-07-1), Isoniazid (CAS # 54-85-3), and 6-aminonicotinamide (CAS # 329-89-5). They were purchased from Sigma Chemical Co. (St. Louis, MO) in bulk quantities from the same lot. One technician individually weighed out the compounds using a recently calibrated analytical balance. The amount was then placed in 100 ml serum vials (Fisher) and capped with rubber septa sealed with aluminum. Each vial contained enough material for one day of each experiment; therefore four vials were needed for each test. The chemicals were sent in bulk to the coordinator who then shipped out enough chemical to each laboratory for three complete concentration-response (definitive) experiments. Instructions shipped with each chemical included: 1) the stock concentration, 2) testing concentrations, 3)

concentrations to take analysis samples from, and 4) a Material Safety Data Sheet (MSDS).

Data Analysis

Probit analysis, using Litchfield-Wilcoxin (Tallarida and Murray, 1987) was used to determine the 96-h LC50 (median lethal concentration), 96-h EC50 (concentration inducing gross terata in 50% of the surviving embryos), and 95% percent confidence intervals. When the homogeneity test failed, the trimmed Spearman-Karber (Hamilton et al., 1977) and EPA probit analyses were used instead of the Litchfield-Wilcoxin probit analysis. Teratogenic potential was determined using a Teratogenic Index [TI=LC50/EC50 (malformation)]. Head-tail length (growth) was measured using an IBM-compatible computer equipped with digitizing software (Jandel Scientific, Corte Madera, CA). For each test, the minimum concentration to inhibit growth (MCIG) was calculated using the t-Test for grouped observations ($p<0.05$).

The coefficients of variation (CVs) for the 96 hr LC50, EC50 (malformation), TI, and MCIG was calculated according to Steel and Torrey (1980). Analysis of the data was also carried out using ASTM E691-87 (Interlaboratory Study) software and methods.

RESULTS

Phase I Protocol

Phase I of the three phase ILS research plan was designed as a training and initial protocol evaluation phase and some variation was expected. Phase I was planned to identify problems early on so that corrections could be made. Because FETAX involves a subjective determination of normal embryos at the beginning of the test and the determination of abnormal embryos at the conclusion of the test, experienced technicians can be expected to provide less variable results than novices. It was expected, therefore, that some variability would be observed in Phase I. Changes in protocol were expected prior to the start of Phase II. When cases of high variability were reported in individual laboratories, embryos were shipped to John Bantle's laboratory for confirmation of results. Also shipped on ice were sample concentrations from the experiment for spectrophotometric analysis. In this manner, some causes of excessive variation might be determined.

Hydroxyurea

Table 1 shows the results of the Hydroxyurea tests. Three complete concentration-response tests were performed by each lab (replicates). The 96-hr LC50, 96-hr EC50 (malformation) and MCIG were determined for each test. The TI was calculated by dividing the LC50 by the EC50 (malformation). It should be emphasized that the concentration ranges were designed to obtain the LC50 and EC50. Since growth

was usually affected at concentrations considerably below those needed to affect development and survival, there were fewer concentrations designed to define the growth curve. As a result, more variation was expected in the MCIG endpoint. For Hydroxyurea, the intralab coefficients of variation (CVs) for the 96-hr LC50 ranged from 6.7 to 69.8 (Table 1). Only two of the intralab CVs were above 32 (Labs 3 and 5). In both cases a single experiment seemed to cause the higher CV. For Lab 4, the data in the second replicate was not adequate to generate an LC50. The 96-hr EC50 (malformation) showed better results as the CVs ranged from 6.4 to 57.2 with only a single CV above 27. The CVs for the TI ranged from 8.1 to 53 based on the calculated values (Table 1). The mean MCIG was 0.25 mg/ml and the MCIG values ranged from 0.1 to 0.7 mg/ml. The intralab CVs for the MCIG ranged from 23-37. The mean 96-hr LC50, 96-hr EC50 (malformation), and TI for Hydroxyurea were 0.75, 0.18, and 4.23 mg/ml, respectively. The interlab CV values for the 96-hr LC50, 96-hr EC50 (malformation), TI and MCIG were 74, 45, 57 and 64, respectively.

Figs. 1-2 show a graphic analysis of the data that establishes a central tendency and 1 and 2 standard deviation lines. The stars indicate the lab mean of the 3 replicates while the solid circles indicate the individual replicate values. Those individual values that fall outside two S.D. units can be considered to be excessively variable. For the LC50, only two points were outside two S.D. (Fig. 1). Experiments exceeding the EC50 (malformation), TI and MCIG limits were 2, 2 and 1, respectively.

Figs. 3-4 show a graphic analysis using ASTM methodology. Intralab variability was given by the k values (Figs. 3a and 3b). Only values above the dashed line were

considered to have deviated excessively and only Lab 3 showed excessively high variation. The h values presented in Figs. 4a and 4b show the interlab variation. All measurements were within acceptable limits.

During the hydroxyurea test, it became apparent the the 6-aminonicotinamide positive control was not performing as expected in the ASTM New Standard Guide (Bantle and Sabourin, 1991). This reference toxicant experiment was based on two dishes of 25 embryos each of 5.5 mg/l 6-aminonicotinamide and two dishes of 25 embryos each of 2500 mg/l 6-aminonicotinamide. These were the EC50 (malformation) and LC50 of 6-aminonicotinamide based on historical data (Bantle et al., 1989). In order for a test to be valid, malformation should have been from 20-80% in the 5.5 mg/l dishes and mortality should have been between 20-80% in the 2500 mg/l dishes. Even though excellent concentration-response data were obtained for the test material (discussed later) and the negative control (FETAX solution only) was within acceptable limits, the positive control would often fail. It was decided that 6-aminonicotinamide should be tested as a phase I compound and adequate concentration-response data be collected.

Isoniazid

Table 2 shows the results of the tests with Isoniazid. For Isoniazid, the intralab coefficients of variation (CVs) for the 96-hr LC50 ranged from 2.6 to 84.9. Only a single intralab CV was above 15.5 (Lab 3). In this Lab two experiments yielded abnormally low LC50 values while all other ILS LC50 values were very close. The 96-

hr EC50 (malformation) showed similar but more variable results as the CVs ranged from 3.6 to 82.4 with a single CV above 32.1 (Table 2). The CVs for the TI ranged from 1.2 to 612.5 based on the calculated values (Table 2). The high value of 612.5 for Lab 3 was a result of combining the highly variable LC50 and EC50 values. The mean MCIG was 0.89 mg/ml and the MCIG values ranged from 0.05 to 7 mg/ml. The intralab CVs for the MCIG ranged from 0-119. The mean 96-hr LC50, 96-hr EC50 (malformation), and TI for Isoniazid were 7.86, 0.24, and 33.22 mg/ml, respectively. The interlab CV values for the 96-hr LC50, 96-hr EC50 (malformation), TI and MCIG were 49.8, 71.5, 229 and 81.4, respectively.

When a graphic analysis method was used employing the central tendency and two standard deviation units of variation similar to Figs. 1 and 2, only a single experiment fell outside the line for the LC50, 1 for the EC50 (malformation), none for the TI and, as expected, three for the MCIG (data not shown). Most mean values fell within the limits except for the high TI value for Lab 3.

Figs. 5-6 show a graphic analysis using ASTM methodology. Intralab variability was given by the k values (Figs. 5a and 5b). Only values above the dashed line were considered to have deviated excessively and only Lab 3 showed excessively high variation. The h values presented in Figs. 6a and 6b show the interlab variation. Once again, Lab 3 showed the only variation above acceptable limits.

6-Aminonicotinamide

6-aminonicotinamide was tested using a revised protocol designed to eliminate some of the variation observed in the earlier experiments. By premixing the test material and FETAX solution (diluent) in a larger volume prior to aliquoting to the Petri dishes, it was hoped that there would be less variation in concentrations. It should also be noted that a new lab technicians began work in Lab 3 and the new technician was trained in a special session.

