THE USE OF THE SOUTH AFRICAN CLAWED FROG, XENOPUS LAEVIS, IN SHORT-TERM GENOTOXICITY AND TERATOGENICITY TESTING

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PREFACE

The purpose of this study was to develop an inexpensive, short-term assay to determine the effect of chemicals on gene expression in oocytes of the South African Clawed Frog, Xenopus laevis. This assay was designed to complement the teratogenicity testing of these same test compounds using Xenopus embryos in order to define a molecular basis of teratogenicity. This permits the effect of a test agent on gene expression in the oocyte to be compared to the effect of the substance on the growth and development of the Xenopus embryo.

This dissertation is divided into four chapters. An introduction to this work is presented in Chapter I, followed by a review of the literature in Chapter II. Chapters III and IV are separate and complete manuscripts prepared for submission to the journal, Teratogenesis, Carcinogenesis, and Mutagenesis. The use of Xenopus oocytes as a short-term genotoxicity assay is presented in Chapter III while the use of Xenopus embryos in short-term teratogenicity testing is presented in Chapter IV.

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CHAPTER I

INTRODUCTION

Statement of Problem

All living organisms, with the exception of many viruses, contain DNA. The arrangement of the four nucleotide bases composing DNA, adenine, guanine, cytosine, and thymine, into units called genes, determines the blueprint for proper cellular function. Genes are transcribed into RNA, and if the RNA is the messenger type, the sequence of its bases will determine the order of amino acids composing a protein. Changes in the nucleotide sequence of DNA or in the ability of the DNA to be properly expressed can have deleterious effects on the cellular products that the DNA encodes. Therefore, it is important that the genetic information of a cell be replicated, transcribed, and translated in a precise manner. This is especially crucial during embryonic development since the rapid growth, multiplication, and differentiation of cells depends on the accurate replication of DNA and the expression of specific genes. Agents which interfere with the informational flow of DNA to protein may cause abnormal development or be teratogenic to developing embryos.

Because of the increasing number of chemicals being introduced into the environment each year, it is important to develop short-term tests which can identify agents that alter the normal genetic

expression or composition of DNA in living organisms which may lead to abnormal development. While a substance may not itself be hazardous, chemical decomposition in the environment or metabolism within the organism may produce a harmful product. Many tests have already been developed to identify substances in the environment that are mutagenic to living organisms in that they are able to cause a heritable change in the structure of DNA. Compounds that cause mutations or genetic alterations in the DNA have been positively correlated with carcinogenicity (1-3). The term genotoxic in the traditional sense refers to substances that cause changes in the DNA which may lead to cancer in the organism (4). However, this definition may be somewhat limiting. There are many steps involved in gene expression including transcription, processing and transport, and translation. At each of these levels a substance could interrupt the normal flow of information from gene to product. Therefore, a genotoxin will be defined in this study as a substance that interferes with the normal expression of a gene into its final product.

Objectives

Few, if any assays, have specifically been designed to study the effect of test agents on gene expression as a biochemical basis of teratogenicity. The purpose of this study was to develop an inexpensive, short-term genotoxicity assay to determine the effect of test compounds on gene expression in oocytes of the South African Clawed Frog, Xenopus laevis. The assay was designed to complement the data obtained from the teratogenicity testing of these same test compounds using Xenopus embryos. This permits the effect

of a test substance on gene expression in the oocyte to be compared to the effect of the test compound on the growth and development of the Xenopus embryo.

Experimental Design for Detecting Genotoxic Agents

General Strategy

The genotoxicity assay described in this dissertation used the cloned herpes thymidine kinase (TK) gene as a molecular probe to examine the effects of known inhibitors of DNA, RNA, and protein synthesis on the expression the viral TK gene in Xenopus oocytes. The TK gene was selected not only because it was commercially available as an insert in an Escherichia coli plasmid, but also because it had been extremely well characterized and shown to be expressed in the Xenopus oocyte. The Xenopus oocyte will transcribe the viral TK gene into RNA and translate the RNA into functional protein. The ability of a test compound to change the expression of the TK gene in Xenopus oocytes was determined by measuring the amount of functional TK produced in two different tests referred to as the Gene Exposure Assay and Oocyte Exposure Assay.

Gene Exposure Assay

The Gene Exposure Assay measures the ability of a test agent to alter the expression of the TK gene by damaging the DNA. Such damage could result from the intercalation of the substance into the DNA or possibly by altering the nucleotide sequence composing the TK gene. In

the Gene Exposure Assay, the plasmid DNA containing the TK gene (pHSV106) was exposed for two hours to the test compound prior to injection. After this time, any test substance not bound to the DNA was removed by gel filtration chromatography. The DNA was concentrated by lyophilization. The oocytes were then injected with the DNA in the nucleus and allowed to incubate overnight to allow the synthesis of detectable quantities of TK. After incubation, the amount of TK produced in oocytes injected with DNA previously exposed to the test compound was compared with the amount of TK synthesized in oocytes injected with untreated DNA.

Oocyte Exposure Assay

The Oocyte Exposure Assay tests the ability of a substance to change the expression of the TK gene by inhibiting RNA or protein synthesis without necessarily affecting the DNA directly. For this assay, pHSV106 DNA was injected directly into oocyte nuclei. The plasmid DNA was not exposed prior to microinjection. The oocytes were then exposed to different concentrations of the test substance. After 24 hours, the amount of TK synthesized in oocytes exposed to the test agent was compared to the amount of TK synthesized in oocytes injected with DNA not exposed to the test compound.

Significance of the Gene Exposure and
Oocyte Exposure Assays and Relevance
to Teratogenicity Testing

The proposed Gene and Oocyte Exposure Assays (referred to collectively as the Xenopus Oocyte Assays) are useful because

jointly they identify substances which damage the DNA as indicated byaltered expression of the TK gene and because they identify agents which affect transcriptional and translational processes. Most, if not all, current genotoxicity assays measure only DNA damage that results, either directly or indirectly, from mutational events. In this sense, the Xenopus Oocyte Assays are not traditional genotoxicity tests since DNA damage detected in the Gene Exposure Assay may not necessarily be due to a mutation and changes in gene expression observed in the Oocyte Exposure Assay may not be due to DNA damage at all. However, the Xenopus Oocyte Assays are novel in that they can monitor the flow of genetic information into protein. Compounds which produce changes in gene expression may be teratogenic or cause abnormal development. Because the oocyte is destined on fertilization to divide and differentiate into an embryo, we can confirm with teratogenicity tests whether the deleterious effect of a test substance on gene expression in the oocyte is predictive of malformation and death of the organism later in development. Therefore, the Xenopus Oocyte Genotoxicity Assays are relevant to Xenopus teratogenicity tests as well.

By evaluating both the genotoxic and teratogenic potential of a test substance in Xenopus, the chance that a hazardous compound will escape detection is reduced. This makes the proposed Xenopus system a more predictive indicator of toxicity and decreases the frequency of false negatives or positives which may result from using the molecular assay alone. Moreover, the molecular assay strongly complements the teratogenicity assay since it may reveal genetic damage that is not expressed as an observable physical abnormality.

Experimental Design for Detecting Teratogenic Agents

Xenopus laevis embryos were used by Dumont and coworkers as an organism for teratogenicity studies for several years (5-7) prior to the use of Xenopus embryos in teratogenicity experiments in this laboratory. Dumont et al. (7) named this system FETAX (Frog Embryo Teratogenesis Assay-Xenopus). The development of the FETAX assay provides a strong foundation for the building of ancillary tests which attempt to define a biochemical basis for teratogenicity in Xenopus. In order to measure the teratogenic potential of a substance, Xenopus embryos in the late blastula to early gastrula stage were placed in different concentrations of toxicant. Malformation, growth, and lethality were measured every 24 hours for 96 hours. The teratogenicity index (TI) was calculated by dividing the LC50 (lethal concentration which kills 50% of the embryos) by the EC50 for malformation (effective concentration which causes malformation in 50% of the embryos) according to Dumont et al. (7). A high TI indicates that a compound is teratogenic since it causes malformation at much lower concentrations than are required to kill the organism. A TI of 1 means that the substance is not teratogenic since death and malformation occur at identical concentrations. In this case, the substance should be considered cytotoxic.

In order to determine whether the teratogenic action of a test agent can be linked to its ability to interfere with gene expression, the oocytes are exposed to a concentration similar to the LC50 value obtained during the teratogenicity testing of the compound. If the

LC50 concentration observed in the FETAX assay does not affect gene expression in the Gene Exposure Assay or Oocyte Exposure Assay, then this suggests that the test substance does not act as a teratogen by affecting gene expression. If gene expression is affected by the teratogen, then the substance is also a genotoxicant. The Gene Exposure Assay will identify teratogens which damage DNA while the Oocyte Exposure Assay will identify teratogens which act by affecting either RNA and/or protein synthesis.

CHAPTER II

LITERATURE REVIEW

The Short-Term Genotoxicity Assays

Approaches to Short-Term Testing

Many short-term genotoxicity assays are currently available to identify genetically hazardous substances in the environment. All have advantages and disadvantages. For this reason, a battery of short-term assays are generally performed to evaluate the toxicity of a substance. The battery approach is considered superior to the tier strategy (see 8 for review). In the tier system, chemicals are initially screened with an inexpensive short-term test. On the basis of this single test those substances yielding positive results are then subjected to longer, more expensive studies. Since a false negative could occur during initial screening, the likelihood that a mutagen will escape detection is higher with the tier approach than in battery testing where the results of many different short-term assays are used to assess toxicity.

The short-term genotoxicity assays can be divided into four general classes. These include assays for gene mutation, chromosomal aberrations, DNA repair, and oncogenic transformation. Both *in vivo* and *in vitro* gene mutation assays have been developed using microbial, mammalian, and insect species. The following

discussion of short-term assays is to provide the reader with an overview of the most commonly used genotoxicity assays. The discussion should not be considered inclusive of all assays presently available.

Prokaryotic Gene Mutation Assays

The earliest described microbial gene mutation assays used streptomycin-dependent strains of *E. coli* which were back mutated to streptomycin-independence if the chemical was mutagenic (9). This test was improved by Inyer and Szybalski (10). However, it was later determined that base-pair substitution mutations could be detected with this method but frameshift mutations could not (11). Other *E. coli* systems have also been described (see 12 for review) which are based on the induction of mutations in the forward or reverse direction due to base-pair, frameshift, or small deletion mutations as detected by dependence or independence of the bacteria to streptomycin, tryptophan, arginine, galactose, or 5-methyltryptophan.

The most recognized of the microbial procedures for detecting gene mutations is the Ames test (1-3). The Ames test in its simplest form measures the ability of a chemical to cause a histidine deficient *Salmonella* strain to revert. The appearance of histidine-independent colonies is scored as a mutational event. Different types of *Salmonella* mutants are used thus permitting the type of mutation (base-substitution versus frameshift) caused by a chemical to be determined. There are many variations of the *Salmonella* mutagenicity assay system (13).

The Ames test has several recognized advantages. It is a very rapid, inexpensive test, and can identify many mutagenic substances and

link mutagenicity with carcinogenicity (1-3). However, the Ames assay is far from a definitive test for carcinogenicity (14) and also yields some false positive and false negative mutagenic responses (13). In addition, as in all bacterial assays, the Ames test measures mutations at only one gene locus and uses prokaryotes to extrapolate the effect of chemicals on higher organisms.

The Limitations of Prokaryotes as Indicators of Genotoxicity to Mammals. It is important to realize the problem of using prokaryotes as indicators of toxicity to mammals. The eukaryotic genome is regulated quite differently from the prokaryotic genome. Prokaryotic genes are typically arranged in operons while eukaryotic genes are not (15). Recombinant DNA work has demonstrated the presence of intervening noncoding sequences in the middle of protein-coding sequences of eukaryotic genes (16). Many sites unique to eukaryotic DNA seem to regulate gene expression. These sites include DNA sequences which control transcription initiation and termination (17), splicing (16), and polyadenylation (17). There are many post-transcriptional and translational controls which regulate gene expression in eukaryotes such as pre-mRNA cleavage and splicing, 5' mRNA capping, mRNA methylation, polyadenylation, and mRNA recognition at the ribosomes (18). A chemical may not be toxic to the comparatively simple prokaryote but extremely deleterious to the eukaryote since it may act on any one of the many unique regulatory mechanisms involved in eukaryotic gene expression. Therefore, with the goal of being more relevant to higher eukaryotes, mammalian systems have been developed to screen for genotoxic chemicals.

Mammalian Gene Mutation Assays

In Vitro Mammalian Gene Mutation Assays. There are many *in vitro* mammalian gene mutation assays and all involve cell culture. One of the most popular systems utilizes the CHO or V79 cell lines derived from the Chinese hamster ovary and lung tissue, respectively (see 19-20 for review). Most studies using these cell lines employ the hypoxanthine-guanine phosphoribosyltransferase (HPGRT) gene loci to monitor mutational events by measuring the resistance of the cells to the purine analogs 8-azaguanine or 6-thioguanine (19-20). The HPGRT gene loci and ouabain resistance have been used as markers for mutant selection in mouse lymphoma L5178Y and human lymphoblast cells (19-20). The thymidine kinase gene has been used for gene mutation studies in mouse lymphoma L5178Y cells (19-20).

While the mammalian cell culture assays may identify some mutagenic compounds the Ames test does not (21), these test systems, like microbial assays, may be insensitive to some mutagens due to the highly specific nature of the lesion required to produce a response (20). Mutational events occurring along the DNA at points other than at the marker gene locus will go undetected. In addition, cell culture lines can be difficult and expensive to maintain. While mammalian cell culture systems provide data faster than long-term studies, a considerable amount of money, time, and labor is involved which reduces the number of chemicals that can be investigated at a particular time.

In Vivo Mammalian Gene Mutation Assays. Several *in vivo* mammalian gene mutation assays are available. Two common assays are the somatic cell spot test and the specific locus test (see

19, 22 for review). The somatic cell spot test detects mutations at several coat color loci in strains of mice. Females are treated with the test compound ten days into pregnancy. Three to five weeks after parturition the offspring are scored for coat color spots due to mutation at the coat color loci (22). The spot test has several advantages. It can identify, using a conservative number of animals, mutations at more than one marker locus and provides information on the ability of a compound to cross the placental blood barrier (22). However, there are also disadvantages with this system. The test is long for a short-term assay, requiring three to five weeks for completion. Identification of true recessive spots can be difficult and the time of exposure of the animal to the test substance is critical since changes in the mutational frequency will result (22).

The specific locus test crosses wild-type mice treated with the test compound with mice carrying multiple recessive traits. The F1 offspring will exhibit the wild-type phenotype unless a mutation has occurred. Additional tests are performed on mice possessing mutant phenotypes to determine the nature and heritability of the mutagen. This test only identifies mutagens that cause phenotypic changes and it is difficult to determine the nature of the mutation in phenotypically altered mice (22). Moreover, large sample sizes are required and the test takes several weeks to perform.

Another *in vivo* gene mutation assay uses *Drosophila melanogaster* and is called the SLRL assay since it detects induction of sex-linked recessive lethals in the second generation of treated individuals (see 12 for review). This test detects point mutations of all types (19) and offers several advantages. It covers a

wide range of genetic damage from point mutations to major deletions in the DNA, utilizes a eukaryotic organism, and activates test compounds to their mutagenic form. The major problem with the assay is quantitating the dose actually administered to the flies during feeding. In order to solve this problem, extensive preliminary toxicity and uptake studies are required to ensure that the chemical has been taken up by the flies (19).

Assays Which Detect Chromosomal Aberrations

Somatic Cell Assays. There are many somatic cell assays which measure the ability of a substance to cause chromosome damage. Three examples of somatic cell assays detecting DNA damage are somatic cell cytogenetics, the micronucleus test, and sister chromatid exchange (see 22 for review).

As reviewed by Soares (22) there are many cytogenetic assays utilizing somatic cells with well defined karyotypes. For example, human, chinese hamster, mouse, syrian hamster embryo, and rat liver cells have been used. Metaphase chromosomes are examined for the presence of aberrations including breaks, gaps, deletions, translocations, and ploidy changes (22). While these assays offer the advantage of providing direct observation of chromosome damage, they cannot detect mutagenic compounds that cause microlesions in the DNA. Moreover, chromosomal aberrations in these assays may not be the result of direct action of the test compound on the chromosomes. Thus, interpretation of the data is difficult (22).

Another in vivo short-term assay used to detect chromosome damage is the micronucleus test (22). Reticulocytes or polychromatic

erythrocytes from bone marrow are removed from animals treated with the test substance and then scored for chromosomal fragments in the cytoplasm of interphase cells. An increase in the number of micronuclei in experimental animals over controls suggest that the substance causes genetic damage. The test is simple, but like with the cytogenetic assays, interpretation of the data is difficult. While the presence of micronuclei does suggest genetic damage, such chromosome aberrations may not necessarily be a direct result of treatment with the test substance (22).

Sister chromatid exchange (SCE) measures the number of crossovers between sister chromatids which occur after chemical exposure. SCE protocols detect the ability of a compound to induce DNA rearrangements, presumably toward the goal of repair. These exchanges apparently result from chromatid breakage and subsequent reunion. The significance of SCE is poorly understood. Apparently, the greater the DNA damage, the higher the frequency of cross-overs (repairs). Mutagens not causing DNA breakage may not be observed with this assay, placing great doubt on negative results for a test substance when the mechanism of action of the mutagen is unknown (22).

Germ Cell Assays. Many germ cell assays for detecting mutagenesis in vivo by way of chromosomal aberrations have been developed. These include the dominant lethal assay, heritable translocation assay, and sperm morphology assay (see 19, 22 for review).

The dominant lethal assay measures the number of living and dead embryos in mouse uteri following mating with a male treated with the

test substance. An increase in dead implantations is indicative of induced dominant lethal mutations in the sperm cells of the male. This assay has been reported to identify compounds that most likely cause chromosomal damage although lethality could possibly be due to single gene mutations (22). This assay has several other disadvantages. It is relatively insensitive due to spontaneous lethals. Moreover, the relevance of the test is questionable since dominant lethals cause no genetic threat to future generations (22), although there is concern for identifying substances that cause fetal loss in humans.

The heritable translocation assay detects chromosome aberrations in the form of translocations which have occurred in the germ cells of the test organism. Usually male mice are exposed to a chemical and allowed to breed with a female. The male offspring are then placed with a female for mating. The female is killed 17 days after mating and the dead and live embryos are scored. An increase in dead embryos in treated over control groups suggests a mutational event. The presence of a translocation in the male germ cells is verified by cytogenetics. This test has the advantage of being more sensitive than the dominant lethal assay and detects heritable genetic defects (22). However, small mutations in the DNA may go undetected if they do not affect fertility. In addition, the test requires 10 weeks and the cytogenetics involved requires special skill (22).

The sperm morphology assay detects the mutagenic capabilities of a compound in the sperm cells of male mice treated with the test compound for 2-10 weeks. The number of abnormal sperm is recorded. While the test is simple with sensitivity approaching that of the Ames test in

detecting mutagens (22), changes in sperm morphology are not necessarily due to genetic damage (22).

DNA Repair Assays

DNA repair assays include SCE and unscheduled DNA synthesis (UDS) tests. SCE assays have already been described as a somatic cell technique for detecting chromosome damage. UDS assays measure the synthesis of DNA in nondividing confluent monocultures after chemical exposure. The amount of tritiated thymidine incorporated into DNA is measured by autoradiography. UDS repair assays are burdened with technical difficulties since numerous controls are required to distinguish UDS from spurious repair (19).

Cell Transformation Assays

There are many cell transformation assays which measure the ability of a substance to oncogenically transform a mammalian cell line (see 23 for review). Hamster, mouse, rat, and human cell lines have been used. However, while most of these assays show promise as good methods for identifying carcinogens, a great deal of work must be done before these assays will be of practical use (23). Many carcinogenic chemicals are not metabolized to their active intermediates by cells in culture. Standardization of cell lines is also a problem because the frequency of spontaneous transformation varies. Moreover, the identification of transformed cells tends to be subjective since it is often based on the morphological appearance of the cells. This demonstrates the need for the development of biochemical markers as

indicators of transformation (23). In addition, cell culture systems are difficult and expensive to maintain.

