DEVELOPMENT OF A CARCINOGEN ASSAY SYSTEM UTILIZING ESTUARINE FISHES

by

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FOREWORD

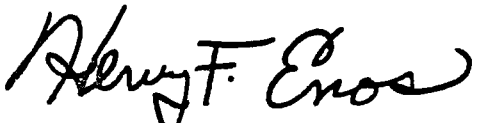
The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants requires that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The EPA Environmental Research Laboratory, Gulf Breeze, contributes to this information through research programs aimed at determining:

the effects of toxic organic pollutants on individual species and communities or organisms;

the effects of toxic organics on ecosystem processes and components;

the significance of chemical carcinogens in the estuarine and marine environments.

The development and use of select species of fishes as carcinogen assay subjects offer new tools for the assessment of carcinogenic risks in the environment. This report summarizes data on the sheepshead minnow (Cyprinodon variegatus) as such a test organism. The results concern the response to known carcinogens from subcellular to organismic levels, and thus should help provide a rational basis for use of fishes in carcinogen risk assessment.



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ABBREVIATIONS AND SYMBOLS

BaP	-- benzo(a)pyrene
BEN	-- benzidine dihydrochloride
cm	-- centimeter or centimeters
DCM	-- dichloromethane
DENA or DEN	-- diethylnitrosamine
DOC	-- dissolved organic carbon
HBSS	-- Hank's Balanced Salt Solution
HCG	-- human chorionic gonadotropin
i.p.	-- intraperitoneal
LC-50	-- concentration resulting in 50% mortality in 96 hours
M	-- molar
mg	-- milligram or milligrams
ml	-- milliliter or milliliters
MS222	-- tricaine methane sulfonate, Sigma
ng	-- nanogram or nanograms
PAS	-- periodic acid-Schiff
PFU	-- Plaque-Forming Units
ppb	-- parts per billion
ppm	-- parts per million
ppt	-- parts per thousand
µl	-- microliter or microliters
µm	-- micrometer or micrometers

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ACKNOWLEDGMENT

This report was supported by cooperative agreement (#CR 806212) in the NCI/EPA Collaborative Program, Project No. 3 "Effects of Carcinogens, Mutagens and Teratogens in Non-human Species (Aquatic Animals)" administered by the Gulf Breeze Environmental Research Laboratory.

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ABSTRACT

The overall objective of this research was the development of systems previously devised in our laboratory to assay the effects of chemical carcinogens on marine teleosts. The results include the following.

The LC-50 (96 hours) for benzidine dihydrochloride (BEN) with respect to Cyprinodon variegatus was determined to be ca. 64 ppm.

Exposure of C. variegatus to weekly contaminations of 1 ppm BEN caused some individuals to develop liver lesions at 25-29 weeks. The livers of these individuals contain large fibrotic regions within which a proliferation of various types of tubular profiles may be observed.

Efforts to accomplish long-term exposure of teleosts to BEN or diethylnitrosamine (DENA) via contaminated food met with limited success because of the toxicity of these compounds at the concentrations used. Intraperitoneal injections of these compounds also proved to be toxic over a wide range of concentrations.

Exposure of early C. variegatus embryos to BEN at various concentrations produced abnormalities at concentrations of 50 ppm and above. Anomalies in the order of frequency of occurrence were: tubed heart syndrome with distended pericardia, poor circulation, sparse distribution of melanophores around yolk, inability to hatch, abnormal head morphology, scoliosis, and faint RBC pigmentation.

Acute toxicity concentrations were established for benzo(a)pyrene (BaP), BEN and DENA with respect to a cell line from Archosargus probatocephalus (the sheepshead). Long-term exposures provided evidence that BaP and BEN have mutagenic effects on this cell line.

A dechorionation technique was developed to observe better detailed cellular and subcellular activities during early embryonic development of C. variegatus. Employment of this technique to observe inverted blastoderms provided evidence that the ectodermal cells that cover the yolk travel from the superficial blastoderm via a pathway along the blastoderm floor.

Detailed studies of the gross and histological structure of digestive tract and histological studies of the peripheral blood cells of C. variegatus were conducted.