Table 3 shows the results of the tests with 6-aminonicotinamide. For 6-aminonicotinamide, the intralab coefficients of variation (CVs) for the 96-hr LC50 ranged from 1.8 to 29.6. This time it was Lab 2 that showed the highest variability. The 96-hr EC50 (malformation) showed similar but slightly more variable results as the CVs ranged from 5.7 to 62 with only a single CV above 30.1 (Table 3). The CVs for the TI ranged from 5.8 to 322.7 based on the calculated values (Table 3). The high value of 322.7 for Lab 3 was once again a result of combining the LC50 and EC50 values. When Lab 6 was removed from the study, the intralab coefficients of variation (CVs) for the 96-hr LC50 ranged from 4.2 to 29.6 as before but the CVs for the EC50 (malformation) was 5.7 to 30.1. The deletion of Lab 6 dramatically changed the interlab variations. With Lab 6, the interlab CVs for the 96-hr LC50, 96-hr EC50 (malformation), and TI was 20.45, 183.5 and 120.87, respectively. Without Lab 6, interlab CV values for the 96-hr LC50, 96-hr EC50 (malformation), TI and MCIG were dramatically improved at 21.7, 31.1, 41.5 and 70.5, respectively. The concentration of 6-aminonicotinamide in

selected test samples from all the labs was determined spectrophotometrically. Stage 46 test larvae from Lab 6 were also reevaluated in John Bantle's lab. Neither analysis prove sufficient to explain the results obtained.

When a graphic analysis method was used employing the central tendency and two standard deviation units of variation similar to Figs. 1 and 2, only a single experiment fell outside the line for the LC50, 1 for the EC(50) malformation, none for the TI and, as expected, three for the MCIG (data not shown). Most mean values fell within the limits except for the high TI value for Lab 3.

Figs. 7-8 show a graphic analysis using ASTM methodology. Intralab variability was given by the k values (Figs. 7a and 7b). Labs 2 and 6 showed excessively high variation any of the endpoints of FETAX. The h values presented in Figs. 8a and 8b show the interlab variation (h values). Once again, Lab 6 showed the only variation above acceptable limits.

Fig. 9 shows typical concentration-response curves for mortality, malformation (Fig. 9a) and growth (Fig. 9b). For most experiments, there was a good fit of the data points to the curve. These experiments showed that 6-aminonicotinamide tested no differently from any other compound and could be used as a reference toxicant. Because the concentrations were chosen to define the mortality and malformation curves, a break occurs in the growth-response curve in the area where the MCIG should occur. This figure shows some of the difficulty in determining the MCIG.

DISCUSSION

Sources of Variation in FETAX

Genetic variation and differences in laboratory procedures contribute to the variation observed in this study. While genetically-defined strains of *Xenopus* can be purchased, they are not available in sufficient numbers for developmental toxicity testing. Consequently, the only way to minimize the variation from using outbred animals is to use the best husbandry practices possible. These practices are covered in a number of different sources (Bantle and Sabourin, 1991; Bantle et al., 1991, Dawson et al., 1992; Kay and Peng, 1992 and Deuchar, 1975). With proper feeding, care, embryo selection and eliminating sick animals from the colony, variation in embryo quality can be minimized resulting in only natural genetic variation affecting the test. This genetic variation is minimized in a single test as all embryos come from the same parents.

The main causes of variation in laboratory procedures are microbial contamination and technician training. Attempts to sterilize embryos prior to culture have been unsuccessful. Additional problems arise when the test material can serve as an energy source. Once contamination begins, each additional dead embryo contributes to the energy source. The problem frequently manifests itself when there is differential death in dishes at the same concentration of test material. The FETAX solution can also become turbid due to the growth. Antibiotics such as penicillin and streptomycin are effective in controlling contamination and are relatively non toxic. However, they are not routinely used in FETAX because of the possibility of interaction with the test material. When contamination is observed to affect the results, it is possible to repeat the experiment using antibiotics and note that this is a departure from standard procedures.

The use of antibiotics is mandatory when the *in vitro* metabolic activation system is used in conjunction with standard FETAX (Bantle et al., 1991).

The ASTM New Standard Guide (Bantle and Sabourin, 1991), the "Atlas of Abnormalities" (Bantle et al., 1991) and the annual FETAX workshop help technician training. However, it became apparent as Phase II began that experience played a role as FETAX data became more uniform than observed in Phase I. All other variations in laboratory procedure contribute to the observed variation in the data. Important among these other factors is the selection of the number of concentrations used to define the LC50 and EC50 (malformation). When a test material is used that has a large separation between the two curves, fewer concentrations are usually chosen to define each curve. When these concentrations are outside the 16-84% response, more variation can occur. For test materials that have overlapping mortality and malformation curves, the same concentrations can help define both curves. In this study, at least five concentrations were typically used to define each curve as each compound used in Phase I had mortality and malformation curves that were significantly different.

Hydroxyurea

Hydroxyurea was selected as the first test material for the ILS because it had been previously tested in two separate laboratories although with slightly different protocols. The main differences in protocol were different diluent, the use of jellied embryos and a 20°C test temperature by Sabourin et al. (1985) compared with dejellied embryos and 23 °C by Courchesne and Bantle (1985). Sabourin et al. (1985) reported an LC50, EC50 (malformation) and MCIG of 0.382, 0.085 and 0.0076 mg/ml, respectively. Courchesne and Bantle (1985) reported 1.82, 0.43 and 0.3, respectively. Although test procedures and lots of Hydroxyurea differed, the TI for Sabourin et al., (1985) was 4.5 and 4.2 for

Courchesne and Bantle (1985). For the present study the interlaboratory LC50, EC50 (malformation) and MCIG were 0.75, 0.18 (Table 4) and 0.25 mg/ml, respectively. The TI was 4.2 showing a remarkable constancy for this value for Hydroxyurea.

Some training difficulties were reported from lab three during this phase of the study and the intralab variation suggests this might be the case. Another indication of problems was a relatively high rejection rate of tests due to unacceptable malformation rates in controls. The current protocol requires <10% mortality and 7% malformation in controls (Bantle and Sabourin, 1991). Very often high control mortality and malformation is a reflection of embryo selection during test set up.

Isoniazid

Dawson et al. (1989) found that Isoniazid had a mean LC50, EC50 (malformation), TI and MCIG of 9.8, 0.26, 38 and 0.0012 mg/ml, respectively. In a later study in the same laboratory, Fort and Bantle found that Isoniazid had a LC50, EC50 (malformation), TI and MCIG of 9.99, 0.28, 35.7 and 0.02 mg/ml, respectively using the same protocol as the previous study. This constancy in historical data recommended its use in Phase I of the ILS. The mean ILS values for Isoniazid were 7.86, 0.24, 33.2 and 0.89 mg/ml for the LC50, EC50 (malformation), TI and MCIG, respectively (Table 4). Except for the MCIG which was affected by only two very high values in the ILS, there is a remarkable constancy in the data despite 4 years between the first and last tests.

In the ILS study, Lab 3 showed high intralab variability and Labs 3 and 5 showed interlab variability. Test reviews, particularly from Lab 5 which had LC50 values ten fold lower than other study participants (Table 2), showed that the technicians from Labs 3 and 5 had not misinterpreted the data. Spectrophotometric analysis of sample

concentrations showed that Lab 5 had properly made up test solutions. It was concluded that either genetic variation, poor glassware washing or bacterial contamination caused the lowered LC50.

6-Aminonicotinamide

Dawson et al. (1989) found that 6-Aminonicotinamide had a mean LC50, EC50 (malformation), TI and MCIG of 3.07, 0.0055, 560 and 0.0031 mg/ml, respectively while Rayburn et al. (1991) reported values of 2.5, 0.0055, 455 for the LC50, EC50 (malformation), and TI. The only difference in protocol was the use of antibiotics by Dawson et al. (1989) which may have helped survival and raised the LC50. The EC50 (malformation) values were the same. 6-Aminonicotinamide was tested in the ILS using slightly modified test solution procedure previously described. When Lab 6 was removed from the ILS study as an outlier, the mean LC50, EC50 (malformation), TI and MCIG were 2.18, 0.005, 428.6 and 1.15 mg/ml, respectively (Table 4). The ILS was performed without antibiotics. With lab 6 included, the EC50 (malformation) was raised to 0.013 and the TI dropped to only 174. This increase was caused by only two tests from Lab 6 having EC50 (malformation) values 10 fold higher than those reported from other labs. Independent analysis of embryos from Lab 6 confirmed that the number of malformations were properly recorded and that variation in test concentrations could not account for the observed results.

Bacterial contamination was not thought to be a cause of the variability observed in these tests because the LC50 was within the expected values. With bacterial contamination, the LC50 is greatly reduced. It may be that genetic variation was the cause of these results as the technician in Lab 6 had long experience with FETAX.