The Need For the Development of Additional Short-term Genotoxicity Assays

All short-term genotoxicity assays have serious limitations. However, when assessing the toxicity of a chemical, these limitations can be minimized when a battery of tests are used (8). All of the short-term genotoxicity discussed were designed to identify only mutagenic agents. These tests were not designed to identify compounds which affect gene expression by nonmutagenic DNA damage or through alterations in RNA or protein synthesis. Furthermore, present genotoxicity assays cannot link the molecular events involved in gene expression to the embryonic development of the organism. It is known that environmental agents can cause either congenital or nonheriditary malformations in development. Such substances are called teratogens. The correlation between the teratogenic and mutagenic effect of chemicals in mammals has been known for many years (24). However, tests which attempt to define a biochemical basis of teratogenicity through changes in gene expression or DNA damage using a single organism have yet to be developed.

The Development of *Xenopus laevis* as a Test Organism

It has been stated that *Drosophila melanogaster* is the best genetically characterized and standardized test organism with the

house mouse ranking a distant second (22). However, Xenopus laevis, the South African Clawed Frog, ranks highly when compared to both of these organisms with regard to the level of characterization of gene organization, gene expression, growth and development. The stages of morphological events associated with the development of Xenopus from the fertilized egg through metamorphosis have been well documented (25). The changes in the synthesis of the various classes of nucleic acids during oogenesis, fertilization, and early development in the frog embryo have been determined (26). Dumont has characterized the stages of Xenopus oocyte development, from the immature follicle to the mature cell (27). Xenopus oocytes can be surgically removed by the hundreds without the death of the animals and the oocytes survive for at least 48 hours in simple salt solutions (28). The frogs are commercially available from many vendors and are easily and inexpensively maintained in the laboratory. It seems then, that Xenopus may be an excellent organism for toxicity testing.

The potential of using Xenopus embryos for toxicity testing was recognized by several investigators but most notably by Greenhouse (29-31). Dumont and coworkers later standardized and validated the use of Xenopus embryos in a true teratogenicity assay and named this system FETAX (Frog Embryo Teratogenesis Assay, Xenopus) (5-7). Dumont suggested the use of the teratogenicity index (LC50/EC50 malformation) as a numerical representation of the teratogenic potential of a substance; the larger the teratogenicity index (TI), the more potent the teratogen (7). The TI may provide a method of extrapolating toxicity data collected for the amphibian to other species, including mammals. However, differences in species

sensitivity to test compounds may cause large variations in teratogenicity indices. Nonetheless, a compound with a high TI for several species is more likely to be teratogenic to man than substances which possess a low TI in different organisms since in the latter case the incidence of malformation would be less.

The effect of various inhibitors of DNA, RNA, and protein synthesis on development has been investigated (see 32 for review). Agents which inhibit DNA synthesis, such as chemotheraputic compounds, are potent teratogens, although the mechanism of teratogenesis may be through cell death rather than through the actual inhibition of DNA synthesis itself (32). RNA synthesis inhibitors such as actinomycin D have also been associated with teratogenesis (32). However, in contrast to DNA and RNA synthesis inhibitors, inhibitors of protein synthesis do not appear to be teratogenic but are certainly cytotoxic (32). This suggests that protein synthesis inhibitors are not specific for a particular cell type (32) unlike what may be the case for DNA and RNA synthesis inhibitors.

The oocyte offers a unique advantage as a molecular system for studying the effects of toxins at the level of the gene because the oocyte represents a cell destined after fertilization to divide and differentiate into an embryo. This permits the effect of a test substance on the gene in the Xenopus oocyte to be compared to the effect of the test agent on the development of the Xenopus embryo as determined by teratogenicity testing. While Xenopus embryos have been previously employed in toxicity tests (5-7), Xenopus oocytes have not. However, the Xenopus oocyte is well known for its ability to transcribe foreign genes which have been microinjected

into the nucleus and translate foreign mRNAs. Thus, it seemed possible to design a toxicity assay to study the effect of chemicals on the expression of a particular gene in the Xenopus oocyte. The characteristics of the oocyte which enable its use in a toxicity assay will now be discussed.

The Xenopus Oocyte

The Xenopus oocyte is a very large cell at maturity (stage 6), being 1-1.2 mm in diameter with a 0.4 mm nucleus (germinal vesicle) (27). The oocyte nucleus is not only remarkable for its size but for its rate of hnRNA and rRNA synthesis as well. It has been estimated that hnRNA is transcribed with an average frequency two to three times greater in the oocyte than the rate normally found in animal cells (33). During oogenesis, each oocyte accumulates massive amounts of diverse mRNA species (34-35). The accumulation of mRNA in the oocyte occurs early in oogenesis and does not occur thereafter (36-37). However, transcription of single copy genes by RNA polymerase II has been demonstrated in full grown oocytes (see 38 for review). Estimates on the rate of mRNA synthesis suggest that this may be due to the rapid production and degradation of mRNA by mature oocytes (33, 39). The possibility that mature oocytes are deficient in some mechanism of mRNA processing seems unlikely since mature oocytes have been demonstrated to be fully capable of several post-transcriptional activities (40). By maturity, an oocyte contains great reserves of RNA and proteins required for embryogenesis. To assure successful development following fertilization the oocyte contains a store of ribosomal 4S and 5S RNAs

(41), RNA and DNA polymerases (42-43), histones (44), and nucleoprotein assembly complexes (45-46).

The Uses of the Xenopus Oocyte in Microinjection Studies

The Oocyte as a Method for Studying Gene Expression

In 1971, Gurdon and coworkers injected mRNA into the cytoplasm of the Xenopus oocyte and found that the oocyte translated this message into protein (47-48). Over the past ten years not only mRNA but cloned and purified genes as well as organelles have been injected into amphibian eggs and oocytes (see 49 for review). Xenopus oocytes represent a living cell which can be used to study purified macromolecules. Xenopus oocytes are extensively used to study different aspects of gene regulation including DNA template requirements, polyadenylation, RNA splicing, base modification, order of RNA processing, partitioning of RNA molecules between the nucleus and the cytoplasm, and post-translational modifications (50).

Microinjection of mRNA Into Xenopus Oocytes

A great deal of information regarding translation has come from mRNA injection studies with Xenopus oocytes. The frog oocyte translates, with varying efficiency, a wide variety of mRNAs from different species and phyla including insects, birds, mammals, viruses, plants, and fishes (see 51-53 for review). It is generally agreed that the translational machinery of the Xenopus oocyte is neither cell type (54), species (48), nor phylum specific (55) since heterologous

messengers (mRNAs other than those of Xenopus origin) are translated. However, Lane (52) has pointed out that this conclusion must be held with caution since most of the mRNA injection experiments used crude mRNA preparations that may have contained species-specific factors which permitted translation. Nonetheless, experiments have shown that all messengers use at least some common translational machinery since competition occurs between different injected messengers (56) as well as between endogenous (oocyte) and injected messengers (57) in oocytes. While the oocyte seems to translate any authentic eukaryotic mRNA (see 52 for review), other mRNAs such as synthetic polynucleotides (58) and bacteriophage mRNA (47, 53) are inactive. There is evidence that a 7-methylguanosine cap is required for translation since removal of the cap from globin mRNA (59) dramatically reduces translation while the addition of a cap to a prokaryotic messenger permits its translation in a eukaryotic system (60). It has been suggested that the role of the cap is to protect the mRNA from degradation (53). However, it is unknown whether many of the mRNAs translated in oocytes actually possess a 5' terminal cap or not (49) since many of these reports have not examined the mRNA termini. The literature suggests that at least one protein, ovalbumin, is apparently transcribed and translated from genes which are deficient in cap site sequences (61).

The role of the poly(A) segment on eukaryotic messengers has been investigated using Xenopus oocytes. In the case of SV40 RNA it has been shown that only adenylated molecules are able to leave the nucleus of the oocyte (40). This does not appear to be true for all transcripts injected into nuclei since the messengers of histone and

the thymidine kinase genes are largely deficient in poly(A) but are translated anyway (62, S. L. McKnight, personal communication to 49). The poly(A) segment has been suggested to play a role in the stability of mRNA molecules in the cytoplasm since some deadenylated mRNAs, for example, globin mRNA, are less stable in Xenopus oocytes than adenylated molecules (see 53 for review). However, not all deadenylated mRNAs appear to be less stable in Xenopus oocytes than adenylated transcripts (63-64). On the basis of this information, it is possible that the poly(A) segment may function differently for different mRNA species.

Translation in the oocyte is not regulated by the supply of mRNA (57) as believed in early studies (65). Therefore, oocytes do not have spare translational capacity since endogenous and injected messengers compete for a limited translational activity (57). The limiting factor appears to be some component of the translational apparatus (56-57).

Xenopus oocytes are also capable of performing post-translational modifications on many foreign proteins (see 52 for review). These modifications include phosphorylation, hydroxylation, glycosylation, acetylation, signal sequence removal, and disulfide bond formation of proteins from a diverse group of organisms including viruses, fishes, insects, amphibians, birds, and mammals (52). This demonstrates that frog enzymes can process foreign proteins which suggests that these enzymes are not species or cell-type specific.

Injection of Purified DNA and Cloned Genes

Replication in Oocytes and Unfertilized Eggs. As a logical extension of mRNA injection experiments, DNA was injected into

Xenopus oocytes and unfertilized eggs. Initial experiments were designed to study gene replication. Purified DNA was replicated when injected into the cytoplasm of unfertilized eggs (66). The mechanism of replication in unfertilized eggs appeared to follow the semiconservative model (67-69). DNA was not replicated when injected in the cytoplasm of oocytes (46, 70-73). When DNA was injected into the nucleus of oocytes, replication occurred only at very low levels (71) or not at all (67). Because DNA is not significantly replicated in oocytes (67, 71), the expression of DNA introduced into Xenopus nuclei reflects the actual number of genes microinjected into the cells (74).

When one considers the changes which occur in the synthesis of various nucleic acids during oogenesis, fertilization, and early development of Xenopus, it is hardly surprising that microinjected DNA will be replicated in unfertilized eggs but will not be replicated in oocytes. DNA synthesis in Xenopus oocytes occurs only at low levels while RNA synthesis proceeds at very high levels. In the case of unfertilized eggs, where cleavage is stimulated artificially by injection (75), DNA synthesis becomes quite active following germinal vesicle breakdown while RNA synthesis is negligible until the late blastula or early gastrula stage (26). Because oocytes will synthesize greater amounts of RNA and protein and are generally more tolerable to microinjection (49), they are routinely selected for the injection of purified DNA and cloned genes.

Transcription in Oocytes. The first indication that pure DNA may be transcribed in Xenopus oocytes came from the work of Colman

who demonstrated that the synthetic nucleotide poly d(AT) stimulated RNA synthesis (76). In 1977, Mertz and Gurdon reported that SV40 DNA was transcribed in Xenopus oocytes (77) while Brown and Gurdon showed that purified Xenopus 5S RNA was faithfully transcribed in oocytes as well (78). In that same year, DeRobertis and Mertz demonstrated that some of the mRNA synthesized after injection of SV40 DNA was translated (79). This was the first report of coupled transcription-translation in the oocyte. At present, numerous purified and cloned genes have been transcribed in frog oocytes by the correct RNA polymerase from plasmid, phage, viral, plant, and animal sources (see 52 for review).

Fate of Injected DNA. Xenopus oocytes will transcribe genes injected directly into the nuclei but not the cytoplasm (77, 80). Microinjection of Xenopus oocytes into the germinal vesicle was first developed by Gurdon (66) but later modified by Kressmann (80) to permit visualization of the nucleus. When DNA is injected into oocyte nuclei it does not appear to be integrated into Xenopus chromosomes (75) and so there is no direct influence of the host cell genes on the expression of the foreign nucleic acid (75). This permits the effect of a test substance on the expression of one particular gene (the gene microinjected) to be determined.

The amount of DNA injected into oocytes dictates the amount of protein (81) or RNA (82) transcribed in oocytes. This suggests that injected genes are transcribed independently of one another since large amounts of microinjected DNA does not compete out endogenous transcription (82). However, competition between different injected

messengers (56) for translation does exist. Thus, it appears that there may be spare transcriptional activity but not spare translational activity (57) in oocytes.

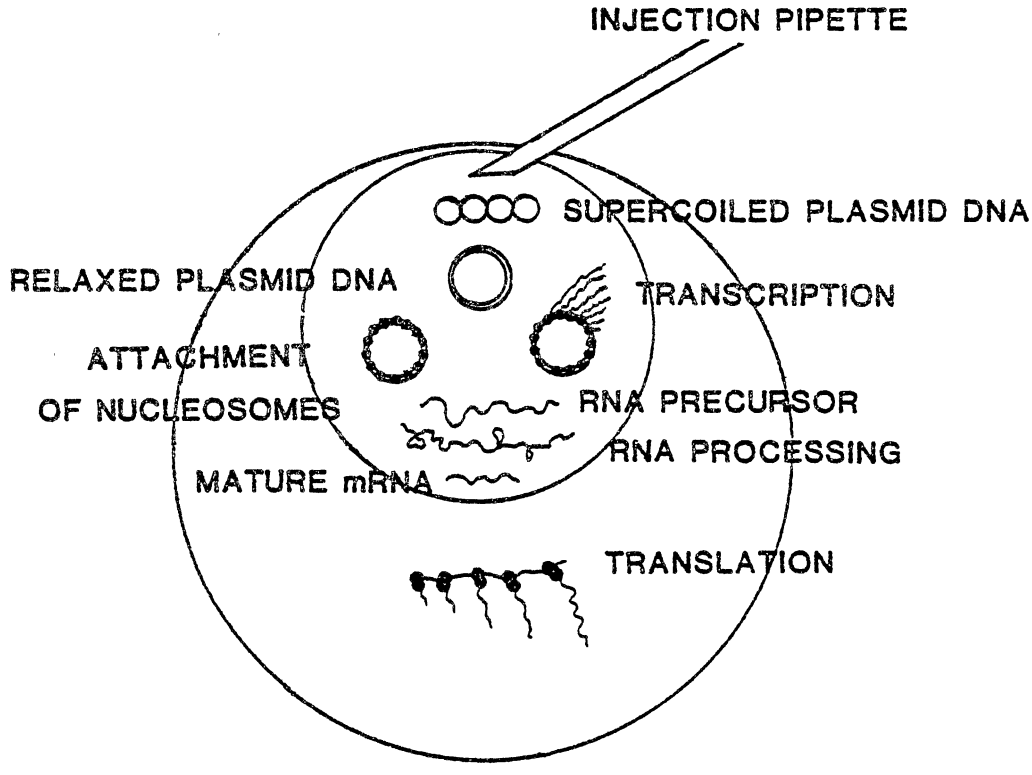
The Importance of Template Topology. The transcription of injected DNA molecules is influenced by the conformation of the template (see 49 for review, 72, 83-84). Circular conformations of DNA are degraded in the cytoplasm (49) but are stable in the nucleus (49). Linear DNA templates are degraded in both the nucleus and the cytoplasm (49). The transcriptional inefficiency of linear templates in the nucleus cannot be due to degradation alone (84). The inefficiency of the protein-coding linear RNA polymerase II templates is far greater than what is observed for linear RNA polymerase III templates (84) which transcribe tRNA and 5S RNA genes. Linear molecules carrying tRNA (80) or 5S RNA (82) are transcribed only slightly less efficiently than circular templates. However, circular templates are preferred, and there is evidence that the template must be double-stranded for transcription, at least in the case of tRNA genes (85). Circular double-stranded DNA templates injected into the nuclei are assembled into nucleoprotein complexes (84, 86) and appear as beaded chains like cellular chromatin (86). Circular DNA injected into the nuclei of frog oocytes is initially relaxed then supercoiled (86). Linear DNA is not assembled into chromatin in oocytes (84). Nucleoprotein complexes of injected circular DNA molecules have been visualized with the electron microscope (87-88). Even mitochondrial DNA, which in nature is not associated with histone and nuclear transcriptional complexes, forms nucleosome like structures when injected into Xenopus oocytes (73).

The bulk of injected circular DNA templates may be transcriptionally inert (88-89), which is supported by the low transcription of heterologous RNA polymerase II genes in Xenopus oocytes (see 49 for review). The overall process by which injected DNA is expressed in the frog oocyte is illustrated in Figure 1.

Transcription of RNA Polymerase III Genes. The best studied of the genes microinjected in Xenopus oocytes have been those genes coding for various tRNAs which are transcribed by RNA polymerase III (90-91). In 1977, Kressmann et. al (80) found that 4S RNA was synthesized by oocytes injected with Xenopus $tDNA_{met}$. Later it was shown that this $tRNA_{met}$ from Xenopus was fully processed and modified after injection (92). Heterologous tDNAs, including nematode (93) and yeast tyrosine tRNA genes (90, 94), are also transcribed in Xenopus oocytes. It is now believed that Xenopus oocytes will transcribe eukaryotic nuclear tRNA genes but neither prokaryotic nor mitochondrial tRNA genes (91).

Frog oocytes microinjected with tRNA genes have been used to analyze RNA processing steps in eukaryotes. tRNA genes injected into Xenopus oocytes undergo post-transcriptional removal of the 5' leader sequence (91), base modifications, addition of the 3' terminal CCA end, splicing of intervening sequences (90, 94), and excision of extra 3' and 5' nucleotides (94). These modifications appear to occur in the nucleus rather than in the cytoplasm (91). The splicing enzymes required for removal of intervening sequences are found in the nucleus (91, 95) and not in the nuclear membrane (95). Only mature tRNA molecules leave the nucleus (91).

Figure 1. Microinjection of the Xenopus oocyte. This figure illustrates the steps involved in the expression of an injected protein-coding gene in the frog oocyte. This figure was modified from an illustration which appeared in Gurdon and Melton (49).


INJECTION PIPETTE
SUPERCOILED PLASMID DNA
RELAXED PLASMID DNA
TRANSCRIPTION
ATTACHMENT
OF NUCLEOSOMES
RNA PRECURSOR
RNA PROCESSING
MATURE mRNA
TRANSLATION

The sequence requirements of RNA polymerase III in oocytes have been studied. The promoter region on the Xenopus $tRNA_{met}$ gene has been investigated (96-99). A tRNA gene of Xenopus laevis requires at least two sites for transcription (97-99). These regions are highly conserved in both prokaryotic and eukaryotic tRNAs (99).

5S RNA genes (transcribed by RNA polymerase III) have also been studied in Xenopus oocytes (78, 82, 87, 100). These include injected purified or cloned 5S DNA from Xenopus (78, 82) as well as heterologous sources such as the water beetle (100). The transcription of cloned Xenopus (87) and water beetle (100) ribosomal genes has been visualized with the electron microscope.

Transcription of RNA Polymerase II Genes. Transcription of protein-coding genes injected into Xenopus oocytes is under the control of RNA polymerase II (62, 101-102). This is also true for the transcription of messengers for endogenous proteins. Many proteins have been synthesized in oocytes injected with either purified DNA or cloned genes derived from various sources including SV40 (40, 79, 103), polyoma (103), adenovirus (81), herpes thymidine kinase (102-104), Drosophila heat shock (105) and histones (79), sea urchin histones (62, 106), and chick ovalbumin (61). Further evidence of transcription of heterologous DNA in injected oocytes has been obtained from studies where the presence of foreign mRNA molecules rather than the identification of the protein product was determined such as in the case of SV40 DNA (40, 50, 77), adenovirus DNA, ColE1 plasmid DNA, bacteriophages ϕX174 and ϕ80pIac DNA, cloned Drosophila histone genes (77), sea urchin histone genes (107-109), and the herpes

thymidine kinase gene (110). mRNAs with proper 3' and 5' termini have been found in oocytes injected with cloned histone genes (109) and adenovirus transcripts (81). In addition, Xenopus oocytes can synthesize and process SV40 transcripts including cleavage at 3' splice sites, formation of 3' terminus, addition of a poly(A) tail, and selective intracellular partitioning such that only those messengers with a mature 3' terminus and poly(A) tail are found in the cytoplasm (40). Splicing is not correlated with the partitioning of SV40 transcripts in the nucleus (40). Xenopus oocytes process the transcripts of injected genes spliced both inside (103-SV40 large T, polyoma middle T; 61-chick ovalbumin) and outside (103-SV40, polyoma capsid protein genes) of the protein coding region as well as genes which are not spliced at all (79-Drosophila histone; 62, 106-sea urchin histone; 102-104-herpes thymidine kinase; 81-E2A gene of adenovirus).

Transcriptional Efficiency of the RNA Polymerase II and III Genes in Xenopus Oocytes. The transcriptional efficiency of genes injected into Xenopus oocytes is lower for the RNA polymerase II genes than for the RNA polymerase III genes as determined by transcriptional rates (see 49 for review). While nematode tRNA is transcribed at a frequency of 27 transcripts/gene/hour (tr/g/hr) (91), Xenopus tDNA, 50 tr/g/hr (92), and Xenopus 5S RNA, 25 tr/g/hr (82), the sea urchin histone gene is transcribed at a comparatively low frequency, 0.1 tr/g/hr (77), as is herpes thymidine kinase, 0.15 g/tr/hr (110). The transcription rate for SV40 DNA and chick ovalbumin is less than 0.1 tr/g/hr (61, 79). The reason for RNA polymerase III

genes are transcribed more efficiently than RNA polymerase II genes in Xenopus oocytes is unknown.