Three novel techniques were developed to study the effects of carcinogens on C. variegatus at the cellular level: an aseptic embryo technique that provides the opportunity to study embryos in a sterile environment, and embryo-primary cell culture technique that incorporates in one system characteristics of both intact embryos and primary cell cultures, and a primary hepatocyte cell culture technique that will be employed to study the effects of carcinogens on teleost hepatocytes.

In order to study the immune system of C. variegatus, standard immunological techniques were miniaturized. Serum electrophoresis disclosed considerable variation between BEN-exposed and unexposed fish, and the presence of antibody-forming cells in spleen suspensions from C. variegatus immunized with human type 0 erythrocytes were demonstrated by a modified immune rosette procedure.

Finally, an improved method for the extraction of DENA from water was developed. This method was used to observe significant decreases in the extractability of DENA with increasing salinity. A loss of DENA by photodegradation in the presence of dissolved organic carbon was demonstrated.

SECTION 1

INTRODUCTION

In recent years, the public has become increasingly aware of the importance of environmental pollutants as cancer-causing agents. With this awareness has come the realization that a major portion of the world's cancer incidence is environmentally related and, consequently, preventable. Thus, the public sector has begun to demand more rigorous environmental safety assessment (1). Although, we have made progress in recent years in our methods for detecting chemical carcinogens and in our understanding of the basic mechanisms of neoplasia, our capability of accurately predicting the human health hazards that may result from the myriad of chemical pollutants entering the environment has not kept pace with our increasing needs in this respect.

Murine assay systems continue to be the mainstay for testing chemical carcinogens; however, there is a recognized need for alternate systems. Since existing systems are not particulary amenable to assessment of the aquatic environment, an environment that is experiencing an ever increasing quantity and a variety of potentially dangerous pollutants, it is imperative that we develop valid carcinogen test systems appropriate for this environment.

Teleost fishes are obvious candidates for this role, and there is considerable evidence for the susceptibility of teleosts to carcinogenic agents (2, 3). Thus, in a previous project, whose purpose was the study of the effects of certain carcinogenic compounds that are components of petroleum products, we developed systems for long-term exposures of Cyprinodon variegatus (the sheepshead minnow) at laboratories in inland locations (4). This species was selected because it is a common species in the Gulf Region, its biological characteristics seemed ideal, and it had already been employed extensively in toxicologic assays. This initial project demonstrated that C. variegatus was a suitable model for chronic testing and provided evidence that such a system, when fully developed, could be employed extensively for carcinogen assay.

Therefore, the principal objective of the present project was the comprehensive development of the C. variegatus assay system as a tool for the biological assessment of suspect carcinogens in the aquatic environment. An additional goal was to conduct a search for biochemical or cellular changes that might indicate the usefulness of this species as an "early warning detector" of teratogenic, mutagenic, or carcinogenic substances in the estuarine environment.

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

This project demonstrates the feasibility of employing small estuarine teleosts in laboratory assays of suspect carcinogens. The relatively low cost of maintaining such fish and their apparent short latency period (possibly only 5 to 6 months) provide an economic advantage over traditional murine bioassay systems. Experience with C. variegatus indicates that a requirement for success with such systems is that the fish must be parasite-free and healthy, and their state of health must be carefully maintained during the course of the exposures. The fish normally tolerate the stress resulting from the experimental conditions; however, any additional stress due to poor health or to less than an optimum environment, or both, may produce a sufficient amount to quickly compromise the system. Thus, feral fish brought into the laboratory must be treated for ectoparasites and be in an excellent state of health prior to their use in experiments. Furthermore, all experiments must be designed and conducted in a manner that insures the constant maintenance of a high quality environment. In fact, our experience suggests that the best approach may be to use feral fish only as breeders and to conduct all laboratory experiments with laboratory-bred individuals.

The toxicity problems encountered in this project indicate that one should always determine the LC-50 of a compound as a point of reference prior to making decisions concerning concentrations to be used in long-term chronic exposures.

Food contamination did not produce observable lesions and the i.p. injections, even over a wide range of concentrations, proved, in most cases, to be toxic; therefore, additional research will be necessary to accurately assess these modes of exposure of C. variegatus to carcinogens.