Conclusions

Table 5 shows that the MCIG was the most variable endpoint tested in the study based on a comparison of CV values. As previously discussed, the test concentrations were chosen to define the LC50 and EC50 (malformation) and not the MCIG. As expected, the EC50 (malformation) the next most variable endpoint in two of the three tests. This was probably due to the subjective nature of the evaluation. However, the CVs obtained were reasonable. The LC50 is the most objective endpoint because the cessation of heartbeat is an unambiguous sign of death.

Parkhurst et al. (1992) have reviewed the performance characteristics of effluent toxicity tests and classified the tests according to their design. Under this classification system, the FETAX ILS is closest to Interlaboratory studies involving acute toxicity tests on single chemicals. Therefore, Phase I FETAX performance will be compared to this type of data only. However, Parkhurst pointed out that chronic test results with single tests were as repeatable and reliable as acute tests. Parkhurst et al. (1992) listed interlaboratory CV values ranging from 22 to 143 with a mean of 47% for 48-96 hr acute toxicity tests employing *D. magna, P. promelas, M. bahia and C. variegatus.* Only 7 of 22 studies were conducted among at least six laboratories. By comparison, FETAX interlaboratory CV values range from 21 to 183 with a mean of 74 for the LC50 and EC50 (malformation) endpoints. If Lab 6 is removed from the 6-Aminonicotinamide test, the mean would have been 48.9. Phase II of the FETAX ILS is nearly complete and the preliminary interlaboratory CV values for the four compounds tested ranged from 9.1-45 with a mean of 22.4. Some of these improved results were probably due to two of the test materials being nonteratogens and relatively non-toxic. However, the other two materials were strong teratogens. The improved performance was mainly due to their being no outliers and the increased experience of the technicians performing the test.

The use of 6-Aminonicotinamide as a reference toxicant and the way it is used is currently under review by the FETAX ASTM task force. It is likely that a running average as outlined by the EPA (1989) will be employed (Figs. 1-2) in the future. Another protocol change will be the acceptance of a 10% malformation rate in FETAX solution controls instead of 7%. Many of the experiments that were rejected in this study because of the malformation rate, provided excellent concentration-response data.

In summary, Phase I of the FETAX ILS demonstrated that the repeatability and reliability of FETAX was sufficient to warrant further testing. Changes in the protocol and further assay development may decrease that variability even further. Preliminary Phase II results indicate FETAX is capable of performing as well as other acute and chronic toxicity tests.

References

1. J.N. Dumont, T.W. Schultz, M.V. Buchanan and G.L. Kao, Frog embryo teratogenesis assay: *Xenopus* (FETAX) - A short-term assay applicable to complex environmental mixtures. In *Symposium on the Application of Short-Term Bioassays in the Analysis of Complex Environmental Mixtures III*, ed. by M. D. Waters, S.S. Sandhu, J. Lewtas, L. Claxton, N. Chernoff, and S. Nesnow, pp. 393-405. Plenum Press, New York, (1983).

2. J. A. Bantle and D. A. Dawson, Uninduced rat liver microsomes as an *in vitro* metabolic activation system for the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). In *Aquatic Toxicology and Hazard Assessment*, Vol. 10, ed. by W.J. Adams, G. A. Chapman and W. G. Landis, ASTM STP 971, pp. 316-

326. American Society for Testing and Materials, Philadelphia, 1988.

3. J. A. Bantle, D. J. Fort and B. L. James, Identification of developmental toxicants using the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX). *Hydrobiol* **188/189**, 577-585 (1989).

4. D. A. Dawson, D. J. Fort, G. L. Smith, D. L. Newell and J. A. Bantle, Evaluation of the developmental toxicity of nicotine and cotinine with Frog Embryo Teratogenesis Assay: Xenopus (FETAX). *Teratogen. Carcinogen. Mutagen.* **8,** 329-338 (1988).

5. D. J. Fort, D. A. Dawson and J. A. Bantle, Development of a metabolic activation system for the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX). *Teratogen. Carcinogen. Mutagen.* **8,** 251-263 (1988).

6. Fort and Bantle, Use of frog embryo teratogenesis assay - *Xenopus* and an exogenous metabolic activation system to evaluate the developmental toxicity of diphenylhydantoin. *Fund. Appl. Toxicol.* **14,** 720-733 (1990a).

7. D. J. Fort and J. A. Bantle, Analysis of the mechanism of isoniazid-induced developmental toxicity with the Frog Embryo Teratogenesis Assay - *Xenopus*(FETAX), *Teratogen. Carcinog. Mutagen.* **10,** 463-476 (1990b).

8. D. J. Fort, J. R. Rayburn, D. J. DeYoung and J. A. Bantle, Assessing the efficacy of an aroclor 1254-induced exogenous metabolic activation system for FETAX. *Drug Chem. Toxicol.* **14**(1&2), 143-160 (1991).

9. D. J. Fort, J. R. Rayburn and J. A. Bantle, Evaluation of acetaminophen-induced developmental toxicity using FETAX, *Drug Chem. Toxicol.* **15**, 329-350 (1992).

10. J. A. Bantle, FETAX - A developmental toxicity assay using frog embryos. In: *Fundamentals of Aquatic Toxicology*, ed. by G. M. Rand and S. R. Petrocelli, Hemisphere Publishing, N.Y., (1991).

11. J. N. Dumont and T. W. Schultz, Effects of coal-gasification sour water on *Xenopus laevis* embryos. *J. Environ. Sci. Health* **A15,** 127-138 (1980).

12. D. A. Dawson, C. A. McCormick and J. A. Bantle, Detection of teratogenic substances in acidic mine water samples using the Frog Embryo Teratogenesis Assay-*Xenopus*(FETAX). *J. Appl. Toxicol.* **5**(4), 234-244 (1985).

13. D. A. Dawson, E. Stebler, S. A. Burks and J. A. Bantle, Teratogenicity of metal contaminated sediment extracts to frog (*Xenopus laevis*) and fathead minnow (*Pimephales promelas)* embryos. *Environ. Toxicol. Chem.* **7**(1), 27-34 (1988).

14. J. A. Bantle, D. J. Fort and D. A. Dawson, Bridging the gap from short-term teratogenesis assays to human health hazard assessment by understanding common modes of teratogenic action. In *Aquatic Toxicology and Hazard Assessment*, Vol. 12, ed. by W. G. Landis, W. H. Van der Schalie, ASTM STP 1096, pp. 46-58. American Society for Testing and Materials, Philadelphia, (1989).

15. D. B. Wake and H. J. Morowitz, Declining amphibian populations-a global phenomenon, report of a workshop

sponsored by the Board on Biology, National Research Council, held at Irvine, CA, February 19-20, (1990).

16. D. B. Wake, Declining amphibian populations. *Science* **253**, 860 (1991).

17. J. H. K. Pechman, D. E. Scott, R. D. Semlitsch, J. P. Caldwell, L. J. Vitt and J. W. Gibbons, Declining amphibian populations: The problem of separating human impacts from natural fluctuations. *Science* **253,** 892-895 (1991).

18. Science Briefings, New task force on declining amphibians. *Science* **253,** 509 (1991).

19. J. A. Bantle and T. D. Sabourin, New standard guide for conducting the Frog Embryo Teratogenesis Assay-

Xenopus (FETAX). American Society for Testing and Materials, Philadelphia, (1991).

20. J. A. Bantle, J. N. Dumont, R. Finch and G. Linder, *Atlas of abnormalities: a guide for the performance of FETAX*. Oklahoma State Publications Department, (1991).

21. R. J. Tallarida and R. B. Murray, *Manual of Pharmacologic Calculations with Computer Programs*. 2nd Edn, Springer-Verlag, New York, (1987).

22. M. A. Hamilton, R. C. Russo and R. V. Thurston, Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Tech.* **11**(7), 714-718 (1977).

23. G. D. Steel and J. H. Torrie, *Principles and Procedures of Statistics: A Biometrical Approach*. 2nd Edn, ed. by

C. Napier and J.W. Maisel. McGraw-Hall Book Company, New York (1980).

24. United States Environmental Protection Agency, *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*. EPA-600/4-89-001 (1989).

25. American Society of Testing Materials, Standard practice for conducting an interlaboratory study to determine the precision of a test method, ASTM E691-87, *Annual book of ASTM standards*, **14.02**, 419-438, Philadelphia, (1988).

26. D. A. Dawson, T. W. Schultz and E. C. Shroeder, Laboratory Care and Breeding of the African Clawed Frog. *Lab Animal* **21**, 31-36 (1992).

27. B. K. Kay and H. B. Peng, *Xenopus laevis:* Practical uses in cell and molecular biology. In: *Methods in Cell Biol.* **36**, Academic Press, New York, (1992).