Transcriptional Fidelity of the RNA Polymerase II and III Genes in Xenopus Oocytes. While there is ample evidence for the coupled transcription-translation of RNA polymerase II genes, the degree of transcriptional fidelity may be limited, unlike the RNA polymerase III genes (109). The transcription of the ovalbumin gene has been visualized and there is an extremely low frequency of regularly transcribed genes in oocytes (88). While Wickens et. al (61) reported that chick ovalbumin protein is synthesized in oocytes injected with the complete ovalbumin gene, an incomplete ovalbumin gene that lacks the leader sequence, cap site, TATA region, and 5' flanking sequences directs the synthesis of ovalbumin with the same efficiency. This raises the possibility that the normal production of functional mRNA from some genes does not require initiation at a precise sequence (61). However, this seems unlikely for all RNA polymerase II genes since the expression of many protein-coding genes is dependent on specific regions of the DNA serving as transcriptional control signals as has been shown for adenovirus E2A (81), herpes thymidine kinase (104), and sea urchin H2A histone (107-108). In addition, the oocyte does transcribe all five sea urchin histone genes with correct RNA termini, albeit with large differences in the efficiences (109). Therefore, the poor transcriptional fidelity of the ovalbumin gene in Xenopus oocytes does not seem to be characteristic of all RNA polymerase II genes.

Some Genes Are Not Transcribed or Are Erroneously Transcribed in Xenopus Oocytes. Not all genes are transcribed in Xenopus oocytes. These include the L2-L5 protein coding genes of adenovirus (81), H1 and H4 sea urchin histone genes (62), and prokaryotic or mitochondrial tRNA genes (91). This implies that there is some transcriptional deficiency in these injected genes. This appears likely for the H1 and H4 genes of the sea urchin since these genes lack well defined TATAAATA and cap sequences upstream from the structural region (109).

Some sea urchin histone DNA is erroneously transcribed from the antisense strand in oocytes (62). The extent to which injected DNAs are transcribed from the correct coding strand has been reviewed (49). Transcripts are not synthesized from the noncoding strand of 5S (82), oX174 (77), or thymidine kinase genes (S.L. Mcknight, personal communication to 49) but are synthesized from the noncoding strand of injected histone (62) and ovalbumin genes (61). While genes transcribed by RNA polymerase III are apparantly specific for the coding strand, RNA polymerase II genes appear to vary in strand specificity (49). There is some evidence to suggest that erroneous transcripts of the noncoding strand are sequestered in the nucleus as in the case of sea urchin histone genes (62). This observation is supported by an additional report which demonstrated that incorrect or incomplete SV40 RNA transcripts also did not reach the cytoplasm (40).

The Regulatory Sequences of RNA Polymerase II Genes Injected Into Oocytes. The regulatory sequences of protein-coding genes have been studied in the case of the sea urchin H2A histone gene

(107-108) and the herpes thymidine kinase (TK) gene (104, 111-112). The control region of the H2A histone gene can be functionally separated into three DNA segments (108). Deletion of the TATA box in the H2A gene does not abolish transcription but serves to create transcripts with different 5' ends and so may serve as a specificity element for a start signal (107). Transcription of the TK gene involves at least three distinct DNA sequence signals as well, all within 105 nucleotides upstream from the mRNA cap site (111).

The Herpes Thymidine Kinase Gene

The herpes TK gene is a particularly useful segment of DNA to microinject into Xenopus oocytes for gene regulation studies and because of this it can be used in Xenopus oocytes as a marker gene to measure the genotoxic potential of a test substance. Besides the fact that the herpes TK gene is transcribed and translated in the oocyte (102, 104) with reasonable efficiency (104), a great deal of information is known regarding the sequence and regulation of the gene itself. The gene has been completely sequenced and the coding regions defined (113-114). The gene does not contain intervening sequences (113-114) and the orientation of transcription has been determined (115). The molecular weight of the enzyme is 40-45,000 as determined by SDS gel electrophoresis (116-117). Knowledge regarding the substrate specificity of the herpes TK enzyme has permitted the detection of the enzyme in sensitive and specific assays (118-119) which are able to differentiate viral and endogenous TK (118). In addition, the TK enzyme, which catalyzes the phosphorylation of deoxythymidine to 5' deoxythymidine monophosphate, can be detected by a

simple assay which measures the amount of tritiated deoxythymidine phosphorylated in vitro.

The herpes TK gene has also been microinjected and transcribed in other cell types besides frog oocytes including mouse eggs (120) and mouse somatic cells (121-122) as a method of studying gene regulation. In addition, the herpes TK gene can transform TK- cells to the TK+ phenotype (123-130) including human cells (124), mouse L cells (125, 127-129) and teratocarcinoma cells (130). This demonstrates that the herpes TK gene can function in other high eukaryotic cell types besides the frog oocyte. The idea of using the TK gene to measure the mutagenic capabilities of test substances is not new since the gene has previously been used in mutation studies in mouse lymphoma L5178Y cells (19, 20). Therefore, it seems that the TK gene injected into frog oocytes could be used to detect not only the mutagenic capabilities of a test substance but how a test compound affects the various steps involved in gene expression as well. Because the TK gene has been demonstrated to function in many different cell types, including human cells, the regulatory mechanisms involved in gene expression of the TK gene in the frog oocyte assay can be extrapolated to mammalian organisms. It is also important to note that the frog oocyte assay system for genotoxicity is not limited solely to the use of the TK gene. Any cloned or purified gene could theoretically be used. However, the TK gene offers the advantage of being extensively studied in regard to the regulatory mechanisms governing its expression and the gene is commercially available from Bethesda Research Laboratories. The potential of using the oocyte to examine the effects of a test compound on several different genes whose regulatory mechanisms are

understood may provide a means of comparing the effects of test compounds on genes that may be regulated differently from one another.

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CHAPTER III

DETECTION OF GENOTOXIC CHEMICALS USING STAGE 6 OOCYTES OF *XENOPUS LAEVIS* MICROINJECTED WITH THE HERPES THYMIDINE KINASE GENE

by

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ABSTRACT

We have developed a short-term genotoxicity assay involving the microinjection of the cloned herpes thymidine kinase (TK) gene into Xenopus oocytes. The ability of the test substance to affect the expression of the TK gene was determined in two assays: the Gene Exposure Assay and the Oocyte Exposure Assay. The Gene Exposure Assay measures the ability of a test agent to bind or possibly fragment the plasmid DNA and thereby prevent the expression of the TK gene. The Oocyte Exposure Assay measures the ability of a test substance to affect the expression of the TK gene at any level. In order to validate these two assays, we have tested the effect of known inhibitors of DNA, RNA, and protein synthesis on TK expression in microinjected oocytes using concentrations close to the 96 h LC50 values obtained during the teratogenicity testing of these substances. In the Gene Exposure Assay, actinomycin D gave positive results in that it completely inhibited TK production in oocytes. Hydroxyurea, cytosine arabinoside, cycloheximide, and ethidium bromide yielded negative results since these inhibitors did not prevent the synthesis of TK in oocytes. In the Oocyte Exposure Assay, actinomycin D and cycloheximide inhibited TK production in the expected manner while hydroxyurea and cytosine arabinoside appeared to stimulate TK production. Ethidium bromide did not affect the synthesis of TK. The Gene Exposure Assay and Oocyte Exposure Assay identify agents which alter the expression of the herpes TK gene in frog oocytes. Since the

genotoxic potential of teratogens can be tested, information regarding the biochemical basis of teratogenicity may be obtained using this system.

Keywords: Xenopus, oocytes, genotoxicity, teratogenicity, thymidine kinase.

INTRODUCTION

Normal development depends on the timely expression of genes and the precise replication of genetic information. Genotoxic agents that interfere with these fundamental processes may be teratogenic. Screening assays that measure the potential of a substance to act as both a genotoxin and a teratogen should provide a better assessment of the teratogenic risk of a compound than a teratogenicity assay alone. The term genotoxin in the traditional sense refers to substances that cause changes in the DNA either directly or indirectly (1) which may be mutagenic and ultimately carcinogenic to the organism (2). However, this definition may be somewhat incomplete. There are many steps involved in gene expression including transcription, processing, transport, and translation. At each of these levels of the processing pathway a substance could interrupt the normal flow of information from gene into product. Therefore, it may be more appropriate to define a genotoxin as a substance that interferes with the normal expression of a gene at any level.

The purpose of this investigation was to develop a short-term assay to determine the effect of a test substance on gene expression in the Xenopus oocyte and to correlate the results of such studies to the ability of these compounds to act as teratogens during the development of the Xenopus embryo. The genotoxicity assay presented in this paper is not a gene mutation assay although it can

identify substances that cause DNA damage which affects gene expression.

The South African Clawed frog, Xenopus laevis, was chosen as a test organism for this assay. There were several reasons for our choice. The animals are easy and inexpensive to maintain in the laboratory, the teratogenic potential of substances can be assessed in a 96 h whole embryo bioassay, and the Xenopus oocyte is capable of expressing a wide variety of foreign eukaryotic genes which have been microinjected into the nucleus. The Xenopus oocyte is an ideal system to study the ability of genotoxic agents to affect the expression of a specific gene. Since the Xenopus oocyte can transcribe numerous heterologous genes, although with variable efficiency, the oocyte appears to possess regulatory components that are similar to those found in a diverse array of species (3). This makes the data regarding the effects of substances on the regulatory processes involved in gene expression in Xenopus extrapolatable to other species, including man.

To validate the use of the Xenopus oocyte as an indicator of the genotoxic potential of a substance, we have exposed the herpes thymidine kinase (TK) gene to known inhibitors of DNA, RNA, and protein synthesis either before or after microinjection of the gene into the nucleus of the oocyte. We have tested the ability of hydroxyurea, cytosine arabinoside, ethidium bromide, actinomycin D, and cycloheximide to interrupt the expression of the TK gene either directly by DNA damage or indirectly through inhibition of RNA synthesis or translation. Although any well characterized gene could be used for this assay providing that it was utilized by the oocyte,

the herpes TK gene has previously been shown to be successfully transcribed and translated into functional protein in the Xenopus laevis oocyte (4, 5). Moreover, the gene is commercially available (Bethesda Research Laboratories) and therefore does not require propagation prior to microinjection. This saves considerable time. The results presented in this report show that the Xenopus oocyte has great promise as a short-term assay for identifying genotoxic substances in the environment and provides data which can be correlated to the results of the 96 h Xenopus whole embryo teratogenicity bioassay described in a separate report from this laboratory (6). Together, both the Xenopus teratogenicity and genotoxicity assays provide complementary evidence on the potential hazards of specific compounds and therefore serve as a useful biomonitoring method.

MATERIALS AND METHODS

Preparation of Oocytes

Xenopus laevis females were anaesthetized in MS 222 (Cresent Research Chemicals®) at a concentration of 1.5 g/l in dechlorinated tap water. Oocytes were removed according to the procedure described by Kressmann and Birnstiel (7). A 1 cm incision was made in the skin slightly off-center of the midventral line. Using aseptic technique, the peritoneum and the muscle tissue were cut and a small portion of the ovary was pulled out. The cluster of oocytes was removed using scissors and placed in a Petri dish in modified Barth's solution (MBS-H) containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 10 mM HEPES, 10 mg/l benzylpenicillin, 10 mg/l streptomycin sulfate, pH 7.6 (8). The remaining ovary was then pushed back into the body cavity and the incision closed making sure that the skin was sutured separately from the muscle layer and peritoneum.

Stage 6 oocytes (9) were manually separated from the ovarian tissue using watchmaker's forceps and incubated in MBS-H at 20°C for approximately 18 h prior to microinjection. To facilitate injection directly into the nucleus, the oocytes were aligned in a 1 mm^2 screen which had been attached with silicone sealant to the bottom of a polyethylene lined scintillation vial cap and centrifuged in a swinging bucket rotor at approximately 1000 x g ave. for 30 min at 20°C. This procedure, developed by Kressman et al. (10) causes a displacement of

the pigment such that a brown concentric ring marks the nucleus. Centrifugation of the oocytes does not significantly affect their transcriptional ability (11).

Microinjection Apparatus

A siliconized glass capillary tube was initially tapered to a point using a microelectrode puller. To obtain an injection needle with an orifice of 10-20 μm, needles were placed on a microscope stage and bumped against a flat surface while observing under 10X magnification. A calibrated ocular micrometer was used to measure the size of the orifice. After filling with plasmid DNA, the needle was attached to a 10 μl gas tight Hamilton® microsyringe using rigid walled tubing. The syringe and tubing were filled with light paraffin oil. A syringe pump was used to push the oil which in turn forced the contents of the needle into the oocytes at a rate of approximately 2.5 nl/sec. The needle was held by a pin vise (Curtin Mathis Scientific®) connected to a mechanical microscope stage. This allowed the tip of the needle to be accurately guided into the oocytes during injection. A dissection microscope was used to observe the microinjection process.

Gene Exposure and Oocyte Exposure Assays

The ability of a compound to inhibit macromolecular synthesis directly through DNA damage or indirectly by some other inhibitory effect of gene expression was investigated by the use of two assays: the Gene Exposure Assay and the Oocyte Exposure Assay. In both tests the plasmid pHSV-106 (Bethesda Reasearch Laboratories®), which contains a 3.4 kb insert of the entire herpes thymidine kinase (TK) gene, was microinjected into Xenopus stage 6 oocytes.

For the Gene Exposure Assay, the pHSV-106 DNA was exposed *in vitro* to various concentrations of known inhibitors of macromolecular synthesis for 2 h prior to injection at 20-25°C in MBS-H. After this time, the DNA was separated from the test substance by gel filtration chromatography (See "Test Substances and Injection Solutions"), and injected into oocytes. After injection (25 nl of 0.15 µg/µl pHSV-106 per oocyte), the oocytes were incubated in MBS-H at 23°C for 18-24 h then processed for TK activity as described below.

For the Oocyte Exposure Assay, pHSV106 was injected directly into the germinal vesicle (nucleus) of stage 6 oocytes (25 nl of 0.15 µg/µl pHSV-106 per oocyte). The pHSV106 DNA was not exposed to the test substance prior to microinjection. The oocytes were then exposed to different concentrations of known inhibitors of DNA, RNA, and protein synthesis in MBS-H (10-20 oocytes/group, 3 groups/ concentration) at 23°C for 18-24 h. After this time, the oocytes were processed for TK activity.

Determination of TK Activity

Oocytes were processed for the determination of TK activity using a slight modification of the assay used by Brinster et al. (12). Oocytes were homogenized (10 µl/oocyte) in 10 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 1 mM ATP, 10 mM NaF, 50 mM e-aminocaproic acid. The homogenate was centrifuged at 15,000 x g ave, 10 min, 0-4°C, in an HB-4 rotor (Sorvall®). 37.5 µl of the supernatant was combined with 187.5 µl of the assay (A) buffer containing 150 mM Tris-HCl, pH 7.4, 10 mM ATP, 10 mM $MgCl_2$, 25 mM NaF, 10 mM ß-mercaptoethanol, 5 µCi ^{3}H-thymidine (ICN®). The mixture was then incubated for 4-5 h at 37°C in 1.5 ml conical micro-test tubes. Every hour during the course

of incubation, 30 µl samples were spotted on Whatman® DE-81 filters. The filters were then washed three times in 95% ethanol, 5 min per wash. The filters were dried and the radiation measured by liquid scintillation spectrometry using a toluene based cocktail containing 4.0 g/l PPO and 0.1 g/l POPOP at 20% counting efficiency. This procedure measures the amount of ^{3}H-thymidine converted to ^{3}H-TMP.

Determination of TK Activity in the Presence of Inhibitors

To test for the possibility that the inhibitors used in this study may have changed the catalytic efficiency of the TK enzyme, 37.5 µl of the supernatant from control oocytes was added to each of several 187.5 µl aliquots of A buffer in which all but one mixture contained a different inhibitor. The control oocytes used in these experiments were injected with pHSV-106 DNA and homogenized 18-24 h later. The amount of TK enzyme present in these oocytes is that which has been synthesized in the absence of inhibitory substances. The amount of ^{3}H-thymidine phosphorylated to ^{3}H-TMP in the presence and absence of the inhibitors was determined every hour for 4-5 h as described above. The concentrations selected for all inhibitors were in excess of the highest concentration required to see an effect in the Gene Exposure or Oocyte Exposure Assay.

Test Substances and Injection Solutions

For the Gene Exposure Assay, the inhibitor was resuspended as a concentrated stock in distilled water. In the case of cytosine arabinoside, the pH of the stock was adjusted to 7.6 with NaOH to prevent a large change in the pH of the injection solution. This was not necessary for all other inhibitors used in this study. The concentration of inhibitor which was selected in the Gene Exposure

Assay was usually the highest concentration used in the Oocyte Exposure Assay to reduce the possiblity of a false negative. The inhibitor was added at the desired concentration to the plasmid DNA in 10 mM Tris-HCl, pH 7.4, 5 mM NaCl, 1 mM EDTA in a total volume of 400 µl. After 2 h at room temperature the inhibitor was removed from the DNA by gel filtration chromatography using G-100 Sephadex (Pharmacia®). The excluded fraction containing the DNA was collected in a 1 ml volume and concentrated by lyophilization. The DNA was resuspended in 10 mM Tris-HCl, pH 7.4, 5 mM NaCl, 1 mM EDTA, filtered through a 0.2 µm polycarbonate filter (Nucleopore®) and injected.

In the Oocyte Exposure Assay, oocytes were also injected with pHSV-106 DNA in 10 mM Tris-HCl, pH 7.4, 5 mM NaCl, 1 mM EDTA. It is strongly advised that the solution be filtered as described above to prevent small particulates from clogging the needle orifice. Inhibitors were dissolved at various concentrations in MBS-H solution. In the case of cytosine arabinoside, the pH of the solution was adjusted with NaOH to 7.4-7.6. For all other inhibitors, it was unnecessary to adjust the pH of the solution which remained that of MBS-H. All inhibitors were purchased from the Sigma® Chemical Company.

RESULTS

DNA Synthesis Inhibitors

The DNA synthesis inhibitors tested in this investigation were cytosine arabinoside, hydroxyurea, and ethidium bromide. Ethidium bromide is also an inhibitor of RNA synthesis. The results expected for the DNA synthesis inhibitors were dependent upon their mode of action. Figure 1 and Table I show that hydroxyurea, an inhibitor of ribonucleotide reductase, had no effect on the production of functional herpes TK enzyme when the plasmid containing the herpes TK gene was exposed to a 10 mg/ml concentration of the inhibitor prior to microinjection into Xenopus oocytes in the Gene Exposure Assay. Concentrations of hydroxyurea ranging from 0.25-2.0 mg/ml were tested in the Oocyte Exposure Assay in order to obtain dose-response type data. However, not even as high as 2.0 mg/ml hydroxyurea inhibited the expression of the TK gene in oocytes (Table II). This was despite the use of a concentration of hydroxyurea sufficient to kill 50% of the embryos in the FETAX bioassay (Table II). This demonstrates that hydroxyurea did not damage the plasmid DNA or affect RNA and protein synthesis directly in the oocytes.

Similar results were obtained for the antimetabolite cytosine arabinoside and ethidium bromide, a DNA intercalator. 6.0 mg/ml cytosine arabinoside and 0.2 mg/ml ethidium bromide did not inhibit the expression of the TK gene in the Gene Exposure Assay (Table I). Cytosine arabinoside appeared to stimulate the production of TK in the

Gene Exposure Assay. Several different concentrations of cytosine arabinoside and ethidium bromide were tested in the Oocyte Exposure Assay ranging from 0.05-2.0 mg/ml for cytosine arabinoside and 0.002-0.2 mg/ml for ethidium bromide. However, even the concentrations of these compounds closest to the LC50 of the FETAX bioassay (6), 2.0 mg/ml cytosine arabinoside and 0.02 mg/ml ethidium bromide, failed to inhibit TK expression in the Oocyte Exposure Assay (Table II). These results suggest that these inhibitors primarily interfere with DNA synthesis, which is absent in the oocyte, and kill embryos by some general cytotoxicity mechanism other than the flow of genetic information into protein.