The repeatable induction of a liver lesion, though its pathological classification is currently problematic, by weekly 1 ppm contaminations of the water with BEN demonstrates the usefulness of this mode of exposure. Thus, it is recommended that this type of experiment be continued until the lesion is completely characterized, its incidence determined, and its latency period accurately established. Additionally, experiments with other known mammalian carcinogens should be conducted to provide comparative data relative to this system.

The variety of anomalies resulting from the exposure of early C. variegatus embryos to BEN indicates that this system is likely to be an effective tool in the study of carcinogenesis and teratogenesis. The

studies of the early embryonic development of normal C. variegatus provide comparative data that is needed to accomplish detailed analyses of the carcinogen induced anomalies. In addition to the continued study of these BEN-induced anomalies, it would seem logical to conduct exposures of this type with a number of other known mammalian carcinogens.

The occurrence of multilayered foci in SHF-1 cell cultures exposed to both BaP and BEN indicates that teleost cell cultures, like mammalian cell cultures, can be employed in this type test. The effectiveness of this system is enhanced by the fact that such exposures are less costly than exposures of mammalian cells.

The anatomy of the digestive tract of C. variegatus proved to be similar to other Cyprinodontid species; however, the detailed study accomplished in this project provides the background data needed to evaluate any lesions induced in the digestive tract. The studies of the morphology of peripheral blood cells provide baseline knowledge that will allow the use of hematology, a traditional method for detecting pathology, in evaluating the effects of carcinogens.

The three cellular techniques (aseptic embryo, embryo-primary cell culture, and primary hepatocyte cell culture) are all innovative techniques that possibly can be developed into useful rapid test methods for carcinogens. Because of the obvious need for development of improved rapid test procedures, and the likelihood that these techniques may have even broader applications, their continued development is recommended.

The miniaturization and adaptation of a variety of standard immunological techniques accomplished in this project provides the tools needed to conduct sophisticated studies of the immune system of C. variegatus. Thus, it is now possible to use this species in the study of the intriguing relationship between carcinogenesis and the immune system. The significant effects of low-level chronic exposure to BEN on the serum profiles of C. variegatus provides a preliminary indication that these techniques are likely to be quite valuable, therefore, it is recommended that considerable effort be devoted to studies of the immune systems of normal and carcinogen-exposed C. variegatus.

SECTION 3

MATERIALS AND METHODS

Facilities

The long-term exposures of this project were conducted in a laboratory located four miles from the main campus of the University of Southern Mississippi. The remote location and design of the facility provides safe conditions for working with carcinogens. The laboratory allows control of the ambient environment and provides for the safe handling and disposal of contaminated water. Specimens are maintained in closed-circulating systems, 950-liter cylindrical fiberglass tanks, 185-liter fiberglass-coated plywood tanks (4), or 10-gallon glass aquaria.

Specimen Care and Collection

Approximately 9600 Cyprinodon variegatus (sheepshead minnows) were utilized during the course of the study. About 86% of these specimens were seined from a marshy tidal entrance at Range Point on Santa Rosa Island near the U.S. EPA Environmental Research Laboratory, Sabine Island, Gulf Breeze, Florida. Approximately 13% were collected from a similar tidal stream on the north side and at the east end of Horn Island on the Mississippi Gulf Coast near Pascagoula, Mississippi. All collected specimens were examined for gross lesions and treated for 30 minutes with 1:4000 formalin to remove parasites. The collected fish were acclimated to 5-15 ppt artificial seawater (Rila Mix, Rila Products, Teaneck, NJ) at least 2 weeks prior to their use in experiments. About 800 of the C. variegatus were hatched and raised in the laboratory.

The 2100 Ictalurus punctatus (channel catfish) utilized were obtained as 1- to 3-inch fingerlings from local commercial fish hatcheries. These fish were also formalin-treated and maintained in the laboratory for at least 2 weeks prior to their use in experiments.

The C. variegatus were fed commercial fish flakes (TetraMin or Monolake Flakes, F.A. Martiny and Sons, New Orleans, LA). The diets of immature specimens were supplemented with live or frozen brine shrimp (Artemia). The channel catfish were fed commercial catfish food (Purina).