28. E. M. Deuchar, *Xenopus: The South African Clawed Frog.* Wiley, New York, (1975).

29. T. D. Sabourin, R. T. Faulk and L. B. Goss, The efficacy of three non-mammalian test systems in the identification of chemical teratogens. *J. Appl. Toxicol.* **5**, 227-233 (1985).

30. C. L. Courchesne and J. A. Bantle, Analysis of the activity of DNA, RNA, and protein synthesis inhibitors on *Xenopus* embryo development. *Teratogen. Carcinog. Mutagen.* **5**, 177-193 (1985).

31. D. A. Dawson, D. J. Fort, D. L. Newell and J. A. Bantle, Developmental toxicity testing with FETAX:

Evaluation of five validation compounds. *Drug. Chem. Toxicol.* **12**(1), 67-75 (1989).

32. J. R. Rayburn, D. J. Fort, R. McNew and J. A. Bantle, Synergism and antagonism induced by three carrier solvents with t-retinoic acid and 6-aminonicotinamide. *Bull. Environ. Contam. Toxicol.* **46**, 625-632 (1991).

33. B. R. Parkhust, W. Warren-Hicks and L. E. Noel, Performance characteristics of effluent toxicity tests: Summarization and evaluation of data. *Envir. Toxicol. Chem.* **2**, 771-791 (1992).

Table. Results of the Interlaboratory Study for Hydroxyurea.

Laboratory	Replicate	LC50	Mean LC50	LC50 CV*	EC50	Mean EC50	EC50 CV	Mean TI§	TI CV
1	1	0.45			0.16				
	2	0.49	0.45	6.7	0.15	0.16	6.4	2.73	13.0
	3	0.41			0.18				
2	1	0.71			0.17				
	2	0.32	0.51	31.5	0.10	0.15	23.6	3.42	17.9
	3	0.50			0.18				
3	1	0.45			0.21				
	2	2.19	1.11	69.8	0.41	0.24	57.2	4.67	53.0
	3	0.68			0.08				
4	1	0.80			0.14				
	2		0.08	25.9	0.22	0.19	19.0	5.66	50.6
	3	1.36			0.21				
5	1	0.23			0.07				
	2	0.12	0.24	43.8	0.12	0.11	26.8	2.21	43.9
	3	0.38			0.14				
6	1	1.82			0.28				
	2	1.24	1.56	15.4	0.33	0.27	21.7	5.85	33.7
	3	1.63			0.19				
7	1	0.55			0.15				
	2	0.40	0.44	18.7	0.12	0.13	10.9	3.36	8.1
	3	0.36			0.12				

* Coefficient of Variation.

§ Mean Teratogenic Index (TI) calculated by dividing the LC50 by the EC50 for each experiment.

Table. Results of the Interlaboratory Study for Isoniazid.

Laboratory	Replicate	LC50	Mean LC50	LC50 CV*	EC50	Mean EC50	EC50 CV	Mean TI§	TI CV
1	1	10.48			0.28				
	2	9.93	9.96	4.1	0.20	0.23	17.7	43.85	12.7
	3	9.48			0.19				
2	1	10.11			0.23				
	2	11.15	10.15	7.9	0.22	0.20	20.5	51.63	17.6
	3	9.20			0.14				
3	1	0.34			0.32				
	2	2.44	3.23	84.9	0.94	0.45	82.4	7.25	612.5
	3	6.92			0.07				
4	1	10.90			0.17				
	2	10.20	10.19	5.7	0.12	0.14	17.3	72.57	13.8
	3	9.47			0.13				
5	1	0.70			0.22				
	2	1.00	0.90	15.5	0.30	0.22	32.1	4.14	49.7
	3	0.99			0.13				
6	1	11.49			0.20				
	2	10.36	11.10	4.7	0.23	0.20	12.0	55.42	16.2
	3	11.46			0.17				
7	1	9.58			0.23				
	2	9.17	9.50	2.6	0.22	0.23	3.6	41.30	1.2
	3	9.75			0.24				

* Coefficient of Variation.

§ Mean Teratogenic Index (TI) calculated by dividing the LC50 by the EC50 for each experiment.

Table. Results of the Interlaboratory Study for 6-aminonicotinamide.

Laboratory	Replicate	LC50	Mean LC50	LC50 CV*	EC50	Mean EC50	EC50 CV	Mean TI§	TI CV
1	1	2.32			0.0052				
	2	1.70	1.98	12.9	0.0039	0.0048	12.7	417.0	8.1
	3	1.93			0.0052				
2	1	1.93			0.0050				
	2	2.54	2.79	29.6	0.0045	0.0045	9.1	620.1	39.9
	3	3.91			0.0040				
3	1	2.27			0.0054				
	2	2.02	2.05	8.2	0.0032	0.0052	25.4	555.8	19.6
	3	1.86			0.0070				
4	1	2.42			0.0049				
	2	2.08	2.25	6.2	0.0021	0.0044	30.1	432.2	31.5
	3	2.26			0.0063				
5	1	1.91			0.0087				
	2	1.83	1.86	1.8	0.0085	0.0077	15.9	240.9	17.8
	3	1.85			0.0060				
6	1	2.46			0.0076				
	2	2.58	2.54	2.1	0.0900	0.0592	62.0	42.8	322.7
	3	2.57			0.0700				
7	1	2.16			0.0050				
	2	2.24	2.14	4.2	0.0045	0.0046	5.7	461.9	5.8
	3	2.02			0.0044				

* Coefficient of Variation.

§ Mean Teratogenic Index (TI) calculated by dividing the LC50 by the EC50 for each experiment.

Table 4. Summary Data for FETAX Phase I Interlaboratory Study.

Compound	LC50 (mg/ml)	LC50 CV*	EC50 (mg/ml)	EC50 CV	TI**	TI CV
Hydroxyurea	0.75	74.7	0.18	45.0	4.2	79.0
Isoniazid	7.86	49.8	0.24	71.5	33.2	81.2
6-Aminonicotinamide	2.23	20.5	0.013	183.5	174.1	29.7
6-Aminonicotinamide***	2.18	21.7	0.005	31.2	428.6	41.5

* CV= coefficient of variation.

** TI= Mean Teratogenic Index found by dividing the LC50 by the EC50 (malformation) for each experiment.

*** Shows how results would appear if Lab 6 is removed from the Study.

Table 5. Comparison of the Variability of FETAX Endpoints.

Compound	Least Variable	Mid-range	Most Variable
Hydroxurea	EC50	MCIG	LC50
Isoniazid	LC50	EC50	MCIG
6-Aminonicotinamide	LC50	EC50	MCIG

FIGURE LEGENDS

Figure 1. Hydroxyurea 96-hr LC50 and 96-hr EC50 (malformation) values obtained in each laboratory. A) 96-hr LC50; B) 96-hr EC50 (malformation), (•) individual values from a single test, (*) mean laboratory value, (s) standard deviation.

Figure 2. Hydroxyurea TI and MCIG values obtained in each laboratory. A) TI; B) MCIG, (•) individual values from a single test, (*) mean laboratory values, (s) standard deviation.

Figure 3. Hydroxyurea k values representing individual intralaboratory variation. (– – – –) acceptable limit of variation. A) Lines reading from left to right for each Lab are: EC50, MCIG, LC50, TI, B) Lines reading from left to right for each endpoint are Labs 1, 2, 3, 4, 5, 6, 7.

Figure 4. Hydroxyurea h values representing interlaboratory variation. (– – – –) acceptable limit of variation. A) Lines reading from left to right for each Lab are: EC50, MCIG, LC50, TI; B) Lines reading from left to right for each endpoint are Labs 1, 2, 3, 4, 5, 6, 7.