It is possible, as in the case of hydroxyurea, that both ethidium bromide and cytosine arabinoside stimulated TK activity in the Oocyte Exposure Assay (Table II) since the amount of activity in experimental groups were higher than control groups. The 2.0 mg/ml value reported on Table II for cytosine arabinoside was the only concentration among the ranges tested (0.05-2.0 mg/ml) where the amount of TK activity was lower than that of the controls. Concentrations of cytosine arabinoside lower than 2.0 mg/ml stimulated TK activity 1.6 fold over controls (data not shown). To study this stimulatory effect further, the amount of endogenous TK activity was measured by placing uninjected oocytes in 10 mg/ml hydroxyurea and 2 mg/ml cytosine arabinoside in MBS-H for 18-24 h. In this experiment, TK synthesis was stimulated 1.7 and 1.4 fold, respectively (data not shown). Thus, both endogenous and viral TK production appear to be stimulated by hydroxyurea and cytosine arabinoside. However, the standard error of the mean reported for cytosine arabinoside and ethidium bromide in Table II suggests that the

differences in TK activity in the Oocyte Exposure Assay were not statistically significant.

RNA Synthesis Inhibitors

Ethidium bromide, and actinomycin D were selected as inhibitors of RNA synthesis. Since ethidium bromide also inhibits DNA synthesis, the effect of this substances on TK expression was discussed in the preceeding section. Actinomycin D is also capable of preventing DNA synthesis but at much higher concentrations than are required to inhibit RNA synthesis. Therefore, the primary effect of actinomycin D is to prevent RNA transcription, and this occurs by the intercalation of actinomycin D into DNA (13).

The ability of actinomycin D to bind to the DNA template and prevent RNA transcription was demonstrated in both the Gene Exposure Assay (Figure 2, Table I) and the Oocyte Exposure Assay (Figure 3, Table II). When the plasmid DNA was exposed prior to microinjection in the Gene Exposure Assay to 0.025 mg/ml actinomycin D, no thymidine kinase activity was observed (Figure 2, Table I). However, when the oocytes were exposed to different concentrations of actinomycin D in the incubation medium after injection in the Oocyte Exposure Assay, the amount of thymidine kinase activity observed varied in a dose-response manner (Figure 3). While 0.13 mg/ml actinomycin D completely prevented transcription of the TK gene, 0.059 mg/ml actinomycin D permitted 25% of the amount of TK activity found in controls and 0.013 mg/ml actinomycin D allowed 39% of the TK activity found in controls (Figure 3, Table II).

Protein Synthesis Inhibitors

The ability of cycloheximide to inhibit protein synthesis in the Xenopus oocytes was investigated. Figure 4 and Table I show the results obtained for the inhibition of TK activity when the plasmid DNA was exposed to 2.8×10^{-3} mg/ml cycloheximide for 2 h then separated from the inhibitor prior to injection. This data also provides evidence that positive results in the Gene Exposure Assay, such as that found for actinomycin D (Figure 2, Table I), were not due to poor separation of the plasmid DNA from the inhibitor since cycloheximide caused no effect.

Following microinjection of oocytes and incubation in concentrations of cycloheximide ranging from 1.4×10^{-5} mg/ml to 1.4×10^{-2} mg/ml, the amount of TK activity observed decreased as the concentration of cycloheximide increased (Figure 5). While 1.4×10^{-2} to 1.4×10^{-3} mg/ml cycloheximide completely abolished TK enzyme activity, 1.4×10^{-4} mg/ml and 1.4×10^{-5} mg/ml cycloheximide permitted approximately 22% and 35%, respectively, of the amount of TK activity produced by controls. Together Figures 4 and 5 show that cycloheximide does not damage the DNA but rather acts at some other level of processing to prevent the expression of the TK gene.

TK Activity in the Presence of Inhibitors

The ability of cytosine arabinoside, hydroxyurea, ethidium bromide, actinomycin D, and cycloheximide to affect the catalytic efficiency of the TK enzyme was determined by placing various concentrations of each inhibitor separately into assay buffer which contained a portion of the supernatant of control oocytes. None of the inhibitors used in this study were able to increase or decrease the

amount of ^{3}H-thymidine converted to ^{3}H-TMP in the absence of inhibitor (Table III). Therefore, an increase in TK activity must be due to an increase in the amount of TK synthesized and not an enhancement of the catalytic efficiency of the enzyme in vitro. Similarly, a reduction in TK activity would be due to a decrease in the amount of TK synthesized and not due to enzyme inhibition.

Summary of Results

A summary of the results for the inhibition of DNA, RNA, and protein synthesis in Xenopus oocytes by known inhibitors of macromolecular synthesis for both the Gene Exposure and Oocyte Exposure Assay is provided in Tables I and II. All inhibitors behaved according to their expected mode of inhibitory action except ethidium bromide.

DISCUSSION

The Development of Xenopus laevis Oocytes as a Genotoxicity Assay

One possible method of performing this assay was to simply microinject radioactive precursors of DNA, RNA, and protein synthesis into Xenopus oocytes in order to identify agents which alter macromolecular synthesis. We intentionally avoided the use of radioactive precursors for such studies because of the possible changes in the endogenous pool size of the precursor which may occur during experimentation. When cells are exposed to toxic agents, changes in membrane permeabilty or compartmentation of the nonradioactive pools may change the amount of incorporation of radioisotopes into DNA, RNA, and protein. This requires measurement of the pool sizes of experimental versus control groups which is too time consuming for a screening assay. Differential leakage of ^{3}H-leucine injected into Xenopus oocytes after exposure to benzo(a)pyrene compared to controls was observed in initial experiments in our laboratory. This resulted in an apparent stimulation in protein synthesis in the oocytes exposed to benzo(a)pyrene due to a change in the ratio of nonradioactive to radioactive leucine available for protein synthesis. In addition, the injection of radioisotopes does not permit the monitoring of agents that are capable of causing DNA damage. We therefore elected not to develop an assay based on the microinjection of radioisotopes.

We have developed an assay which utilizes the herpes thymidine kinase gene cloned into a bacterial plasmid to identify genotoxic substances acting at any level of gene expression. However, this assay will only identify substances that prevent the production of functional TK enzyme. It is not a replacement for gene mutation assays. The system is capable of identifying agents which bind to or fragment the DNA only if they prevent the expression of the TK gene. Such substances need not necessarily be mutagens. It is important to realize, however, that the goal of this assay is different from gene mutation assays such as the Ames Test. Our goal was to develop an inexpensive, short-term assay that would identify genotoxic agents and provide insight on the molecular mechanisms of teratogenesis.

In this assay, the TK gene containing plasmid must be microinjected directly into the nucleus of the frog oocyte in order for transcription to take place (14). The amount of viral TK produced was measured by comparing the amount of total TK activity as measured by the ability of the oocyte homogenate to convert ^{3}H-thymidine to ^{3}H-TMP in injected versus noninjected groups. The level of endogenous TK activity is very low in oocytes (see Figure 1 for example), thus virtually all of the TK activity observed in injected oocytes was assumed to be due to viral TK. Furthermore, the work of McKnight et al. (5) using a substrate specific for herpes TK but not Xenopus TK (15), have shown that the TK activity observed after injection was indeed viral in origin.

We have used the Xenopus oocyte to identify DNA binding agents which prevent transcription as described by the Gene Exposure Assay as well as compounds which prevent the expression of the herpes TK gene

without necessarily causing DNA damage in an assay referred to as the Oocyte Exposure Assay. The importance of using both assays in the routine screening of the genotoxic potential of a substance is well demonstrated in the case of cycloheximide which does not damage DNA or prevent transcription (Figure 4) but does prevent the translation of RNA into protein (Figure 5).

Rationale for Comparing the FETAX LC50 to Similar Test Concentrations in the Genotoxicity Assay

The 96 h LC50 of the Xenopus whole embryo (FETAX) bioassay (6) was used in performing the genotoxicity assay (Tables I and II) rather than the 96 h EC50 (malformation). Xenopus oocytes are more sensitive to toxicants than are early embryos. This difference in sensitivity made it impossible to state that any inhibition of gene expression in the oocyte at the 96 h EC50 (malformation) would definitely be a factor that caused malformation in the whole embryo test. However, if a test substance did not inhibit gene expression in oocytes at the embryo 96 h LC50, then any malformation or toxicity observed in the FETAX test could not be due to genotoxicity since this concentration would surely inhibit gene expression in the more sensitive oocytes. Conversely, substances which inhibited TK synthesis in oocytes would almost certainly affect protein synthesis in embryos, although the extent of inhibition may be less.

The Effect of Inhibitors on Herpes TK Expression in the Oocyte

The inhibitors used in this study were selected on the basis that their mode of action on DNA, RNA, or protein synthesis was known. For the validation of this assay, it is crucial to demonstrate that the

frog oocyte microinjected with the herpes TK gene will respond to such inhibitors in the expected manner.

Most of the inhibitors used in this study behaved according to their known mode of action. The DNA synthesis inhibitors hydroxyurea and cytosine arabinoside were both negative in the Gene Exposure and Oocyte Exposure Assays in that they did not decrease the amount of TK enzyme produced. However, hydroxyurea and cytosine arabinoside may have increased TK production. Since hydroxyurea is known to inhibit ribonucleotide reductase which catalyzes the conversion of ribonucleotides to deoxyribonucleotides (16), and there is virtually no DNA synthesis in mature oocytes (17), a reduction in the transcription of the herpes TK gene or subsequent translation of the RNA into protein was not anticipated. It has been previously documented that while hydroxyurea inhibits DNA synthesis, RNA and protein synthesis continue (16, 18). This is also true for cytosine arabinoside (19) whose mechanism of action has been suggested to be the inhibition of the reduction of CDP to dCDP (20).

The possibility that hydroxyurea and cytosine arabinoside may have stimulated the amount of TK normally expressed in injected oocytes (Table II) is supported by the literature. The same effect for these compounds was observed in cultured cells at concentrations even lower or identical to the concentrations used in this study (18-19). Kim et al. (1967) found that 0.076 mg/ml hydroxyurea increased the amount of TK activity by three fold over controls in HeLa cells after 24 h (18). Kit et al. (1966) found that 0.05 mg/ml cytosine arabinoside stimulated the thymidine kinase activities of green monkey kidney and HeLa cells after 48 h by 5 and 10 fold, respectively, but not mouse lymphoma cells

(19). This increase in TK activity could be prevented by cycloheximide suggesting that this effect was caused by increased amounts of the TK protein production (19). Our data precludes the possibility that these inhibitors increase TK activity without increasing the amount of TK synthesized in Xenopus oocytes since hydroxyurea and cytosine arabinoside did not affect the activity of TK when placed in the assay buffer of control oocytes (Table III). However, our results do not show an increase in TK activity as high as was reported by Kim et al. (18) and Kit et al. (19) after 24 h incubation in hydroxyurea and cytosine arabinoside since 0.25 mg/ml hydroxyurea stimulated TK activity by 1.6 fold while 1.0-2.0 mg/ml hydroxyurea caused a 2.5 fold increase in TK activity. In the case of cytosine arabinoside, 0.05 mg/ml also increased TK activity in microinjected oocytes by 1.6 fold over that of controls. The differences between our data and the results reported by other investigators (18-19) could easily be accounted for by an increase in sensitivity of cultured cells to these inhibitors due to several reasons. Cultured cells are typically active in DNA synthesis while Xenopus oocytes are not. Differences in species sensitivity may also be important. However, it may be possible that the stimulatory effect of hydroxyurea and cytosine arabinoside on TK production in microinjected oocytes may be due, at least in part, to variable amounts of RNA and protein synthesized from the injected template. This problem has been reported by investigators working with Xenopus oocytes (14, 21-22). We are presently performing additional experiments to evaluate this possibility.

Our assay, as presently developed, cannot measure the effect of a test substance on replication. However, if the process of replication

had been assayed, both hydroxyurea and cytosine arabinoside should have had a significant inhibitory effect (16, 18-19). We are presently developing a replication assay using Xenopus oocytes. However, in the meantime, a rise in TK activity may indicate that a compound is inhibitory to DNA synthesis and that the cell has a larger amount of RNA than DNA precursors and therefore makes more RNA and protein than controls. The testing of additional inhibitors of DNA synthesis will determine whether this is a general response or specific to the inhibitors thus far tested.

The response of Xenopus oocytes to ethidium bromide was different from that expected. In both the Gene Exposure (Table I) and Oocyte Exposure (Table II) assays, up to 0.2 mg/ml ethidium bromide did not inhibit TK activity. Ethidium bromide is well known for its ability to intercalate linear double-stranded DNA more readily than circular double-stranded DNA (23). Although it is likely that ethidium did intercalate into the circular double-stranded DNA molecule used in this study, this intercalation was not sufficient to prevent the access of RNA polymerase to the promoter regions of the TK gene. This result is probably related to the conformation of the plasmid DNA. Harland et al. (22) have suggested that the transcription signals of the herpes TK gene in Xenopus oocytes may function not only by the binding of regulatory proteins to sequence-specific regions on the DNA but by the conformation of the DNA as well. In addition, ethidium bromide was also only marginally teratogenic to Xenopus embryos (6). This suggests that ethidium may have also intercalated poorly into native chromatin which is protected by proteins. However, it remains unclear

as to whether the results obtained for ethidium bromide in both this assay and the teratogenicity assay (6) represent a false negative.

The RNA synthesis inhibitor, actinomycin D, was positive in both the Gene Exposure (Figure 2, Table I) and Oocyte Exposure (Figure 3, Table II) Assays. Since actinomycin D is capable of intercalating into double-stranded DNA including native chromatin such results were anticipated (13). Moreover, the oocytes responded to actinomycin D in a dose-response manner. However, we have had difficulty in determining the range of concentration of a substance to use in order to obtain the higher limits of a dose-response curve because of two problems. First, the concentration range needed to elicit a response from 40 to 80% of controls tends to be narrow. Second, the amount of experimental variability in some cases is often high so that the standard errors of the mean from different concentrations overlap. We consider this variability our most difficult problem with the Oocyte Exposure Assay and are currently working on techniques to overcome this difficulty. Other investigators have also reported similar variability problems in the expression of microinjected genes in Xenopus oocytes (14, 21-22)

The results for the protein synthesis inhibitor, cycloheximide, were negative in the Gene Exposure Assay (Figure 4, Table I) but positive in the Oocyte Exposure Assay (Figure 5, Table II). Since cycloheximide is known to disrupt eukaryotic protein synthesis by preventing initiation and elongation, the inability of cycloheximide to bind or damage the DNA in the Gene Exposure Assay was expected. Increasing concentrations of cycloheximide also decreased the amount of

functional TK enzyme produced, yielding dose-response type data with limitations as described for actinomycin D.

Identifying the Molecular Causes of Teratogenicity

The data of Courchesne and Bantle (6) as well as others (24) have shown that DNA synthesis inhibitors are highly teratogenic in diverse species while RNA and protein synthesis inhibitors are not. We are unable to link the results obtained in this study for the DNA synthesis inhibitors to the teratogenic activity of these substances since the process of replication could not be directly measured. However, we can compare the effects of the RNA and protein synthesis inhibitors on TK expression in Xenopus oocytes to their effects on the growth and development of Xenopus embryos. Doses of actinomycin D which caused a significant decrease in RNA synthesis in the oocyte were also lethal to the embryo. With whole embryo teratogenicity tests using Xenopus, 1.89×10^{-2} mg/ml actinomycin D was sufficient to kill 50 % of the organisms in 96 h. In the Oocyte Exposure Assay 1.33×10^{-2} mg/ml actinomycin D was sufficient to reduce TK production by 60%. Therefore it appears that while actinomycin D is not highly teratogenic to the organism it is generally cytotoxic. It has previously been noted that cytotoxicity of actinomycin D in proliferating tissues may be due to an intolerable loss of RNA (25), However, it is reasonable to assume that factors other than RNA synthesis ultimately determine the susceptibiltiy of various cells to inhibition by actinomycin D (25). For example, the loss in RNA has been related to an increase in ribonuclease activity caused by actinomycin (26). In addition, doses of cycloheximide which caused a large decrease in protein synthesis were also lethal to the embryo. A

1.59×10^{-4} mg/ml concentration of cycloheximide was enough to kill 50% of the Xenopus embryos in 96 h and reduce 80% of protein synthesis in the oocyte. Due to the differences in the time of exposure of oocytes (24 h) and embryos (96 h) it may be unwise to imply that a 60-80% decrease in RNA and protein synthesis in the embryo is required before lethality. However, the data imply that embryos may be able to survive in spite of significant decreases in RNA and protein synthesis by perhaps decreasing growth or the rate of development (6).

We have developed the Xenopus Genotoxicity Assay to be used together with, and not separately from, the Xenopus Teratogenicity system described in the proceeding paper (6). When both assays are used together, the possibilty that a biologically hazardous substance will escape detection is minimized. For example, the DNA synthesis inhibitors cytosine arabinoside and hydroxyurea gave negative results for both the Gene Exposure and Oocyte Exposure Assays due to the limitations of the oocyte system at this time to measure replicatory processes. However, teratogenicity testing of these compounds showed them to be potent teratogens.

When analyzing unknown samples for teratogenicity and genotoxicity, the Xenopus teratogenicity (FETAX) assay (6) should be performed first. This procedure determines the 96 h LC50 and EC50 malformation. It also measures the ability of the substance to inhibit growth. The 96 h LC50 concentration should then be used as the first concentration in measuring genotoxic effects using both the Gene Exposure Assay and Oocyte Exposure Assay. Our preliminary experiments suggested that oocytes were more sensitive to inhibitors than early stage embryos of Xenopus. Therefore, the selection of the 96 h

LC50 should be adequate to elicit an effect in the oocytes. If the test substance does not cause a reduction in TK expression in oocytes at the 96 h LC 50 concentration then any teratogenicity or cytotoxicity observed in the FETAX is probably caused by a mechanism other than inhibition of gene expression. Should the test compound enhance the expression of the TK gene, this may be an indication that the substance is an inhibitor of DNA synthesis and DNA replication assays (presently being developed in our laboratory) should be performed to directly test for this possibility. If the substance proves to be an inhibitor of DNA synthesis in oocytes and is teratogenic in the FETAX experiment, then it is highly probable that the compound is a teratogen in other species as well. If a test agent does not affect DNA synthesis but inhibits RNA or protein synthesis in oocytes, then there is a distinct possibility that embryonic growth will be adversely affected in all species. In this manner, test results from both a validated whole embryo bioassay and a molecular test can complement one another to enhance the predictability of each assay. We are continuing to validate this system and feel that it holds great promise as a method of identifying genotoxic agents and understanding their teratogenic potential.

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TABLE I. Effect of genotoxic agents on TK gene expression following exposure of the gene with the agent (Gene Exposure Assay).

Inhibitor	Type of inhibitor	Concentration	cpm of injected control[1]	cpm of experimental[1]	% of control[2]	Results[3]
Hydroxyurea	DNA	10.0	72811 ±12342.(5)	74672 ±19507.(5)	103	NEG
Cytosine Arabinoside	DNA	6.0	167927 ±16598.(3)	273185 ±18330.(3)	163	NEG
Ethidium Bromide	DNA/RNA	0.2	184995 ±86815.(3)	185913 ±91651.(3)	100	NEG
Actinomycin D	RNA	0.025	168968 ±12322.(5)	525 ±371.(5)	0	POS
Cycloheximide	Protein	0.0028	101116 ±9503.(3)	112937 ±4668.(3)	112	NEG

[1] cpm represents the amount of ^{3}H-TMP bound to filter for the last time point during the reaction. Values are corrected for the cpm due to endogenous TK activity. Standard error represents the mean of 3-5 groups of oocytes from the same frog. Oocytes were injected then pooled and randomly divided into groups. Groups varied in number, ranging from 8-20 oocytes. Groups were homogenized in 10 µl/oocyte. See "Materials and Methods" for details.

[2] $\frac{\text{cpm of injected control}}{\text{cpm of experimental}} \times 100$

[3] results based on inhibition of TK expression.

TABLE II. Effect of genotoxic agents on TK gene expression following exposure of the oocyte with the agent (Oocyte Exposure Assay).

Inhibitor	Type of inhibitor	Concentration (mg/ml)[1]	LC50[2]	cpm of injected control[3]	cpm of experimental[3]	% of control[4]	Results[5]
Hydroxyurea	DNA	2.0	1.82	38659 ±4489.(3)	104223 ±64994.(3)	270	NEG
Cytosine Arabinoside	DNA	2.0	6.0	71082 ±33059.(3)	52891 ±26586.(3)	74	NEG
Ethidium Bromide	DNA/RNA	0.02	0.05	71082 ±33059.(3)	103114 ±18765.(3)	145	NEG
Actinomycin	RNA	0.013	0.0189	194718	76434	39	POS
Cycloheximide	Protein	1.41×10^{-4}	1.59×10^{-4}	106731 ±19104.(6)	23099	22	POS

[1]Although different concentrations were tested, the concentration reported on the table was that concentration closest to the LC50 of the FETAX whole embryo bioassay (6). See justification in "Discussion" section.