Benzidine Analysis

The HPLC methodology employed for the chemical analysis of BEN (benzidine dihydrochloride, Sigma, B-3383) was a modification of the techniques described by Riggins and Howard (5). Liquid chromatography on reversed phase columns was employed with detection of BEN by electrochemical oxidation (HPLC-ED) and absorption of light at 214 nm (HPLC-UV).

Toxicity Tests

The toxicity of BEN to C. variegatus was determined by using routine methods for determination of the LC-50. A ten-gallon aquarium was used for each experimental condition, and ten fish were placed in each aquarium. The fish were first acclimated and then BEN was added to provide concentrations of 16, 25, 40, 64, and 102 ppm. The experiment was repeated four times and the data were combined.

Chronic and Pulse Exposures

Stock solutions of BEN were prepared in distilled water. An appropriate amount of the stock solution was then introduced into the systems to obtain the final concentrations desired. Chemical analysis disclosed that when the systems were contaminated with 1 ppm BEN, the concentration began to drop rather rapidly after 2-3 days. It was determined that weekly contaminations at a theoretical concentration of 1 ppm would maintain a concentration of BEN that varied from a high of about 1.5 ppm to a low after 7 days of about 0.5 ppm. All pulse exposures involved a "one time" introduction of BEN into the systems.

Stock solutions of DENA (N-Nitrosodiethylamine, Sigma, N-0756) were prepared in distilled water and introduced directly into the systems.

Acetone was used as a carrier for the introduction of BaP (benzo(a)pyrene, Sigma, B-3500) into the systems because of the low water solubility of BaP. A stock solution was prepared by placing 4.35 mg of BaP in 10 ml of acetone. An appropriate amount of the stock solution was then placed in the systems to obtain the theoretical concentrations desired. Appropriate acetone controls were conducted.

Food Contamination

BEN-contaminated food was prepared by adding 1 gm BEN per 100 gm dry food. Distilled water was added and the food was thoroughly mixed. The contaminated food was stored frozen and thawed prior to feeding.

The DENA-contaminated food was prepared by mixing 1 ml of DENA per 100 gm dry food. This food was prepared and stored utilizing the same procedure as that employed for the BEN-contaminated food.

Injection Experiments

A 27-guage hypodermic needle was employed to make i.p. injections of the specimens along the ventral surface of the abdomen. The volume of the injection fluid was maintained at 50 or 100 µl to avoid producing a traumatizing pressure in the abdominal cavity. Control specimens were sham-injected with equal volumes of distilled water. BEN was injected as an aqueous solution, and DENA was injected as concentrated DENA or as an aqueous solution.

Benzidine Exposure, C. variegatus Embryos

Spawning was induced in female C. variegatus with three i.p. injections of 50 IU of HCG (human chorionic gonadotropin, Sigma) at 48-hour intervals. The experiments were conducted at 13 parts per thousand salinity at 21°C.

BEN exposures were accomplished in finger bowls (10 cm diameter) in 100 ml volumes. The required dilutions were prepared from a stock solution of 5 gm BEN per liter of 13 parts per thousand artificial sea water. Experiments have been conducted at 5, 10, 25, 50, 75, 100 and 500 ppm BEN. All experiments were conducted at 17°-19°C, and the finger bowls were covered with parafilm to reduce fluid loss due to evaporation.

SHF-1 Cell Culture Exposures

A teleost cell line from the sheepshead (Archosargus probatocephalus), designated SHF-1 (6) was employed in studies to determine its suitability for carcinogen assays. SHF-1 was grown in Leibovitz's L-15 medium supplemented 10% with fetal calf serum and 1% with L-glutamine. The cells were cultured in Falcon tissue culture flasks and subcultured weekly.