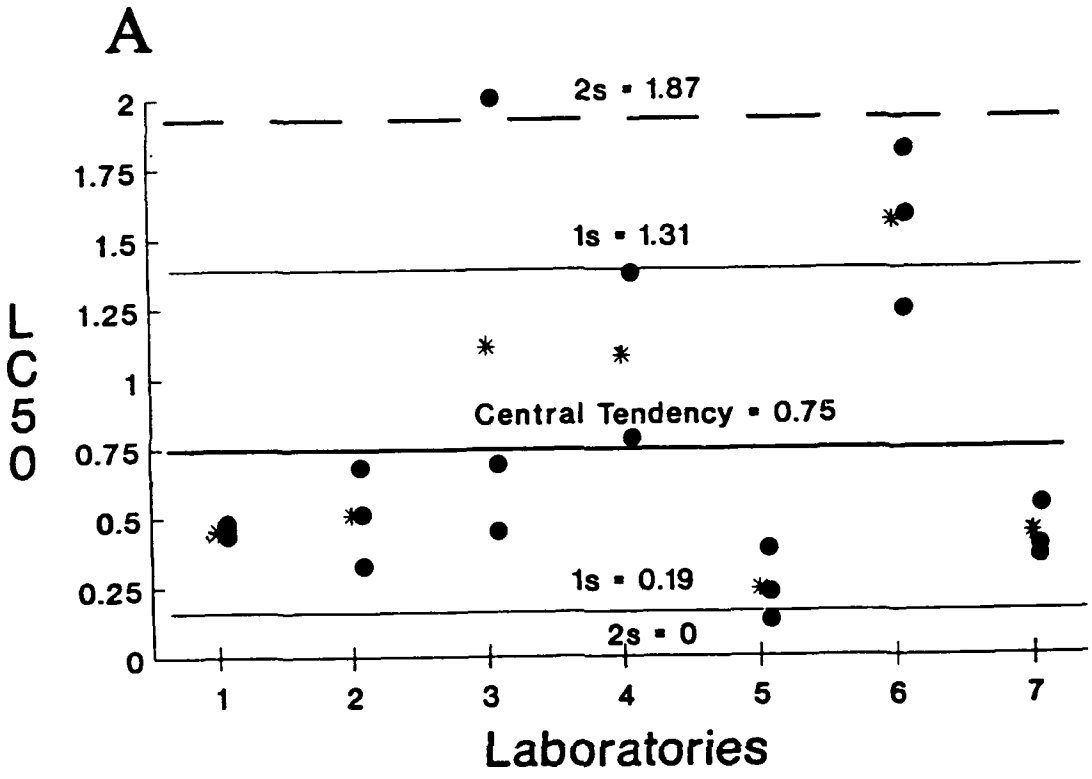
Figure 5. Isoniazid k values representing individual intralaboratory variation. (– – – –) acceptable limit of variation. A) Lines reading from left to right for each Lab are: EC50, MCIG, LC50, TI, B) Lines reading from left to right for each endpoint are Labs 1, 2, 3, 4, 5, 6, 7.

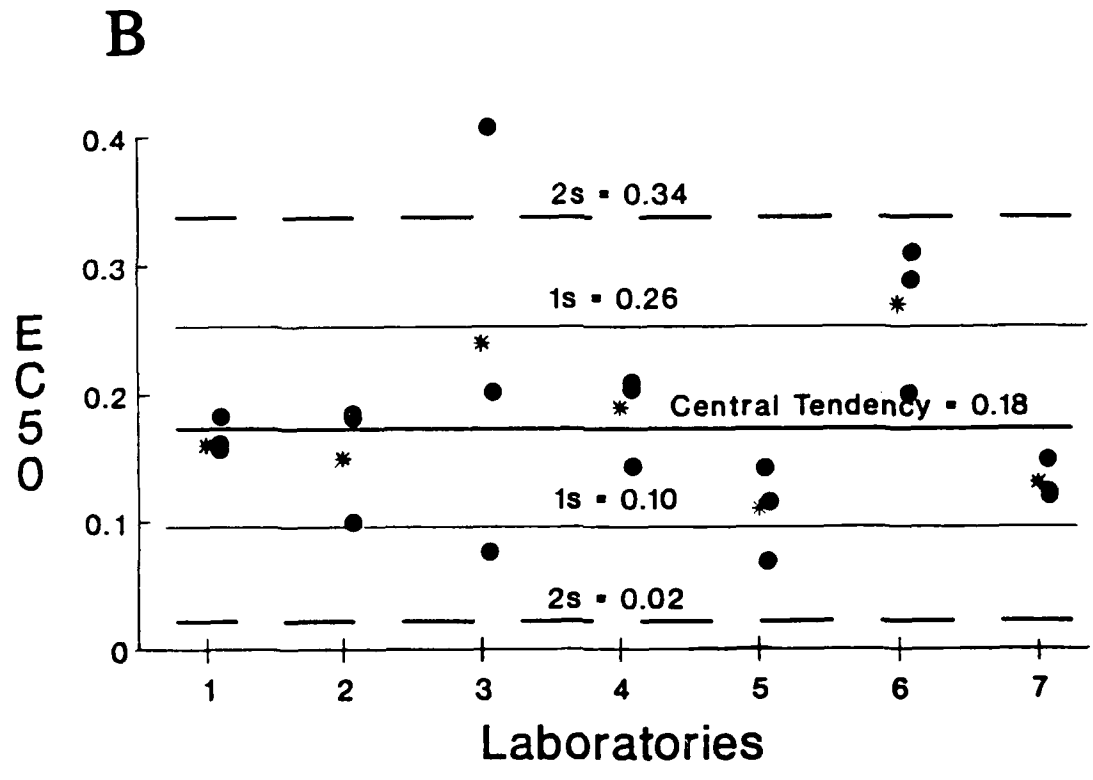
Figure 6. Isoniazid h values representing interlaboratory variation. (– – – –) acceptable limit of variation. A) Lines reading from left to right for each Lab are: EC50, MCIG, LC50, TI; B) Lines reading from left to right for each endpoint are Labs 1, 2, 3, 4, 5, 6, 7.

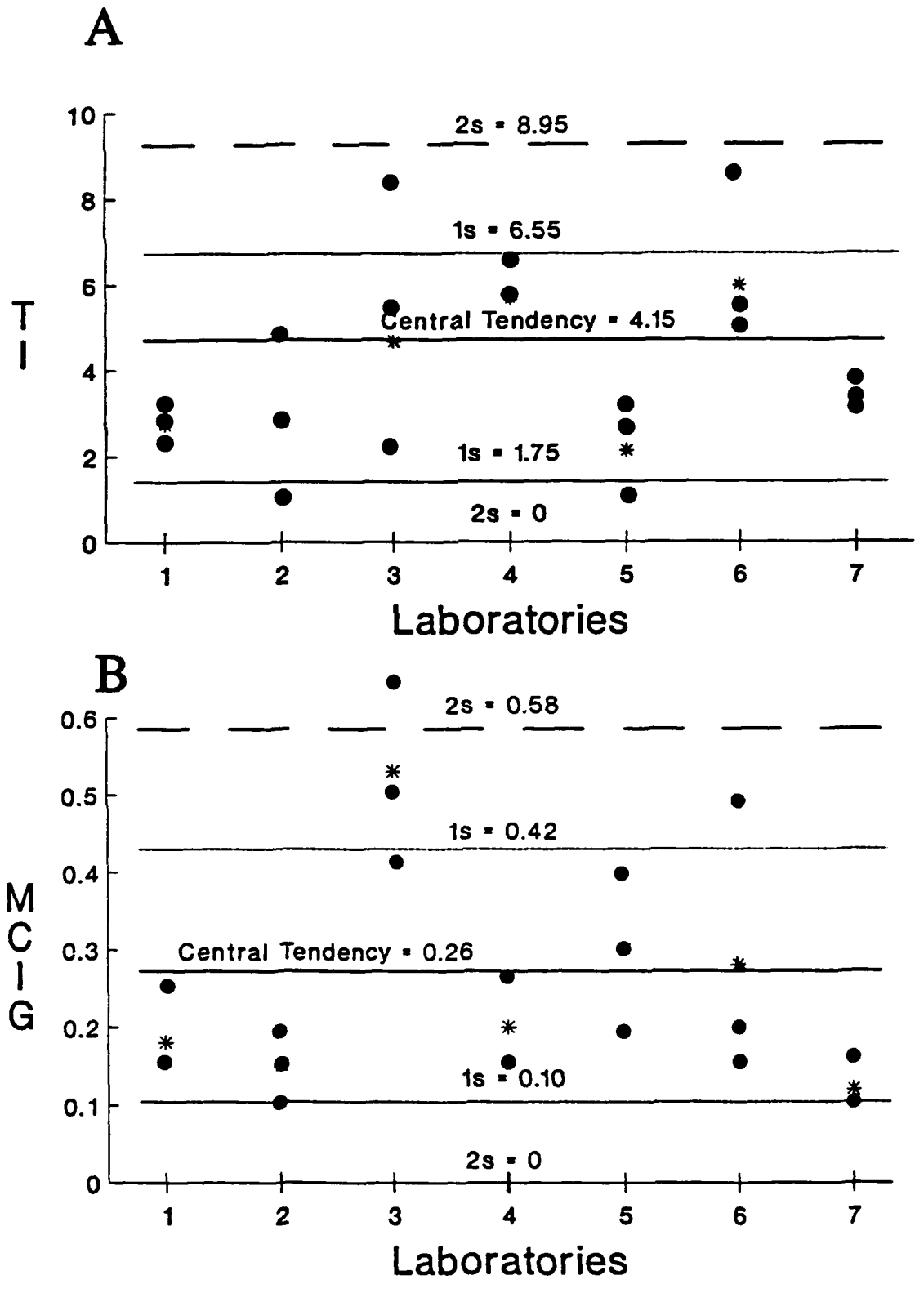
Figure 7. 6-Aminonicotinamide k values representing individual intralaboratory variation. (– – – –) acceptable limit of variation. A) Lines reading from left to right for each Lab are: EC50, MCIG, LC50, TI, B) Lines reading from left to right for each endpoint are Labs 1, 2, 3, 4, 5, 6, 7.