[2]LC50 value in mg/ml from FETAX bioassay (6).

[3]See comment 1 at end of Table I.

[4]See comment 2 at end of Table I.

[5]See comment 3 at end of Table I.

TABLE III. Effect of Genotoxic Agents on Thymidine Kinase Activity.[1]

Inhibitor	Type of Inhibitor	Concentration (mg/ml)	cpm of Control (cpm)	cpm of Experimental (cpm)	% of control (%)	Results
Hydroxyurea	DNA	10.0	93679	101639	108	NEG
Cytosine Arabinoside	DNA	4.0	10321	9082	88	NEG
Ethidium Bromide	DNA/ RNA	0.2	38116	39562	104	NEG
Actinomycin	RNA	0.025	93679	90863	97	NEG
Cyclohexi-mide	Protein	0.028	93679	96901	103	NEG

[1]Ooctyes were injected with the TK gene and incubated for 20 h. Following incubation the oocytes were homogenized and centrifuged as described in "Materials and Methods. The supernatants were divided into groups and the inhibitor added to the supernatant at the concentration indicated in the table. The control consisted of a group that received only buffer instead of inhibitor. Following treatment, the groups were assayed for thymidine kinase activity as described in "Materials and Methods".

Fig. 1. Gene exposure assay for hydroxyurea. pHSV-106 DNA was exposed to hydroxyurea prior to injection into oocytes. Hydroxyurea was removed from the DNA after exposure and the DNA was lyophilized to concentrate. The control group (□) was not lyophilized while the processed control (○) was lyophilized.

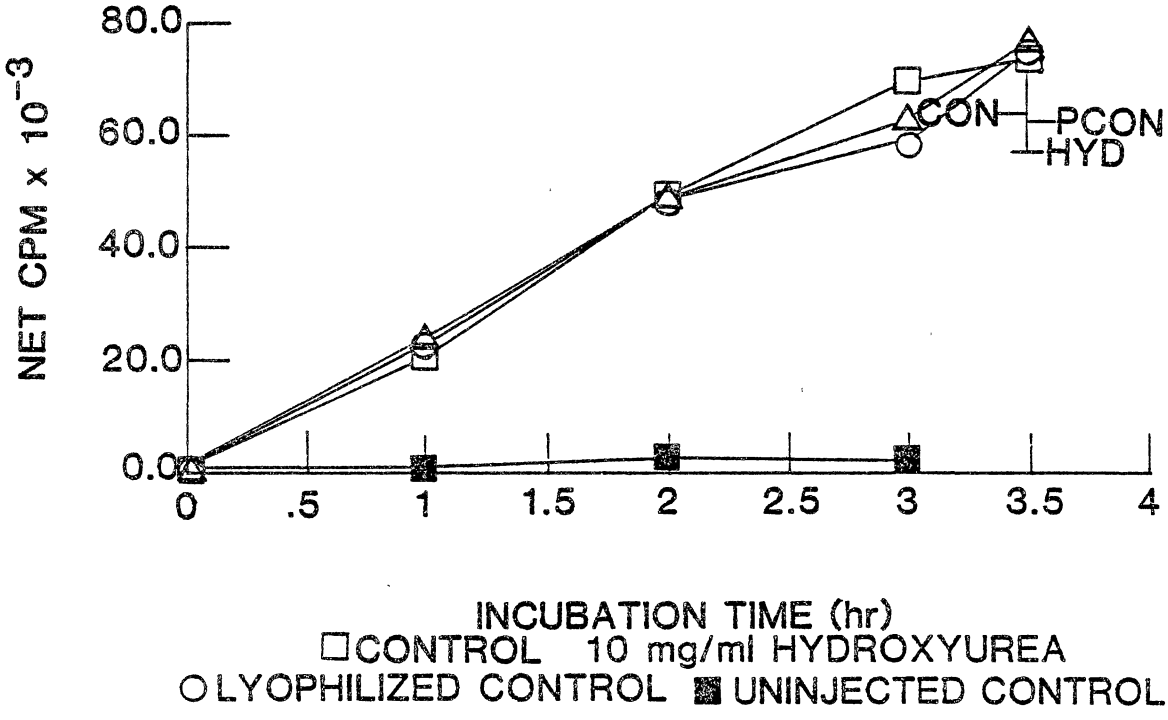

NET CPM x 10^{-3}
80.0
60.0
40.0
20.0
0.0
0
.5
1
1.5
2
2.5
3
3.5
4
CON
PCON
HYD
INCUBATION TIME (hr)
□CONTROL 10 mg/ml HYDROXYUREA
○LYOPHILIZED CONTROL ■ UNINJECTED CONTROL

Fig. 2. Gene exposure assay for actinomycin D. pHSV-106 DNA was exposed to actinomycin D prior to injection. Actinomycin D not binding to the DNA was removed after exposure and the DNA was lyophilized to concentrate. The control group (□) was not lyophilized while the processed control (●) was lyophilized.

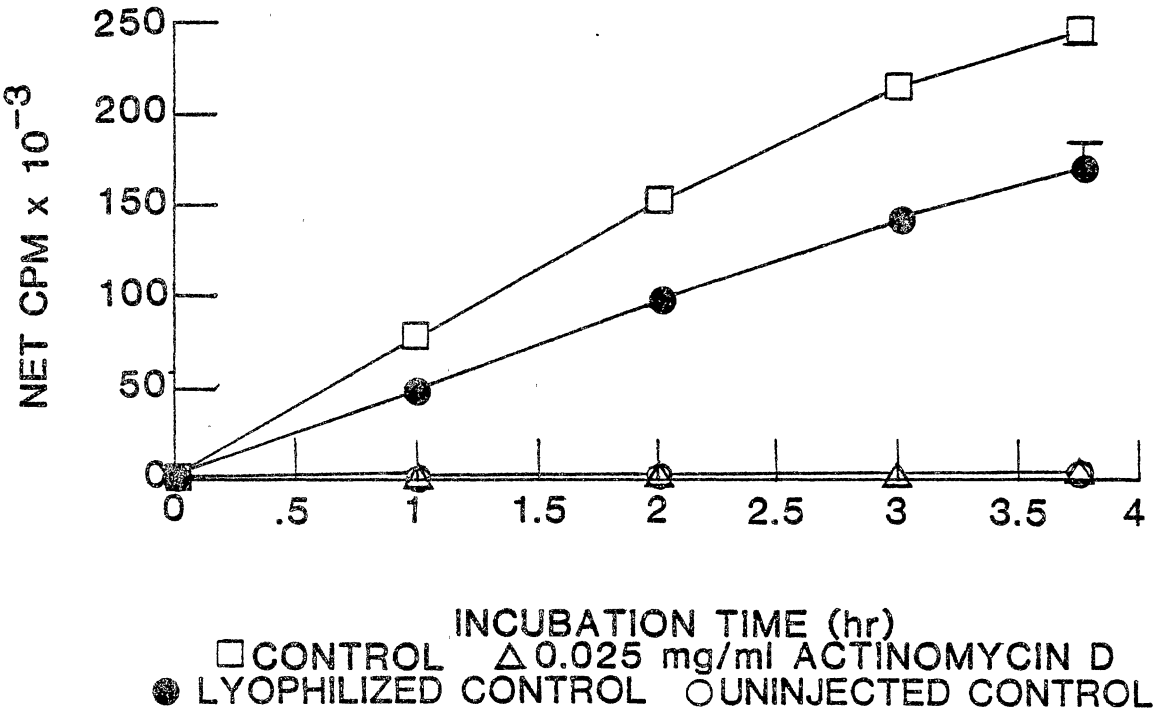

NET CPM x 10^{-3}
250
200
150
100
50
0
0
.5
1
1.5
2
2.5
3
3.5
4
INCUBATION TIME (hr)
□CONTROL △0.025 mg/ml ACTINOMYCIN D
● LYOPHILIZED CONTROL ○UNINJECTED CONTROL

Fig. 3. Oocyte exposure assay for actinomycin D. pHSV-106 DNA was injected into oocytes. The oocytes were then exposed to different concentrations of actinomycin D.

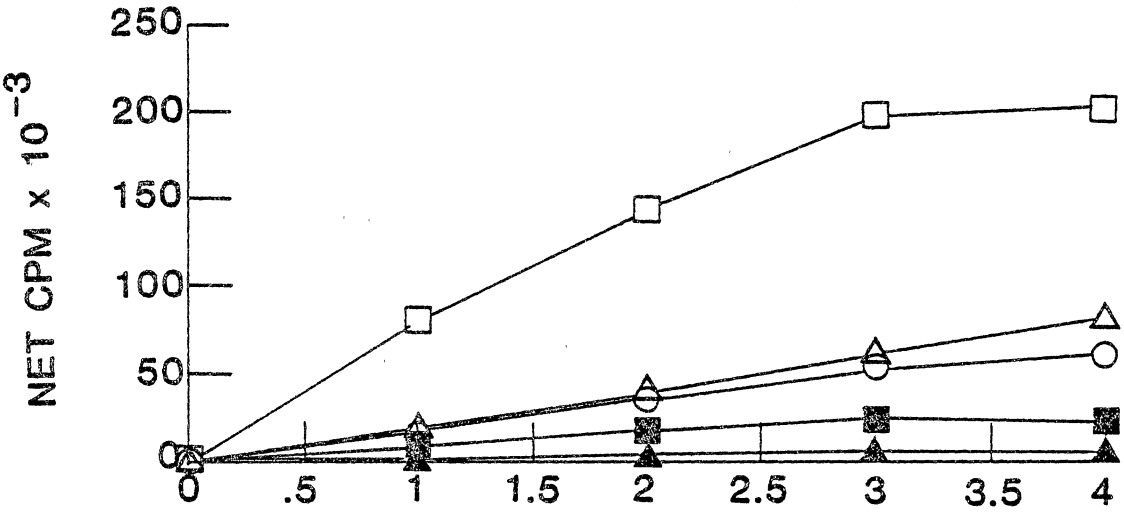

NET CPM x 10^{-3}
250
200
150
100
50
0
0
.5
1
1.5
2
2.5
3
3.5
4
INCUBATION TIME (hr)
□ CONTROL △1.33x10^{-2} mg/ml
○5.94x10^{-2} mg/ml ■1.32x10^{-1} mg/ml
▲UNINJECTED CONTROL

Fig. 4. Gene exposure assay for cycloheximide. pHSV-106 DNA was exposed to cycloheximide prior to injection. Cycloheximide was removed from the DNA after exposure and the DNA was lyophilized to concentrate. The control group (□) was not lyophilized while the processed control (○) was lyophilized.

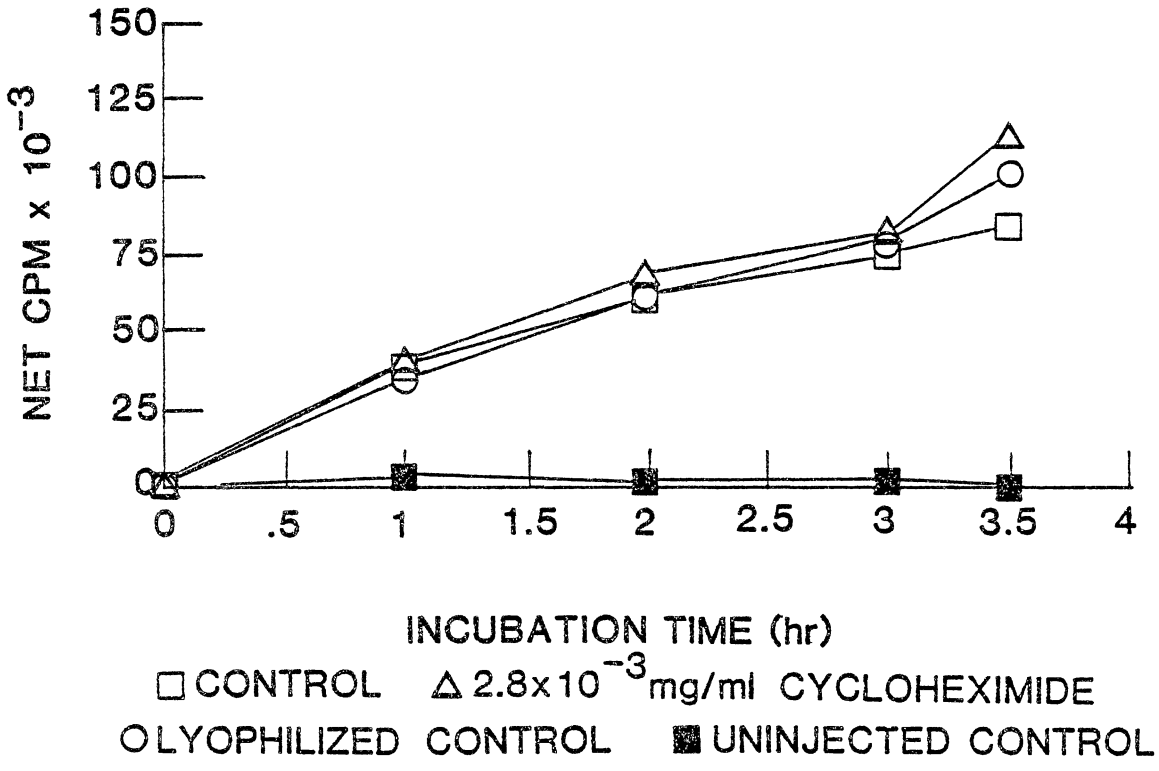
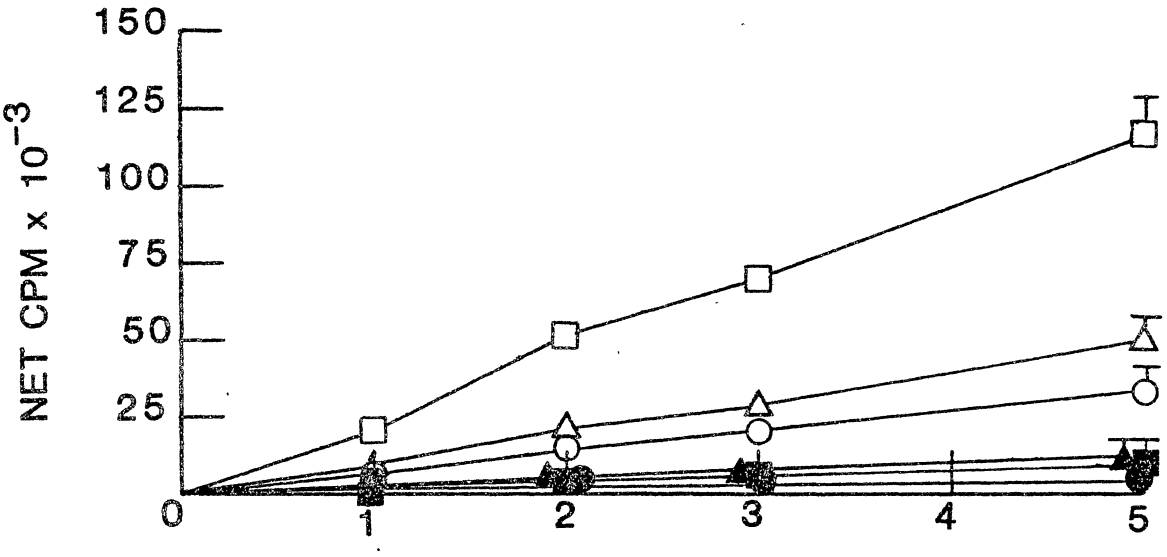

NET CPM x 10^{-3}
150
125
100
75
50
25
0
0
.5
1
1.5
2
2.5
3
3.5
4
INCUBATION TIME (hr)
□ CONTROL △ 2.8×10^{-3} mg/ml CYCLOHEXIMIDE
○ LYOPHILIZED CONTROL ■ UNINJECTED CONTROL

Fig. 5. Oocyte exposure assay for cycloheximide. pHSV-106 DNA was injected into oocytes. The oocytes were then exposed to different doses of cycloheximide.


NET CPM x 10^{-3}
150
125
100
75
50
25
0
1
2
3
4
5
INCUBATION TIME (hr)
□ CONTROL
△ 1.41×10^{-5} mg/ml
○ 1.41×10^{-4} mg/ml
■ 1.41×10^{-3} mg/ml
▲ 1.41×10^{-2} mg/ml
● UNINJECTED CONTROL

CHAPTER IV

ANALYSIS OF THE TERATOGENIC ACTIVITY OF DNA, RNA AND PROTEIN SYNTHESIS INHIBITORS USING *XENOPUS* EMBRYOS

by

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ABSTRACT

The teratogenic and growth inhibiting potential of DNA, RNA and protein synthesis inhibitors was explored using the Frog Embryo Teratogenesis Assay: Xenopus (FETAX). Endpoints measured in 96 h static tests were survival, malformation, ability to swim, skin pigmentation, stage of development and growth. The DNA synthesis inhibitors hydroxyurea, cytosine arabinoside and ethidium bromide proved to be teratogenic as measured by the teratogenicity index (LC50/EC50 malformation). The RNA synthesis inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide were not particularly teratogenic but significantly inhibited growth as determined by head-tail length measurements and staging of embryos. The results were comparable to similar studies using mammalian embryos thereby helping to validate the use of Xenopus embryos as a rapid screening test for teratogenic chemicals found either in the environment or in the workplace. Advantages of using Xenopus embryos as a screening test include rapid data collection, the ability to measure effects at different stages of development and the ability to use large number of embryos to obtain excellent dose-response curves with narrow confidence limits.

Keywords: FETAX, Xenopus, Teratogenicity, Embryo Toxicity, Growth.

INTRODUCTION

Approximately 50,000-70,000 chemicals are currently available in the marketplace with some 300 new chemicals released each year. Many of these chemicals pose a human health risk as well as a hazard to other organisms when these chemicals find their way into the environment. Short-term mutagenicity tests such as the Ames test (1) have been developed to test the mutagenicity and potential carcinogenicity of these compounds in a rapid and cost-effective manner. The development of similar short-term screening assays for teratogenicity has lagged behind mutagenicity assays but several systems are now being developed (2-5). Embryos of the South African Frog, Xenopus laevis, have been used previously as an assay system (6-9) but Dumont and co-workers have recently been responsible for both standardizing and validating this assay (10-12). They have named this the "FETAX" system (Frog Embryo Teratogenesis Assay: Xenopus) and we will also use this term in this work.

Xenopus embryos offer numerous advantages as a screening assay. The frog embryo is a classical developmental system that undergoes many of the same developmental processes as mammalian embryos. Mating is inducible at any time during the year from commercially available frogs and development is external thereby permitting nondestructive analysis of malformation at different stages of development. Most of the endpoints are easily and quantitatively

determined within 96 h using simple conditioned water or buffer solutions to culture the embryos in.

Because of the importance of gene expression in normal development, the possible teratogenic action of DNA, RNA and protein synthesis inhibitors have long been explored using a variety of experimental systems including mammals (13). Because of this previous work, these inhibitors can be used to help validate the FETAX system. For the purposes of this work, we define any inhibitor of normal gene expression as a genotoxic agent whether it works by attacking the structure of the gene or the cellular machinery that expresses the gene. Using this broad definition, all compounds used in this study qualify as genotoxic agents although under a more narrow definition only cytosine arabinoside, ethidium bromide and actinomycin D would be considered genotoxic since they alter DNA structure. Validation by using known teratogenic chemicals is one approach to extrapolating screening assay results from one system to another. Another approach is to determine which molecular mechanism is altered by a chemical and how this molecular mechanism relates to normal development. We have employed this combined approach to assess the teratogenicity of any genotoxic compound (14). By using complementary tests, we hope to raise the scientific and legal defensibility of short-term in vitro tests which have been lowly rated in the past (15).

In this study, we tested the effect of hydroxyurea, cytosine arabinoside, ethidium bromide, actinomycin D and cycloheximide on endpoints such as survival, gross malformation, ability to swim, skin pigmentation and growth after 96 hours of exposure in a static test. We

found that the DNA synthesis inhibitors hydroxyurea, cytosine arabinoside and ethidium bromide were teratogenic to varying degrees while the RNA synthesis inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide were not teratogenic but inhibited growth. The FETAX assay meets most of the criteria set forth by Kimmel et al. (16) for short-term in vitro teratogenicity assays and the data from this study can be integrated with data from other in vitro genotoxicity assays (14). The teratogenicity results of this assay using genotoxic agents were similar to those obtained for these same agents in mammalian systems (13). Complete validation of the FETAX system should provide a rapid, reliable and cost-effective screening assay for teratogenic substances.

MATERIALS AND METHODS

Water Quality

The water for holding tanks of Xenopus adults and larvae was filtered through an activated carbon filter (Barnstead™) and aerated for 48 h prior to use. The water was routinely tested for pH, oxygen content, hardness, heavy metal content and total organic carbon. The test results indicated that the water was nontoxic (17).

Animal Care and Breeding

Adult Xenopus were obtained from Xenopus I®, Ann Arbor, Mich. and stored in glass aquaria for a minimum of 30 days before use. Adults were fed beef liver and lung obtained from a local packing house. The meat was supplemented with baby vitamins (Polyvisol®) injected into the meat. Twelve h prior to mating, the female was injected with 1000 I.U. of human chorionic gonadotropin (Sigma®, St. Louis, Mo) and the male received 400 I.U. into the dorsal lymph sac. Amplexus ensued within 4-6 h and egg deposition 9-12 h from the time of injection.

After breeding, the adults and any fecal material were removed from the tank and the embryos obtained by scraping the eggs into 55 mm plastic Petri dishes. The jelly coats of the embryos were removed by swirling for 2-3 min in a 2% w/v cysteine solution made up in aerated water and the pH adjusted to 8.1 with NaOH.

Normally developing blastulae were initially selected by first removing them from necrotic eggs and abnormally cleaving embryos. A

second selection was then performed to ensure that only normal embryos were used in the test. For each toxicant concentration, there were 2 groups of embryos of twenty each placed into 55 mm glass Petri dishes. These dishes had previously been washed in dilute HCl, rinsed, then washed in dilute NaOH. This washing was followed by extensive rinsing. All glassware used in this study was washed in this manner.

Test Substances

Test substances were dissolved at different concentrations in aerated tap water. All inhibitors were obtained from Sigma®, St. Louis, Mo.. It was necessary to adjust the pH of the cytosine arabinoside solution to 7.8 by adding 20 mM HEPES and adjusting the pH with NaOH. Solutions were changed once every 24 h after each set of observations were recorded. Great care was taken to prevent damage to the embryos during the change. This procedure was different from the technique of Dumont (10) who used the same solution for the entire 96 h test period.

Data Collection

At 24, 48 and 72 h the dead embryos were scored and removed from the dishes. The number malformed and the stage of development according to Neiuwkoop and Faber (18) were recorded. Death at 24 and 48 h had to be ascertained by the embryo's skin pigmentation, structural integrity and irritability while at 72 and 96 h, the lack of heartbeat in the transparent embryo was an unambiguous sign of death.

At 96 h, the number of dead and malformed embryos was recorded for each group. Survivors were rated on their ability to swim and those demonstrating phase two or greater swimming behavior (19) were judged to be defective. Swimming behavior is rated phase two when the larvae

lose buoyancy and sink, when they spiral while swimming or when they swim erratically. The dorsal pigmentation of each embryo was observed and those embryos exhibiting small melanophores or a reduced number of melanophores compared to controls were judged to be abnormally pigmented.

Surviving embryos were fixed in 0.5% formalin and the head-tail length of each embryo was measured using a Radio Shack® digitizer and the data input directly to a Radio Shack ® model 16 microcomputer.

Data Analysis

The Litchfield-Wilcoxon (20) dose-response test was used to analyze the data for survival, malformation, swimming ability and skin pigmentation while the t Test for grouped observations was used to test for a significant reduction in growth. These statistical tests were part of a microcomputer software package (21) adapted by us for the analysis of these experiments. The software can perform all statistical tests, print and plot the results as well as store data relevant to the experiments. Additionally, a Visicalc® data entry model was developed to perform intermediate calculations. Any Radio Shack Model II, 12 or 16 microcomputer equipped with digitizer, and high resolution graphics board can run this software package which is available from the authors. Much of the programming is in Microsoft® Interpretive Basic and can easily be translated for other microcomputers. It will be necessary for the user to purchase Visicalc® from Radio Shack although this software package is optional.

RESULTS

Water Quality Analysis

We designed our modification of the FETAX system so as to test the teratogenicity of any substance using pure substances or undiluted complex mixtures. This testing of complex mixtures made it necessary to use dechlorinated tap water to rear Xenopus embryos, larvae and adults. It was then necessary to routinely analyze the quality of the tap water. Table I shows the results for water quality analysis. Total organic carbon content of the carbon filtered tap water averaged 3.6 mg/L which was was within allowable limits (17). Throughout the entire study, the malformation and death rate of control embryos (80 control embryos/test) averaged less than 5% thereby indicating the water was nontoxic.

Effect of DNA Synthesis Inhibitors on Growth and Development

Inhibitors of DNA synthesis are potent teratogens (13) even though the mechanisms whereby they inhibit DNA synthesis may be different. The DNA synthesis inhibitors used in this study were hydroxyurea, cytosine arabinoside and ethidium bromide. Hydroxyurea inhibits DNA synthesis by blocking the formation of deoxynucleotide triphosphates and has been proven to be a potent teratogen in mammalian systems (22,23). Cytosine arabinoside is a cytidine analog and is incorporated into the base structure of the DNA. It then inhibits DNA polymerase during replication. Cytosine arabinoside is also a potent teratogen in mammals (24,25). Ethidium bromide acts to inhibit replication by

intercalating into double stranded DNA and inhibiting polymerase function (26). However, the tertogenicity of ethidium bromide has not been fully established. As teratogens, DNA synthesis inhibitors may prevent the formation of sufficient cells in tissue primordia to permit proper development. The three compounds were selected not only because they could help validate the FETAX assay, but they would also provide information as to whether markedly different mechanisms of action affected the process of teratogenesis.

Using FETAX to Assay for Teratogenicity

When testing the effect of a genotoxin using the FETAX assay, we first exposed Xenopus larvae to various concentrations of genotoxin for 24 h. This procedure defined the upper limit of cytotoxicity of the test substance and provided an initial basis for establishing concentration ranges for the FETAX assay. Xenopus larvae were excellent indicator organisms of toxicity (19).

The initial concentration range used was very broad for the first experiment in case the substance proved to be teratogenic. In this case, malformation would occur at concentrations of teratogen that were much lower than those causing embryo lethality. A broad concentration range would encompass both events. For hydroxyurea, the first experiment revealed a probable LC50 of about 2 mg/ml (Fig. 1). Two additional experiments were performed using additional groups within a more narrow concentration range. Figure 1 shows the FETAX system gave repeatable results even though different groups of breeding animals were used and the experiments were separated temporally. Usually, the second or third experiment had a sufficient number of groups that gave a response somewhere between 16 and 84% so that the Litchfield-

Wilcoxon probit analysis could be performed on the mortality and malformation data from the third assay. The Litchfield-Wilcoxon test established the 96 h EC50 (malformation) for hydroxyurea of 0.425 mg/ml and the LC50 value of 1.82 mg/ml (Figure 2) with 95% confidence limits of 0.35-0.517 and 1.5-2.7 mg/ml respectively (Table II). From the data it can be seen that the concentrations at which malformation occurred were much lower than those needed to cause embryo lethality. The teratogenicity index for hydroxyurea was 4.3 indicative that a mechanism crucial to development was being inhibited and that general cytotoxicity was not involved in causing abnormal development (10). An index of one would suggest that general toxicity was causing both malformation and death. It should be noted that because of the large number of available embryos, an excellent data fit was possible with good confidence limits. In assaying for teratogenicity, we have found it expedient to follow the sequence listed above for hydroxyurea in performing the FETAX assay for mortality and malformation. This procedure was followed for all the compounds tested even though we only present dose-response data here from the third experiment.

Figure 3 shows the change in the malformation curve through time. Because Xenopus development is external, it was possible to observe mortality and malformation rates through time. This permitted much more data to be collected than in the case of mammalian or avian systems. For hydroxyurea, the slope of the dose-response curve for malformation did not change (Figure 3) but the entire curve was shifted to lower concentrations throughout the course of the experiment. The greatest shift in this experiment was seen between 24 and 48 h into the experiment. This suggested a stage sensitivity to hydroxyurea such

that early stages were more sensitive than later stages. These results may be consistent with the known mode of action of hydroxyurea which is inhibition of DNA synthesis (13) since the rate of cell division in amphibian embryos is very high during the first 48 h of development and then declines. We have also detected a noticeable reduction in the rate of development when inhibitors of DNA synthesis or other genotoxic agents are used. At high inhibitor concentrations, the embryos can lag behind by two full stages of development 48 h post-fertilization even though all other physical parameters are held constant. The effect was very similar to rearing one group of embryos at temperatures several degrees centigrade cooler than another.

The Teratogenic Effects of Hydroxyurea

Table II summarizes the results of the entire study and shows that for all dose-response data that can be analyzed by the Litchfield-Wilcoxon method, narrow confidence limits can be set. For hydroxyurea, the LC50 and EC50 (malformation) data were clearly outside each other's confidence limits. This indicated a significant difference between the two curves and gave validity to the concept that the teratogenicity index was a meaningful measure of teratogenicity.

The Teratogenic Effects of Cytosine Arabinoside

Figure 4 shows the dose-response curve for the effect of cytosine arabinoside on mortality and malformation in buffered water. The LC50 and EC50 (malformation) was estimated to be 5.4 and 0.76 mg/ml respectively. The teratogenicity index was estimated to be about 7.1 (Table II) and clearly showed that cytosine arabinoside was as teratogenic to frog embryos as it was in mammalian systems (24,25). Initial experiments with cytosine arabinoside indicated that high

concentrations lowered the pH of the water to about 3.5. The interaction of low pH and DNA synthesis inhibition yielded an LC50 of 2.45 and an EC50 (malformation) of 0.2 mg/ml (data not shown). Raising the pH artificially above neutrality dramatically increased survival and allowed interpretation of the effect of cytosine arabinoside without regard to the interaction of pH. Although numerous test concentrations were used between 0.25 and 8.0 mg/ml for cytosine arabinoside only the two concentrations above 1.5 mg/ml in the malformation curve shown in Figure 4 exhibited significant death rates. The data for the effect of cytosine arabinoside on mortality and malformation was so disparate that there was no doubt that the two curves were not overlapping and the teratogenicity index was significant.

Effect of Cytosine Arabinoside on Swimming Ability and Skin Pigmentation

Malformation was determined by viewing the entire embryo through a dissection microscope and observing gross malformations. It was possible that damage occurred to the embryo that was not detectable by this method. Swimming behavior and abnormal pigmentation were endpoints designed to show damage at the cellular levels (10). This type of damage was expected to occur at concentrations of teratogen lower than that needed to cause gross malformation. The 96 h EC50 values for swimming ability and abnormal pigmentation were 1.3 and 1.48 mg/ml respectively (Fig. 5). Although there was a close fit of the data, these values were much higher than the 0.76 mg/ml value for gross malformations (Table II). The reduction of pigmentation was estimated by noting the number and size of the melanophores on the back of each

larvae. For cytosine arabinoside and actinomycin D, this procedure yielded good results but none of the other compounds used caused a reduction in pigmentation that occurred in a dose-response manner.

Effect of Hydroxyurea and Cytosine Arabinoside on Growth

Both hydroxyurea and cytosine arabinoside affected embryonic growth at concentrations far lower than the EC50 (malformation) and LC50 (Figure 6). Only 0.3 mg/ml hydroxyurea and 0.8 mg/ml cytosine arabinoside significantly affected growth at the P=0.01 level as measured by head-tail length. Given the ability of each compound to inhibit DNA synthesis and, therefore, cell division, these results were expected. A reduction in growth certainly can compromise the prospects of survival for an organism. At high inhibitor concentrations, embryos were only 60-65% the length of controls. Both curves showed a good dose-response relationship between concentration and the corresponding reduction in growth. Table II also shows that both hydroxyurea and cytosine arabinoside had a multiplicity of effects but that growth was the most sensitive endpoint to these inhibitors followed by the occurrence of congenital malformations.

The Teratogenic and Growth Inhibiting Effects of Ethidium Bromide

Although the teratogenicity of ethidium bromide had not been extensively studied, it was used in this study because it affected DNA synthesis by a mechanism different from hydroxyurea and cytosine arabinoside (26). Table II shows that ethidium bromide did exhibit some teratogenicity as the teratogenicity index is almost 1.5 and the 95% confidence limits for the mortality and malformation were separated from one another. As in the case of hydroxyurea and cytosine arabinoside, the 96 h EC50 (ability to swim) was not more sensitive

than the EC50 (malformation). The effect of ethidium bromide on growth was greater as concentrations as low as 0.001 mg/ml significantly affected growth. As in the case of hydroxyurea, it was not possible to discern reduction in pigmentation (Table II).

The Teratogenic and Growth Inhibiting Effects of Actinomycin D

Actinomycin D is a potent inhibitor of RNA synthesis while DNA synthesis is only inhibited at higher concentrations. Actinomycin D intercalates into the double helix of the DNA and prevents RNA polymerase from reading the genetic code. After a period of time, this would ultimately reduce protein synthesis. Actinomycin D is considered to be slightly to moderately teratogenic in mammalian systems (13). Figure 7 shows the effect of actinomycin D on mortality and malformation. The 96 h EC50 (malformation) and LC50 values were very similar and the teratogenicity index, in contrast to the DNA synthesis inhibitors, was 0.88 (Table II). Because the 95% confidence limits of the EC50 (malformation) overlapped with the confidence limits of the LC50 there was probably no significant difference between the two values and the teratogenicity index was not significant.

Table II shows that actinomycin D affected swimming and pigmentation at concentrations similar to the EC50 (malformation) and LC50. Actinomycin D was required in higher concentrations to reduce growth than for either of the DNA synthesis inhibitors (Figure 8). The minimum concentration needed to reduce growth was 0.0159 mg/ml while the LC50 is 0.0189 mg/ml (Table II). Because this level was so near the LC50, embryo death occurred quickly and the amount of embryonic size reduction was restricted to only 80% of controls. Apparently, once the concentration was high enough so that the actinomycin D

penetrated the cell and nuclear membranes, death quickly ensued. This was in sharp contrast to the 30-40% growth reduction possible in assays using cytosine arabinoside and hydroxyurea (Figure 6). Actinomycin D may have several cellular side affects at or near the concentration needed to inhibit RNA synthesis so that general toxicity rapidly occurred (13).

The Teratogenic and Growth Inhibiting Effects of Cycloheximide

Cycloheximide inhibits protein synthesis at the level of the ribosome. In mammalian systems, cycloheximide is considered not to be teratogenic but rather an inhibitor of embryonic growth (13). This was also true for its effect on Xenopus embryos. Figure 9 shows the effect of cycloheximide on mortality and malformation. There was a close correlation between the two dose-response curves (96 h EC50 (malformation) 1.34×10^{-4} mg/ml; 96 h LC50 1.59×10^{-4} mg/ml) and the teratogenicity index was only 1.17 which was again within the confidence limits of the two curves. The 96 h EC50 swimming was 1×10^{-4} mg/ml and showed that cycloheximide affected swimming behavior at concentrations only slightly lower than the LC50 or EC50 values (Table II). It was not possible to accurately judge the pigmentation pattern of these embryos as all embryos even in low concentrations were whitish in appearance. This does not necessarily mean that they were lacking pigment but that it was difficult to see the pigmentation pattern.

Cycloheximide affects growth in a manner similar to hydroxyurea and cytosine arabinoside (Fig. 10, Table II). The minimum concentration of cycloheximide required to inhibit growth at the $P = 0.01$ level was 5.6×10^{-5} mg/ml which was quite dissimilar from the

LC50 value of 1.59×10^{-4} mg/ml. Small quantities of cycloheximide were then very effective in reducing embryonic growth and this was consistent with findings in mammalian systems (13).

In summary, except for the mortality curve of cytosine arabinoside, all data gave good dose-response results that could be extrapolated to both zero and maximum effects (Table II). The 95% confidence limits were all narrow which was mainly a of result large sample sizes tested at each concentration. In all cases, growth was the most sensitive criterion of toxicity followed by combined congenital malformations. Lastly, the DNA synthesis inhibitors proved to be teratogenic as evidenced by their teratogenicity indices while actinomycin D and cycloheximide proved to mainly inhibit growth.

DISCUSSION

The FETAX System as a Teratogenicity Assay

The assay, as we have employed it, differed from that of Dumont and coworkers (10) in that we used dechlorinated tap water instead of a buffer solution for rearing embryos. We also dejellied the eggs whereas Dumont et al. (10) did not. The toxic chemicals in our assay were changed once every 24 h with data being collected on mortality and malformation at that time or just after 96 h. We also used covered 55 mm glass Petri dishes instead of larger finger bowls. Moreover, we included malformed with normal embryos in the growth length estimates so as to include the entire surviving population. Other than these differences, our assay was essentially the same to that proposed by Dumont et al. (10).

In its present form, this assay meets most of the criteria set forth by Kimmel et al. (16) for validation of *in vitro* teratogenicity assays. Endpoints such as mortality, malformation, absence of swimming and growth are easily quantifiable, capable of being analyzed statistically and of exhibiting a dose-response relationship with the establishment of narrow confidence limits. Since many of the stages of amphibian development are similar to mammalian development, this assay should provide teratogenicity data highly relevant to humans although the problems of maternal metabolism and placental transport must be considered in this as well as all other *in vitro* assays. Using FETAX it is also possible to determine

what stage of development is affected because development is external and the suspected teratogen can be added at any time during development. Further experimentation must be performed before compounds requiring metabolic activation can be used in this assay and this remains a current, but temporary, limitation.

Because of their lack of sensitivity, the use of the ability to swim and skin pigmentation endpoints will produce little additional data unless new criteria are used to estimate swimming ability and skin pigmentation. For swimming ability, it may be necessary to measure the negative phototactic response of the 4 day larvae following treatment instead of merely estimating the ability of each larva to swim normally in a straight line. In the case of skin pigmentation, it will be necessary to quantify both the number and size of the melanophores on the back of each larva. A rapid and straightforward method of analysis is required in order to routinely use this endpoint to predict stress on the organism.

In order to be useful for assaying the numerous compounds present in the environment, a bioassay must be both time and cost-effective. By using the microcomputer software package that we developed for the FETAX, we have significantly cut labor costs as well as standardized data handling and statistical procedures. The time savings makes it practical to carry out the entire assay from set-up to final data analysis in a single 5 day work week. Additional savings in time and costs must be forthcoming in order for teratogenicity screening assays to be routinely employed.

The Teratogenicity Index as a Predictor of Teratogenic Potential

The Teratogenicity Index (LC50/EC50 (malformation)) as proposed by Dumont et al. (10) may well be useful as a measure of teratogenic risk if species sensitivity and differences in metabolic activation are taken into account. Most teratogens will cause both malformation and embryo death. At low concentration, the compound may selectively interrupt a vital morphogenetic process or cause a selective necrosis of cells in a primordium, yet not affect the bulk of the cells that are not specifically engaged in development. In this situation, malformation frequently occurs with little mortality, resulting in a substantial teratogenic risk. At higher concentrations, all cells are similarly affected due to the general cytotoxicity component of the compound and although malformation occurs frequently, the number of dead embryos increases dramatically. In this case, there is much less teratogenic risk because there are very few live births and the problem is primarily one of embryo lethality.

Species sensitivity becomes important when the assay species is tolerant to a particular compound compared to other more sensitive species. One way this may occur is when the teratogen is not rapidly transported into the body of the assay species but is easily taken up by more sensitive species. However, in both cases the teratogenicity index would be likely to remain the same as the low rate of teratogen uptake in the assay species affects both intracellular concentrations at which malformation and mortality occur equally. This same ratio may then exist for a more sensitive species that takes up the compound more quickly from the environment. The compound is still teratogenic by the same ratio although smaller doses are needed to produce the effect.

Regardless of species sensitivity, the chance of producing live malformed offspring is much higher for a compound with a high teratogenicity index than one with a low index where embryo death predominates. Comparisons should be made between diverse species to determine whether the teratogenicity index is the same for each validation compound. If so, this teratogenicity index could play a valuable role in predicting teratic risks. Unfortunately, situations will probably be encountered where differences in metabolism and development will produce a different teratogenicity index from one species to another thus generating false negative and positives. If these differences can be understood by further experimentation, then the predictability of short-term teratogenicity assays can be enhanced.