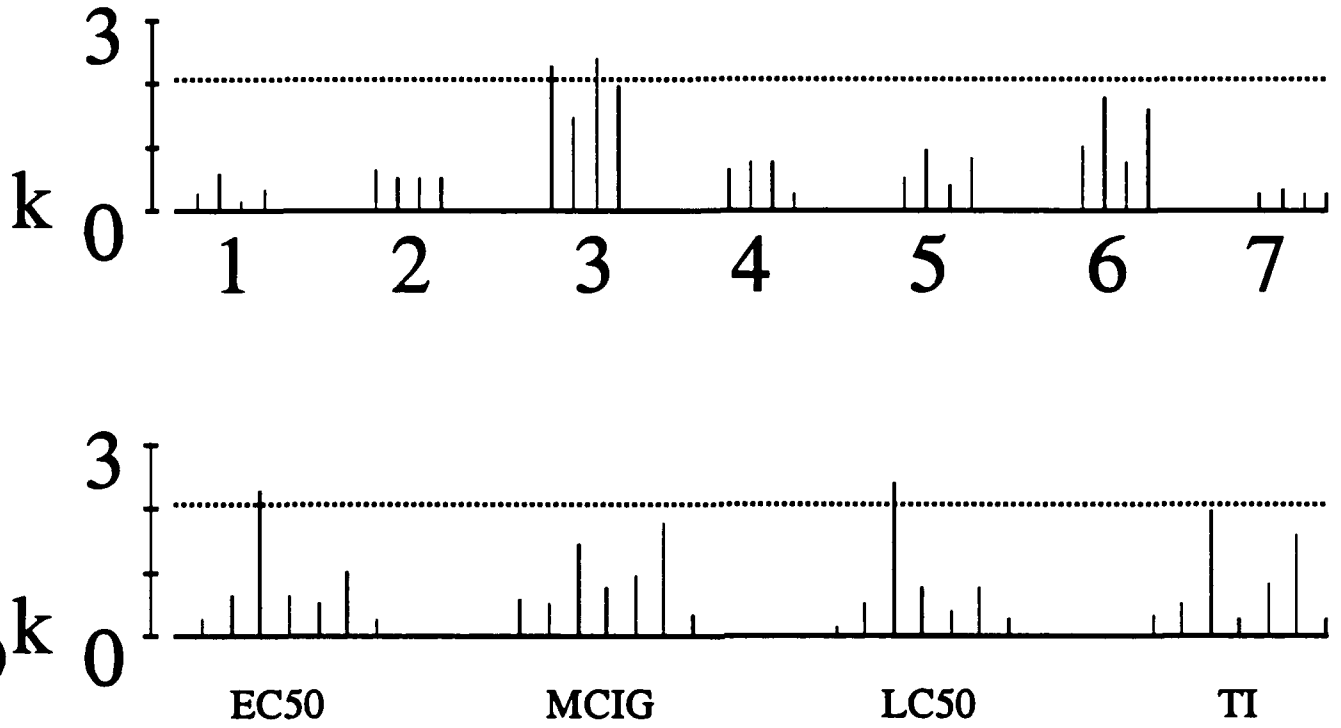
Figure 8. 6-Aminonicotinamide h values representing interlaboratory variation. (– – – –) acceptable limit of variation. A) Lines reading from left to right for each Lab are: EC50, MCIG, LC50, TI; B) Lines reading from left to right for each endpoint are Labs 1, 2, 3, 4, 5, 6, 7.

Figure 9. Concentration-response curves for 6-Aminonicotinamide from a single experiment. A) (•) mortality; (O) malformation; B) Growth.

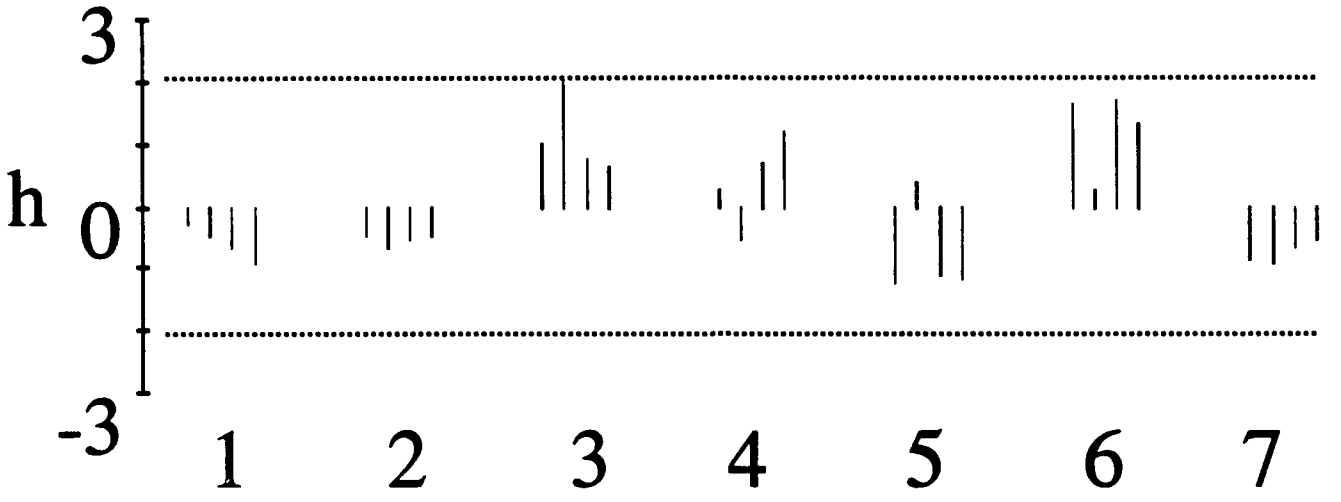

A
2s = 1.87
1s = 1.31
Central Tendency = 0.75
1s = 0.19
2s = 0
LC50
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
1
2
3
4
5
6
7
Laboratories


B
2s = 0.34
1s = 0.26
Central Tendency = 0.18
1s = 0.10
2s = 0.02
EC50
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
Laboratories


A
TI
10
8
6
4
2
0
2s = 8.95
1s = 6.55
Central Tendency = 4.15
1s = 1.75
2s = 0
1
2
3
4
5
6
7
Laboratories
B
MCIG
0.6
0.5
0.4
0.3
0.2
0.1
0
2s = 0.58
1s = 0.42
Central Tendency = 0.26
1s = 0.10
2s = 0
1
2
3
4
5
6
7
Laboratories