Sensitivity of Xenopus Embryos to Test Compounds

The sensitivity of Xenopus embryos to inhibitors of DNA, RNA and protein synthesis was fairly good despite the presence of the rather inert vitelline membrane which was a permeability factor during the first 20 h of development. For hydroxyurea, cytosine arabinoside and ethidium bromide, the minimum concentrations needed to significantly affect growth was 0.3, 0.8 and 0.001 mg/ml respectively while the amount needed to reduce either DNA synthesis or cell growth in cultured cells was 0.04 (27), 0.028 (28) and 5×10^{-5} mg/ml (29). Actinomycin D has a molecular weight of 1255.5 and should therefore pass relatively slowly through cell membranes. Since cultured mouse blastocysts have only a few cells, they should be far more sensitive to this inhibitor than whole Xenopus embryos composed of thousands of cells and this was borne out in this study. It took 0.0159 mg/ml actinomycin D to inhibit Xenopus embryonic growth while only 5 x

10^{-6} mg/ml was needed to inhibit the growth of cultured mouse blastocysts (30). For the lower molecular weight compound cycloheximide, 5.6 x 10^{-5} mg/ml cycloheximide was required to inhibit Xenopus growth while only 1 x 10^{-6} mg/ml was required for cultured mouse blastocysts (30). While somewhat more tolerant than cultured cells and early cultured mammalian embryos, it appears that the FETAX assay promises to be acceptably sensitive to potential teratogens. Most of the differences in sensitivity may be due to the increased number of cells present in late stage Xenopus embryos, the relative impermeability of the early vitelline membrane and the probable detoxification of toxins by late stage embryos.

FETAX Results Are Similar to Those Obtained Using Mammals

The overall conclusions of this study are essentially the same as in similar studies using mammals (13). Inhibitors of DNA synthesis were teratogenic regardless of their molecular mechanism of action. Actinomycin D and cycloheximide exhibited little or no teratogenicity but caused a marked decrease in growth. Most of the mammalian studies that will be compared below with the FETAX study involved single doses of teratogen or a treatment that lasted only a few days while in this study the treatment was continuous. This is one difference that causes some caution in comparing FETAX results with mammalian studies.

Hydroxyurea was as teratogenic in Xenopus as it was in mammals. The cytotoxic component of hydroxyurea reported in rats (22) and monkeys (23) may have been detected in this study. The teratogenicity index for hydroxyurea was 4.3 while that for cytosine arabinoside was 7.1 (Table II). The reduced teratogenicity index for hydroxyurea suggests that hydroxyurea is more cytotoxic than cytosine

arabinoside. In the hydroxyurea tests, malformations occurred in the same concentration ranges as embryo lethality whereas in the cytosine arabinoside tests the ranges were clearly distinct (Figs. 2 and 4).

Cytosine arabinoside was the most teratogenic inhibitor studied. The wide separation of mortality and malformation concentration ranges for cytosine arabinoside was also seen in experiments using rat embryos. Ritter et al., (25) reported that at concentrations where only 21% of the rat embryos died, cytosine arabinoside caused 100% malformation. The low toxicity of this analog clearly increases the risk of producing congenital malformations.

Ethidium bromide was also teratogenic in this study and there is evidence that it causes malformation in other systems (31). Ethidium bromide may not have intercalated efficiently into the embryonic genome and high concentrations could have been required to permit substantial DNA intercalation. At these elevated concentrations, ethidium bromide could have been cytotoxic thereby producing a relatively low teratogenicity index compared to the other DNA synthesis inhibitors.

Actinomycin D caused numerous malformations in Xenopus embryos but it was also quite toxic as well as evidenced by the teratogenicity index of 1 or less (Table II). In rats, acute actinomycin D treatment also causes numerous resorptions as well as significant numbers of malformations (32,33). When the teratogenicity index is calculated from the experimental data for rats (33), the teratogenicity index is less than one. This compares favorably to the results reported in this study. Results of mammalian studies using chronic doses of actinomycin D parallel acute studies but maternal lethality prevents a complete dose-response study from being carried out (32). The FETAX assay has

no such limitation. Actinomycin D also reduces rat embryonic size (13) as it reduces Xenopus embryonic size (Fig. 8).

Cycloheximide also caused malformations in Xenopus embryos only in the range of general toxicity. Its affect on growth was dramatic (Fig. 10) and these results are the same as for other mammalian studies investigating protein synthesis inhibitors (13, 34).

The results show that the FETAX system accurately predicted teratogenicity in this study with no false positives or negatives when compared to mammalian studies. Actinomycin D may be an exception and further tests using mammalian systems should be performed to explore the dose-response relationships that exist between mortality and malformation. The data presented here were more quantitative than most mammalian studies and were based on results from hundreds of animals. Much additional validation with known mammalian teratogens needs to be performed before this system can be a reliable predictor of teratogenicity for mammals.

Validation with known mammalian teratogens is one method that permits extrapolation of test results implying teratic risk to other organisms. Another method is to clearly understand the molecular mechanisms that underlie teratogenesis. DNA synthesis inhibitors routinely seem to be teratogenic (13). Should a compound prove to be a DNA synthesis inhibitor and also cause numerous malformations in the FETAX assay, then this is a more powerful argument for the agent causing human teratogenicity than just the results of the bioassay alone. If the compound causes a reduction in RNA and/or protein synthesis without altering DNA synthesis and the teratogenicity index is from the FETAX assay is approximately one, then growth inhibition

and embryo death are the hazards presented to the embryo. We have therefore combined the FETAX screening assay with another assay that routinely measures whether a compound can interfere with gene expression and whether this interference is due to the agent binding with the DNA or upsetting the metabolic machinery that produces the protein (14). This approach should not only make risk assessment easier but should also expand our data base for the molecular action of chemical teratogens.

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Appendix

Probit Analysis

The probit transformation is commonly used to straighten cummulative curves such as obtained for dosage mortality and malformation data. It is merely a type of regression analysis. A probit is a unit of probability. It is related to the percent area under the normalized probability curve. The percent of animals responding is converted to probits. A 50% response would yield a probit of 5.0, based the fact that the 50% point is equivalent to 0 standard deviations coded by the addition of 5. The 83.14 point on a cummulative curve becomes +1 standard deviation, and the probit value is 6.0. Using this method, cummulative curves can readily be linearized.

Because there are no probit values corresponding to 0 and 100% effects, the problem of analyzing such complete dose-effect curves was addressed by Litchfield and Wilcoxon (20). The method of Litchfield and Wilcoxon was used in this study and this method is available as a computer program (21). In this study, this procedure was programmed into a Radio Shack TRS-80, Model 16, Microcomputer by the investigators. The program then determines the LC50 and EC50 (malformation) values as well as the 95% confidence limits.

Table I. Water quality analysis of carbon-filtered conditioned tap water used to maintain adults and embryos.

Sample[1]	Na (mg/l)	Ca (mg/l)	Mg (mg/l)	K (mg/l)	Fe (mg/l)	Pb (mg/l)	Zn (mg/l)
Sample 1	22	42	12.7	5.6	0.02	<0.005	<0.03
Sample 2	21.8	44.6	13.5	5.5	0.03	<0.005	<0.03
Sample 3	21.8	46.4	12.5	5.4	0.11	<0.005	<0.03
Sample 4	19.8	42.6	12.62	4.6	0.12	0.008	0.002
Sample 5	18.6	41.6	13.07	5.28	0.07	0.007	0.009
average	20.8	43.44	12.88	5.28	0.07	<0.006	<0.02
maximum	22	46.4	13.5	5.6	0.12	0.008	0.03
minimum	18.6	41.6	12.5	4.6	0.02	0.005	0.002

	Cu (mg/l)	Cr (mg/l)	Ni (mg/l)	Cd (mg/l)	Oxygen (mg/l)	Conductivity (μMHOS)	pH
Sample 1	0.04	0.006	0.05	0.001	5.5	229	8.08
Sample 2	0.04	0.023	0.05	0.001	5.8	219	8.1
Sample 3	0.04	0.04	0.05	0.001	5.7	220	8.08
Sample 4	0.008	0.024	0.004	0.001	6.0	212	8.15
Sample 5	0.007	0.005	0.008	0.001	4.8	212	7.8
average	0.027	0.02	0.032	0.001	5.56	218.4	8.04
maximum	0.04	0.04	0.05	0.001	6	229	8.15
minimum	0.007	0.005	0.004	0.001	4.8	212	7.8

[1]Samples for water quality analysis were randomly collected from tap water during the 6 month period that the data was collected for these experiments. Oxygen, conductivity and pH measurements were performed on water samples actually used for experimentation.

Table II. Effects of genotoxic agents on embryo mortality, malformation, teratogenicity index, swimming ability, pigmentation and growth.

Assay	Hydroxyurea (mg/ml)	Cytosine arabinoside (mg/ml)	Ethidium bromide (mg/ml)	Actinomycin D ($mg/ml x 10^{-2}$)	Cycloheximide ($mg/ml x 10^{-4}$)
LC_{50}	1.82	6.0	0.05	1.89	1.59
95% Confidence Interval	1.5-2.7	N.A.	0.047-0.054	1.38-2.61	1.03-2.34
EC_{50} (Malformation)	0.43	0.76	0.035	2.17	1.34
95% Confidence Interval	0.35-0.52	0.63-0.93	0.029-0.043	1.88-2.51	1.06-1.87
Teratogenicity Index[1] (LC_{50}/EC_{50} Malformation)	4.3	7.9	1.43	0.88	1.17
EC_{50} (Swimming Ability)	0.58	1.3	0.05	1.76	1.0
95% Confidence Interval	0.47-0.72	1.13-1.49	N.A.	0.008-3.8	0.52-1.9
EC_{50} (Pigmentation)	-	1.48	-	2.18	-
95% Confidence Interval	-	1.28-1.59		1.68-2.81	-
Minimum Concentration to Inhibit Growth[2]	0.3	0.8	0.001	1.59	0.56
Minimum Concentration to Inhibit Development[3]	0.6	0.7	0.05	1.59	0.28

N.A.: Data not available. Value determined by 2 point method.

[1]Teratogenicity index values are dimensionless

[2]Values are minimum concentrations that inhibit growth (t test for grouped observations at P=0.01.

[3]Values are concentrations at which development is retarded by 1 stage after 24 H development.

Fig. 1. Effect of hydroxyurea on mortality. The number of dead embryos after 96 h exposure was recorded by observing the cessation of heartbeat.

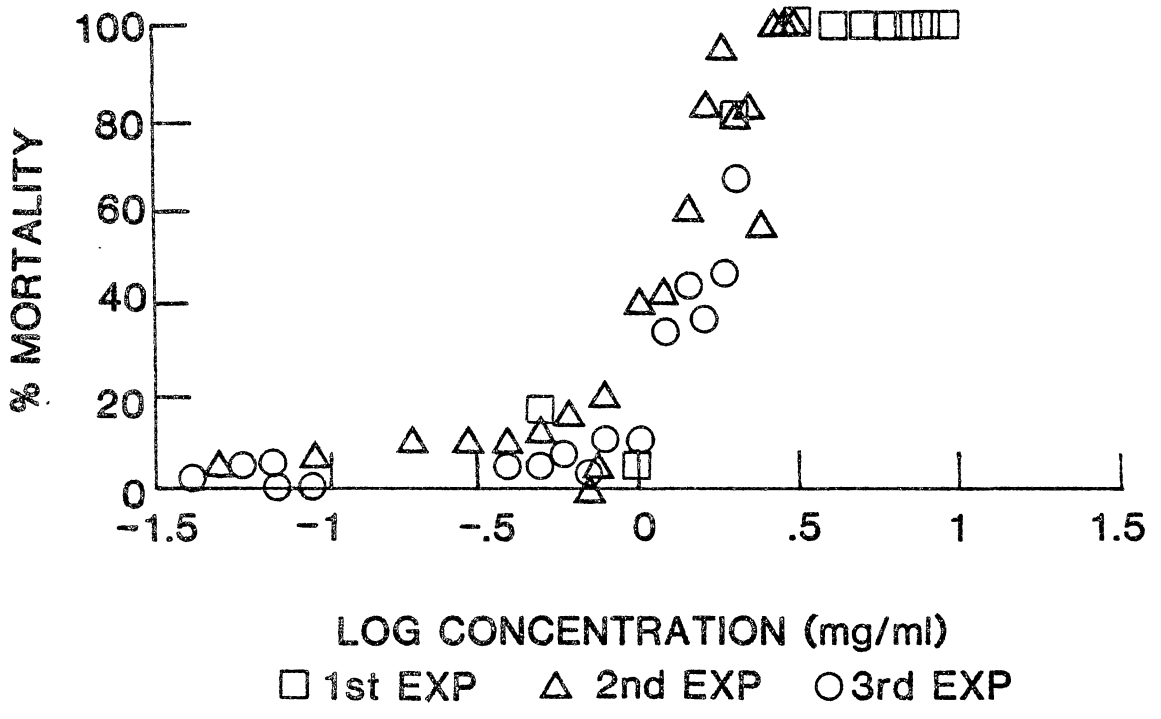

100
80
60
40
20
0
% MORTALITY
-1.5
-1
-.5
0
.5
1
1.5
LOG CONCENTRATION (mg/ml)
1st EXP
2nd EXP
3rd EXP

Fig. 2. Dose-response curve of the effect of hydroxyurea on Xenopus mortality and malformation. The teratogenicity index was 4.3.

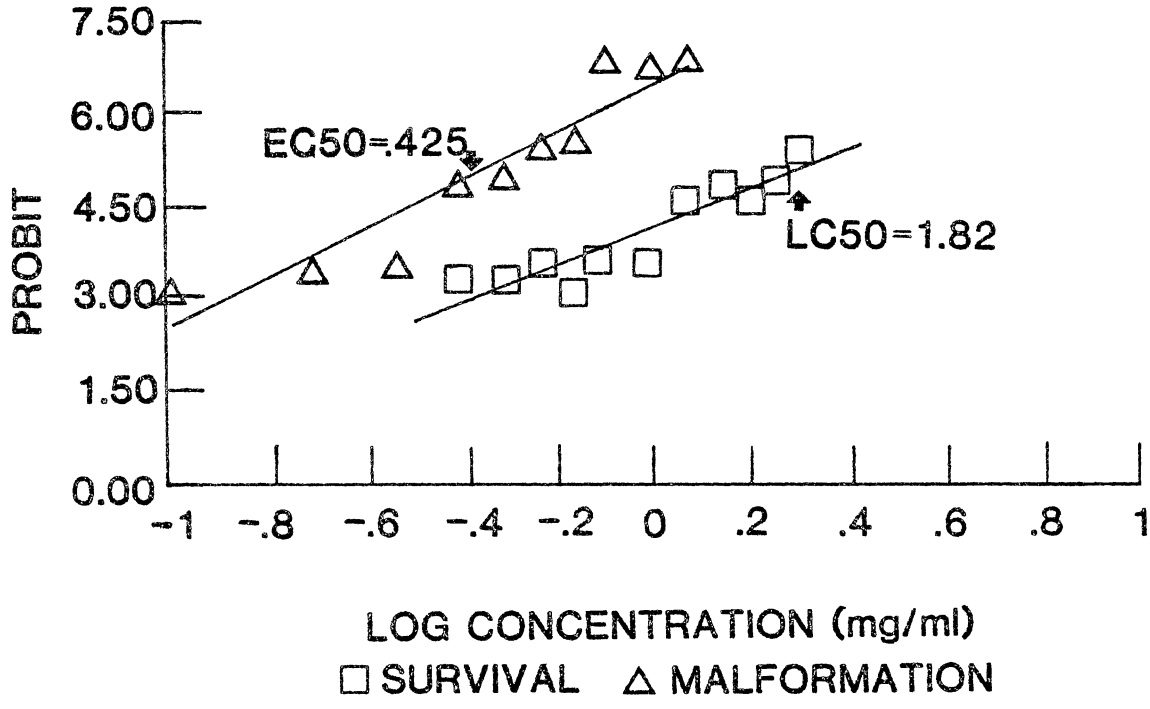

7.50
6.00
4.50
3.00
1.50
0.00
PROBIT
EC50=.425
LC50=1.82
-1
-.8
-.6
-.4
-.2
0
.2
.4
.6
.8
1
LOG CONCENTRATION (mg/ml)
□ SURVIVAL △ MALFORMATION

Fig. 3. Dose-response curve of the effect of hydroxyurea on malformation over time.

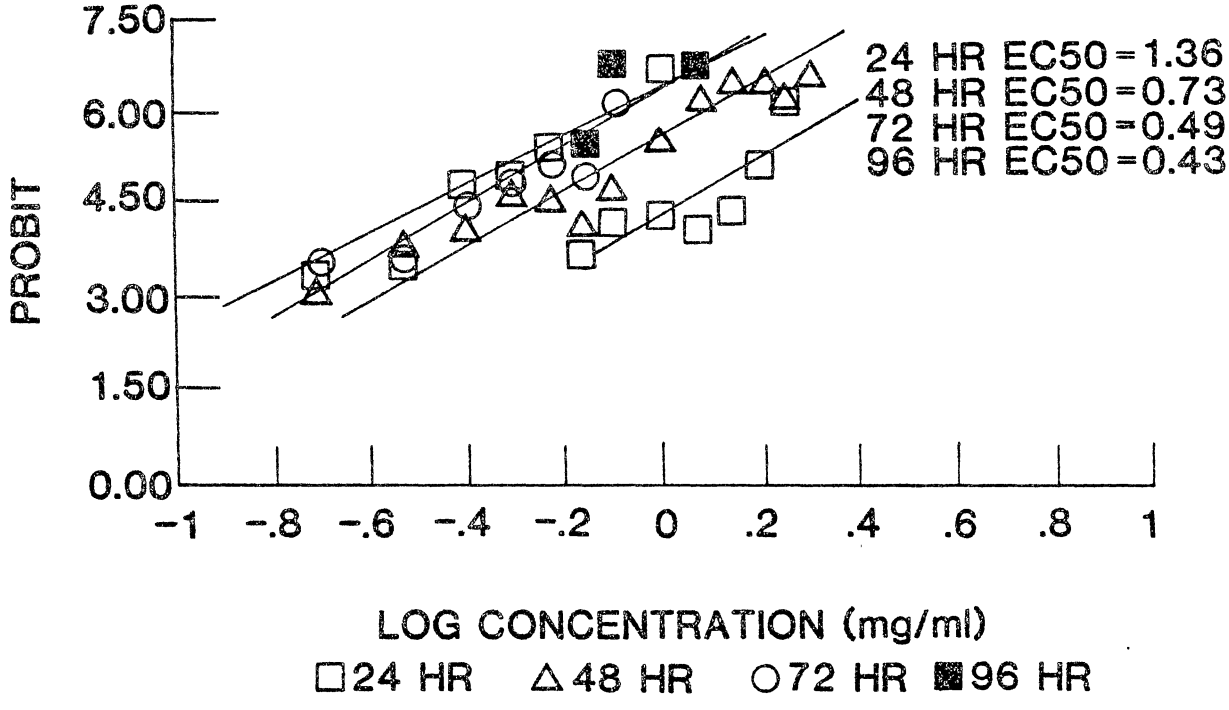

7.50
6.00
4.50
3.00
1.50
0.00
PROBIT
−1
−.8
−.6
−.4
−.2
0
.2
.4
.6
.8
1
24 HR EC50 = 1.36
48 HR EC50 = 0.73
72 HR EC50 = 0.49
96 HR EC50 = 0.43
LOG CONCENTRATION (mg/ml)
□24 HR △48 HR ○72 HR ■96 HR

Fig. 4. Dose-response curve of the effect of cytosine arabinoside on Xenopus mortality and malformation. The teratogenicity index was 7.1. The mortality curve was determined by the two point method due to insufficient data points in the 16-84% mortality region.