3
k
0
1
2
3
4
5
6
7

3
k
0
EC50
MCIG
LC50
TI



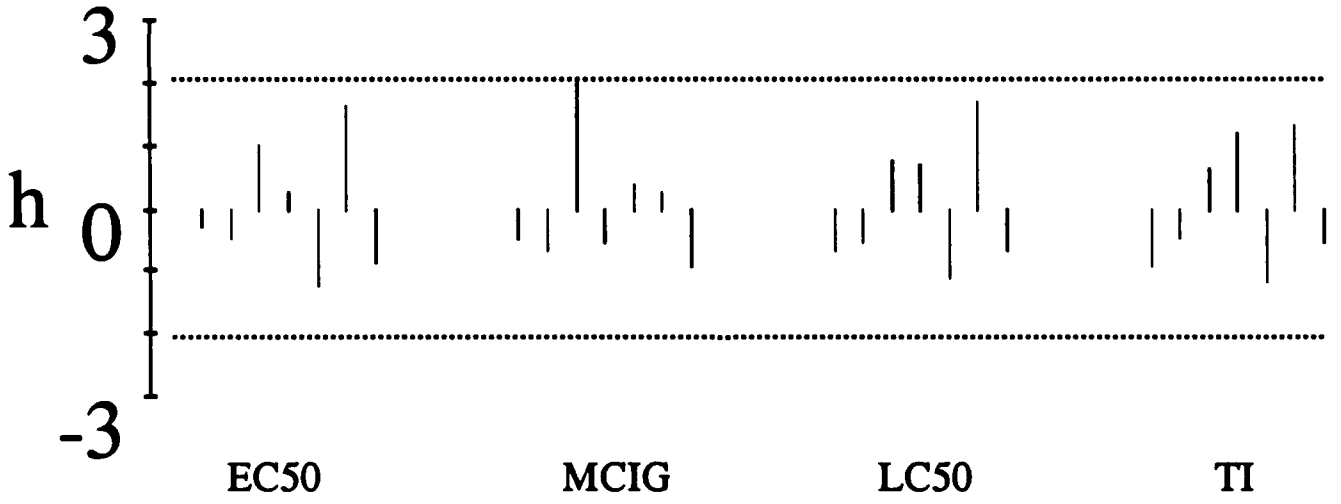


FIG 4

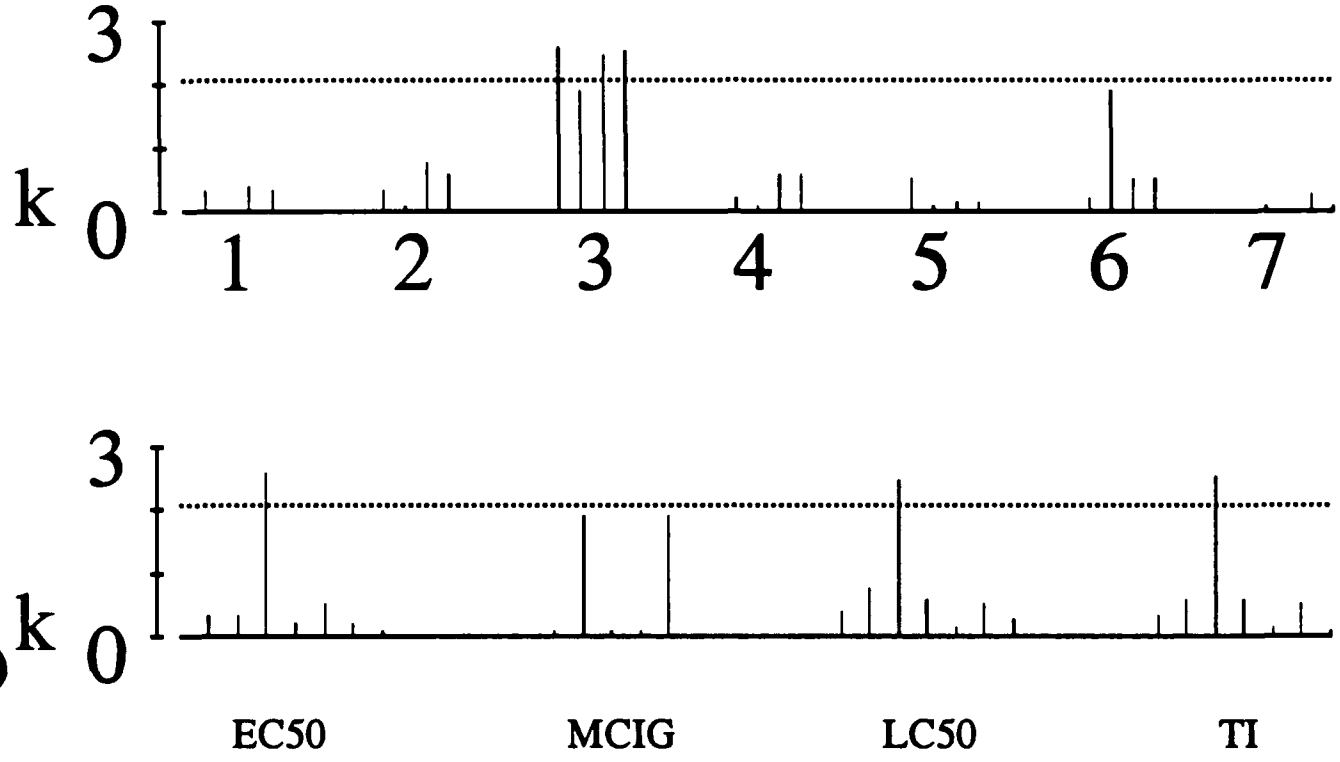
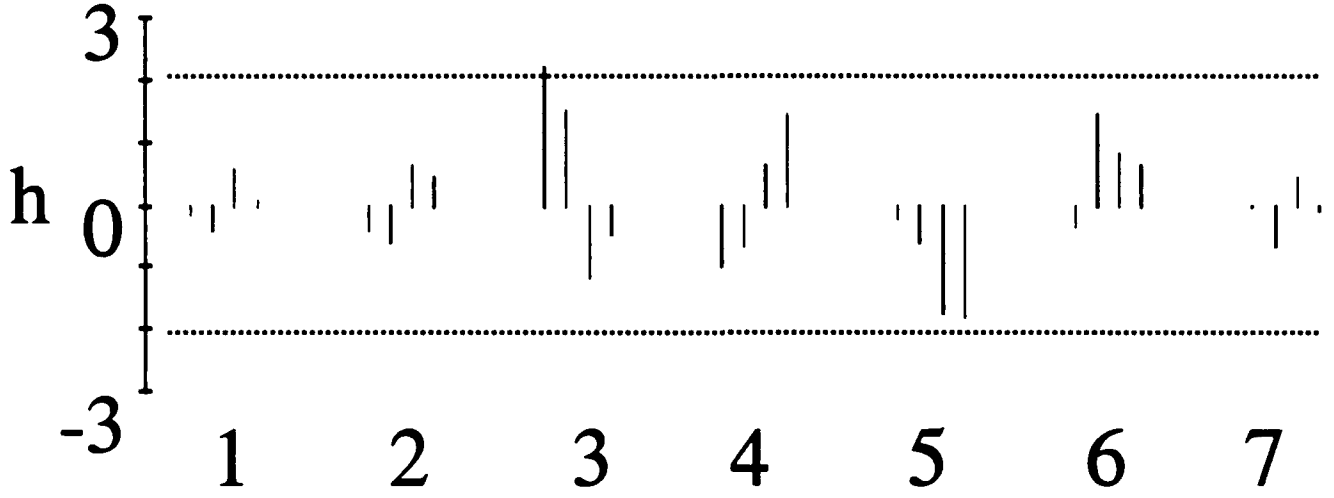
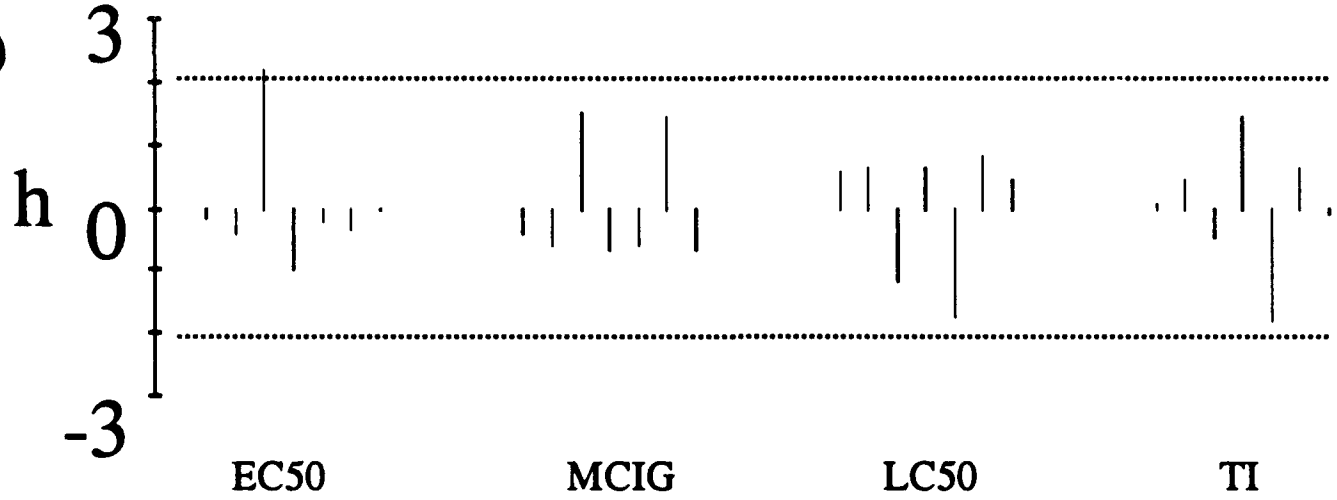
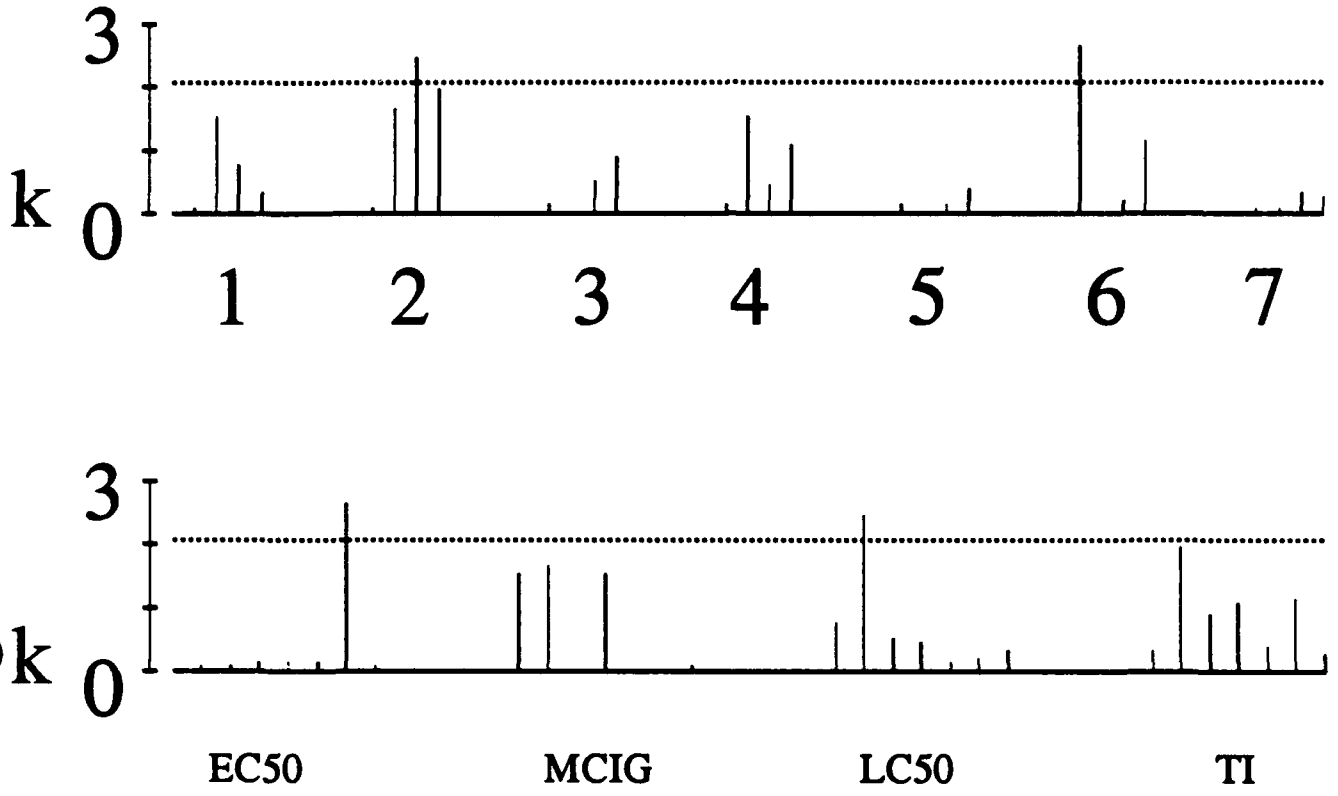