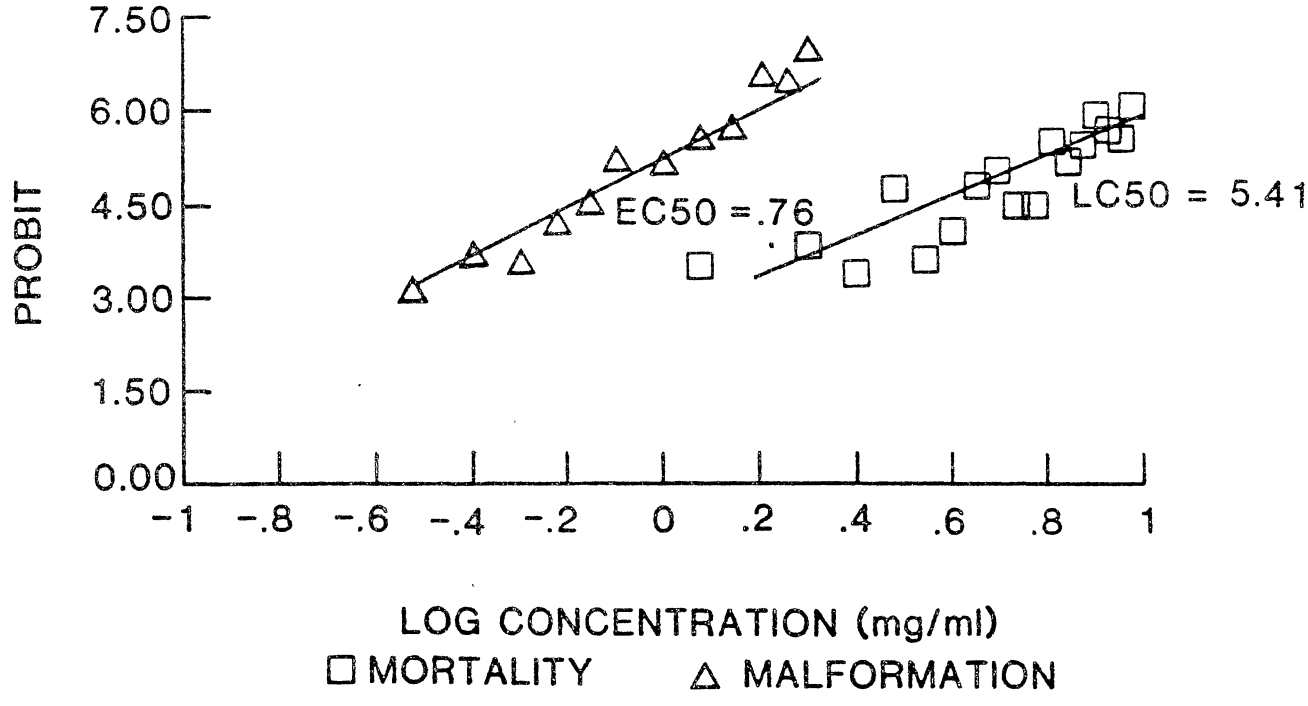

7.50
6.00
4.50
3.00
1.50
0.00
PROBIT
EC50 =.76
LC50 = 5.41
-1
-.8
-.6
-.4
-.2
0
.2
.4
.6
.8
1
LOG CONCENTRATION (mg/ml)
MORTALITY
MALFORMATION

Fig. 5. Dose-response curve of the effect of cytosine arabinoside on larval ability to swim and skin pigmentation after 96 h exposure.

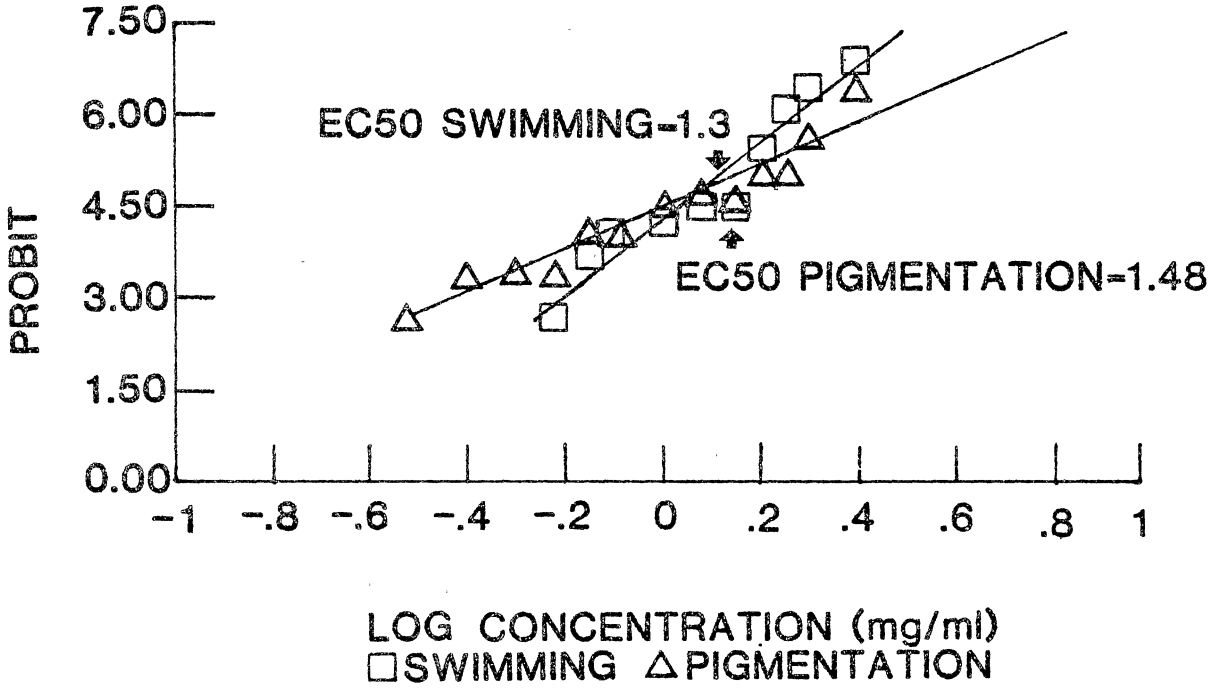

7.50
6.00
4.50
3.00
1.50
0.00
PROBIT
EC50 SWIMMING=1.3
EC50 PIGMENTATION=1.48
-1
-.8
-.6
-.4
-.2
0
.2
.4
.6
.8
1
LOG CONCENTRATION (mg/ml)
□SWIMMING △PIGMENTATION

Fig. 6. Effect of hydroxyurea and cytosine arabinoside on growth as measured by head-tail lengths after 96 h exposure.

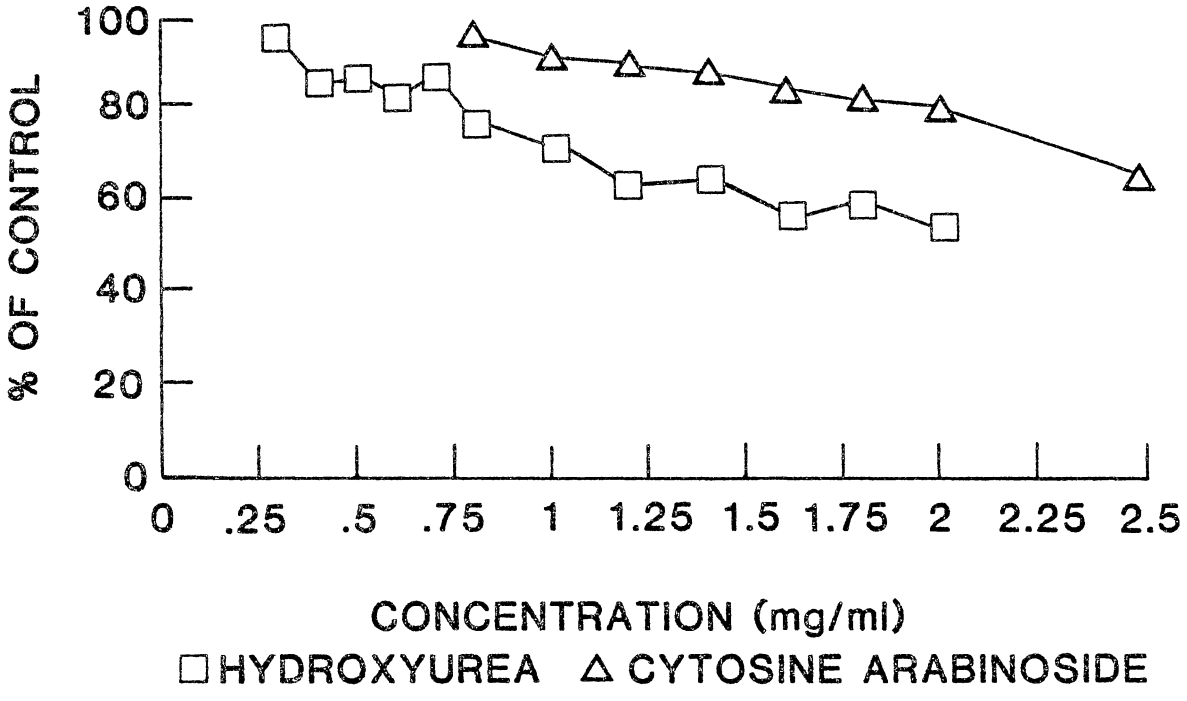

100
80
60
40
20
0
% OF CONTROL
0
.25
.5
.75
1
1.25
1.5
1.75
2
2.25
2.5
CONCENTRATION (mg/ml)
□ HYDROXYUREA △ CYTOSINE ARABINOSIDE

Fig. 7. Dose-response curve of the effect of actinomycin D on Xenopus mortality and malformation. The teratogenicity index was 0.88.

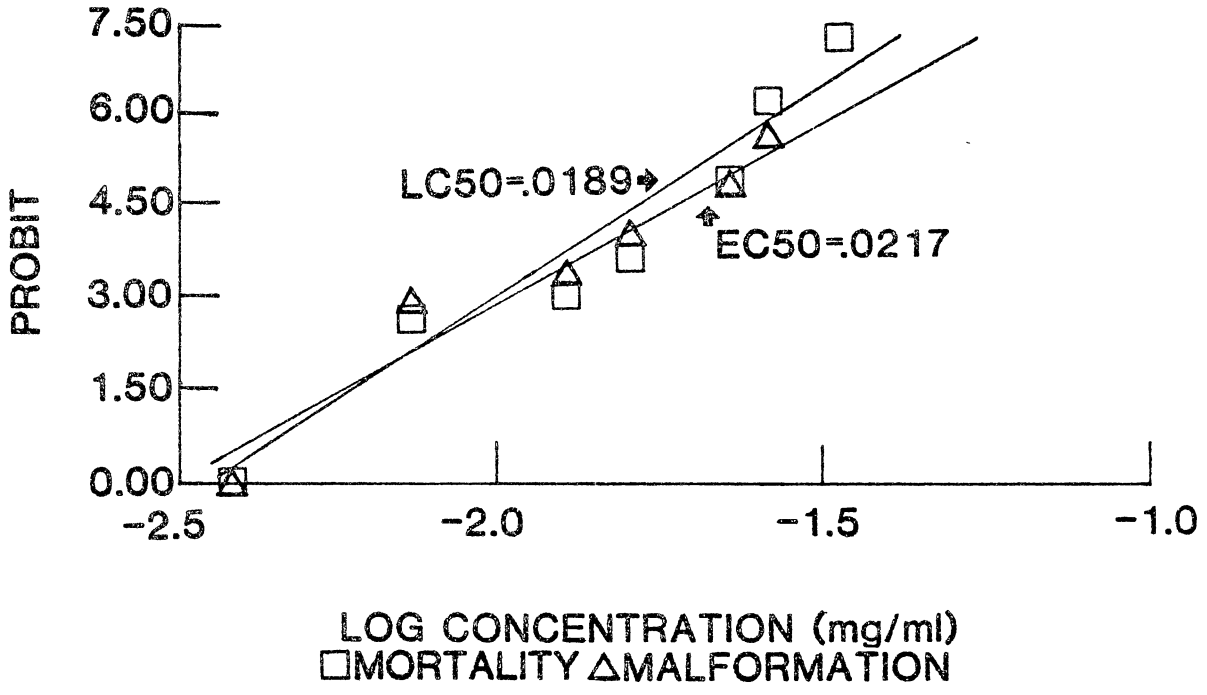

7.50
6.00
4.50
3.00
1.50
0.00
PROBIT
LC50=.0189
EC50=.0217
-2.5
-2.0
-1.5
-1.0
LOG CONCENTRATION (mg/ml)
□MORTALITY △MALFORMATION

Fig. 8. Effect of actinomycin D on growth as measured by head-tail lengths after 96 h exposure.

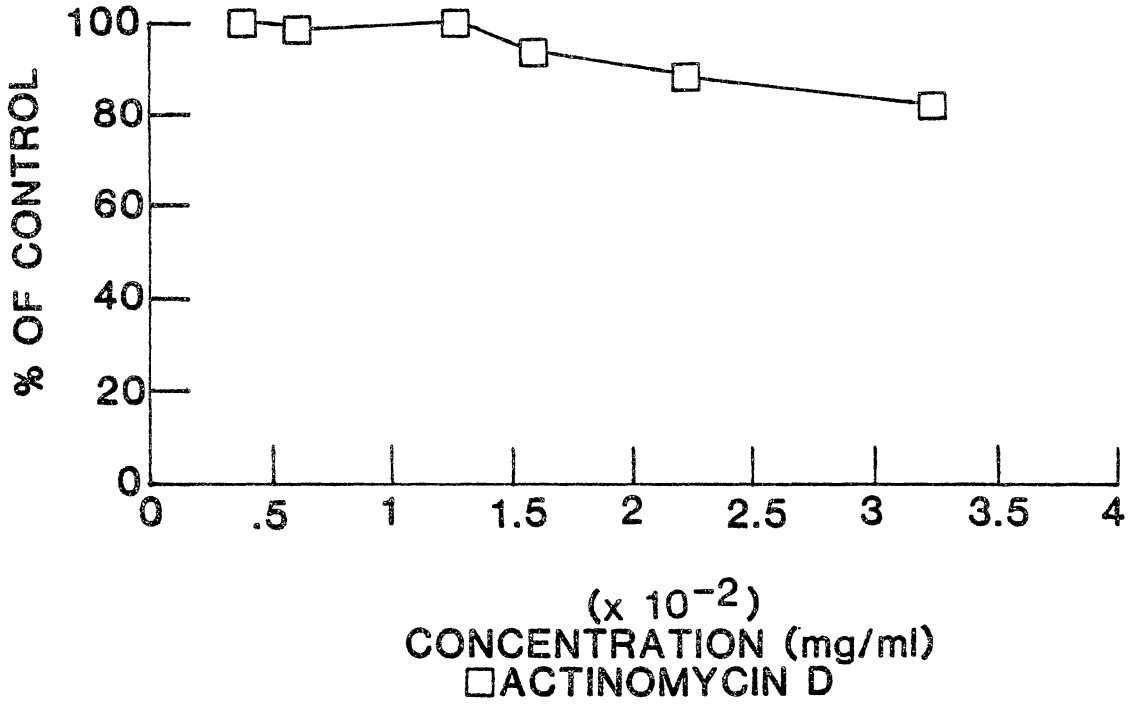

100
80
60
40
20
0
% OF CONTROL
0
.5
1
1.5
2
2.5
3
3.5
4
(x 10⁻²)
CONCENTRATION (mg/ml)
□ACTINOMYCIN D

Fig. 9. Dose-response curve of the effect of cycloheximide on Xenopus mortality and malformation. The teratogenicity index was 1.17.

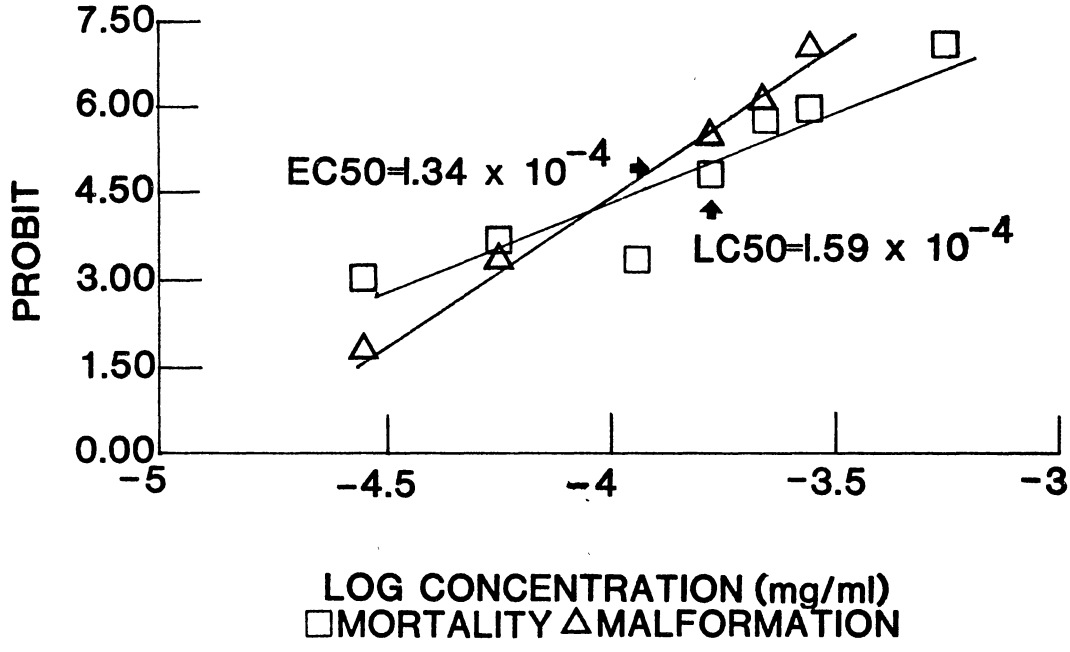
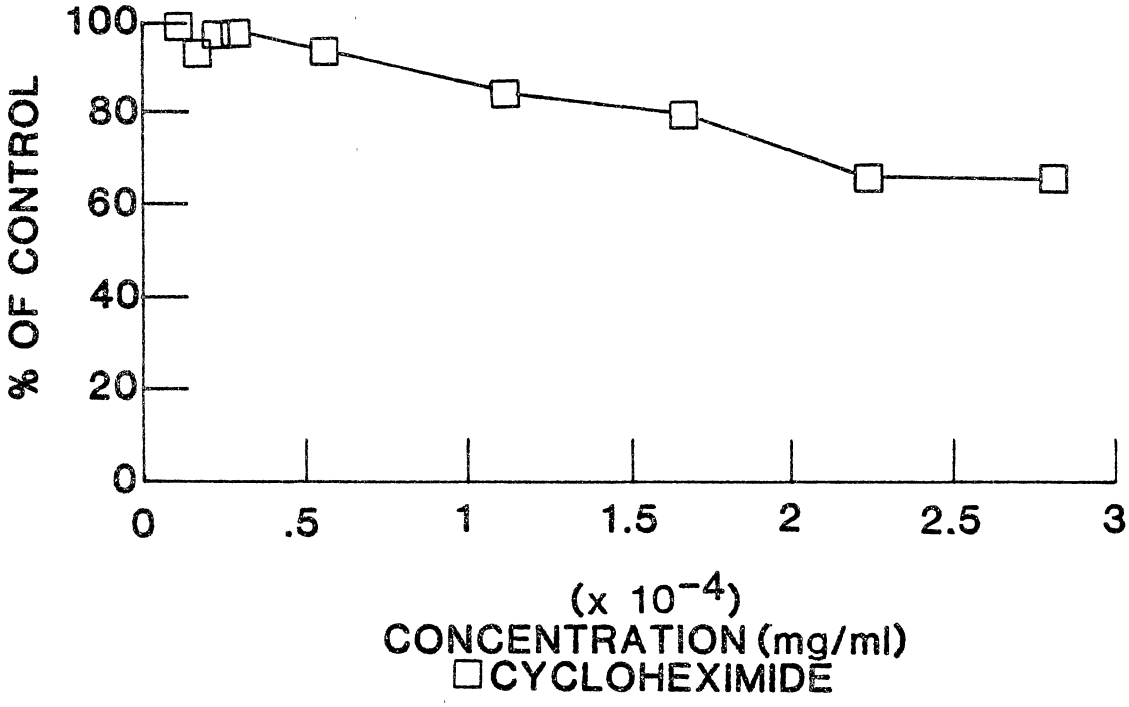

7.50
6.00
4.50
3.00
1.50
0.00
PROBIT
EC50=1.34 x 10^-4
LC50=1.59 x 10^-4
-5
-4.5
-4
-3.5
-3
LOG CONCENTRATION (mg/ml)
□MORTALITY △MALFORMATION

Fig. 10. Effect of cycloheximide on growth as measured by head-tail lengths after 96 h exposure.


100
80
60
40
20
0
% OF CONTROL
0
.5
1
1.5
2
2.5
3
(x 10⁻⁴)
CONCENTRATION (mg/ml)
□CYCLOHEXIMIDE

VITA 2

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Doctor of Philosophy

Thesis: THE USE OF THE SOUTH AFRICAN CLAWED FROG, XENOPUS LAEVIS, IN SHORT-TERM GENOTOXICITY AND TERATOGENICITY TESTING

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