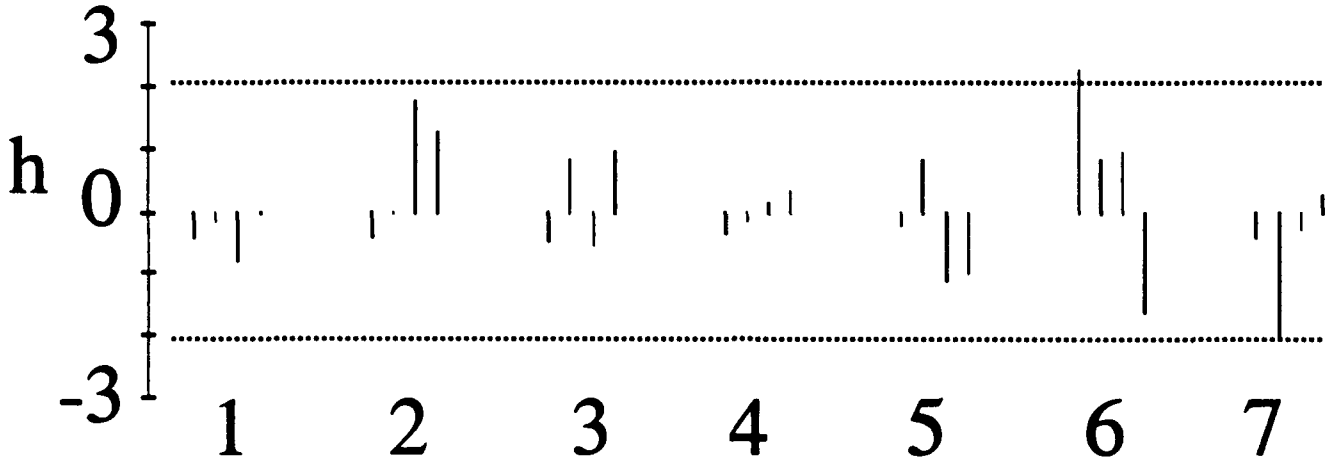
FIG 5


3
h
0
-3
1
2
3
4
5
6
7


3
h
0
-3
EC50
MCIG
LC50
TI


3
k
0
1
2
3
4
5
6
7

3
k
0
EC50
MCIG
LC50
TI



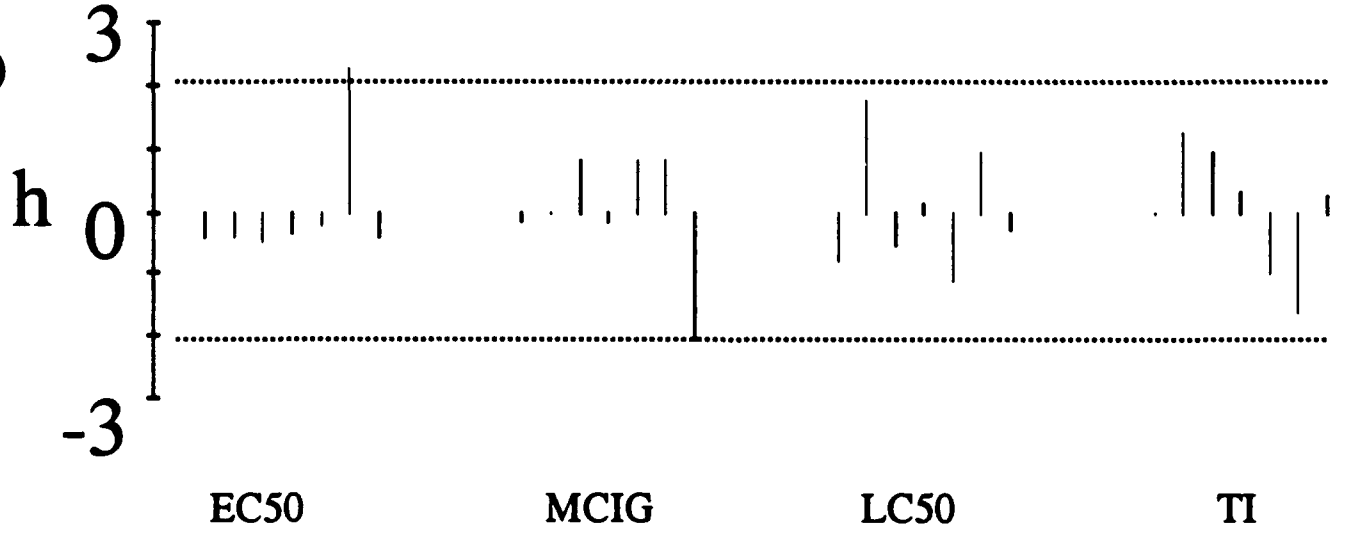


FIG. 8

6-Aminonicotinamide