BaP, BEN, and DENA were used in initial studies. BEN and DENA were quantitated by dilution in distilled water or growth medium. A stock solution of 20% acetone in distilled water containing 0.435 mg BaP per ml of acetone was used to dissolve and quantitate the BaP. In order to determine their toxicity to SHF-1 cells, various concentrations of each compound were added to the growth medium immediately following subcultivation of the cells. A particular concentration of a compound was considered acutely toxic if the cells failed to become attached to the floor of the culture flask, or if the cells showed no growth within seven days and failed to survive the subsequent subcultivation. BaP and BEN exposures were begun at passage 18, and DENA exposures were begun at passage 33. Once acute exposure concentrations for the three compounds were determined, subsequent experiments were performed to observe the response of SHF-1 cells to repeated (one passage after another) exposure. Periodically, chromosome spreads were performed to detect any aberrations that might have occurred in either control or exposed cultures.

Early Embryogenesis, C. variegatus

Spawning was induced naturally to obtain the approximately 2500 embryos utilized in the course of this study by maintaining the breeding stock at 28°-30°C in a 12-hour light - 12-hour dark photoperiod. After collection, eggs were placed in 10 cm diameter finger bowls and maintained at 28°-30°C. Prior to microscopic observation, fertilized eggs were cleaned by rolling them on lens paper and then washing them with tap water. Observations were made both with the chorion intact and after dechorionation. Up to 5 eggs were placed in saline water in each depression of a depression slide and covered with a coverslip.

Dechorionation, though extremely difficult to accomplish, provides increased cellular resolution. Therefore, a technique was developed that allows dechorionation without causing significant damage to the embryonic and extraembryonic membranes. A number 11 scalpel blade is used to hold the egg in place. The sharpened end of a pair of Dumont forceps is then pushed through the chorion (Figure 1). The forcep tip is then rotated, pushed into the perivitelline space, and closed against the opposite tip (Figure 2). The chorion will begin to split when it is rubbed at the lateral edge of the forceps by a scalpel blade (Figure 3). This process is continued until the chorion is excised. Although the technique is quite tedious, with a reasonable amount of practice one can consistently obtain undamaged dechorionated eggs.

Gross and Histological Anatomy of the Post-Pharyngeal Digestive Tract, C. variegatus

For gross anatomical observations, the fish were pithed and abdominal incisions were made to expose the digestive organs. The entire fish was then placed in Davidson's fixative (7) for 5 minutes at 4°C. The visceral walls were cut away to allow examination of the viscera *in situ*.

For histological observations, the entire post-pharyngeal digestive tract was cut into 1 cm lengths and placed in Davidson's fixative for 24 hours, washed, dehydrated, and embedded in Paraplast. The tissue blocks were serially sectioned at ca. 6 µm and consecutive sections were stained with one of three staining techniques; routine Mayer's hematoxylin and eosin, Mallory's connective tissue stain (8), or periodic acid-Schiff (PAS) counter-stained in aqueous fast green.

Peripheral Blood Cell Morphology, C. variegatus

Blood Collection: Blood must be taken very carefully because it clots quite rapidly and excessive stress may damage or distort thrombocytes. Two methods were used to remove blood for light and electron microscopy. In the first method, samples were collected by allowing blood to flow freely into the top of a heparinized capillary tube from the aorta after severing the caudal peduncle posterior to the anal vent. Blood to be used for electron microscopy was pooled in a centrifuge tube containing a cold solution of 2%

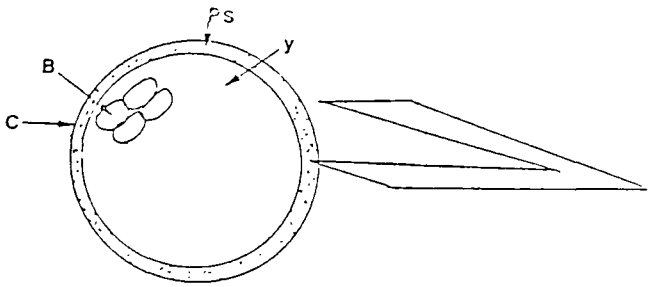


Figure 1

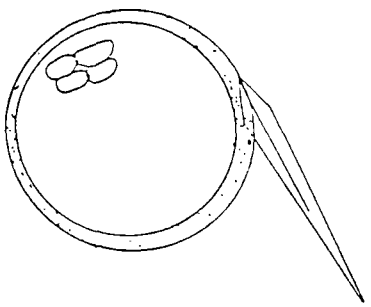


Figure 2

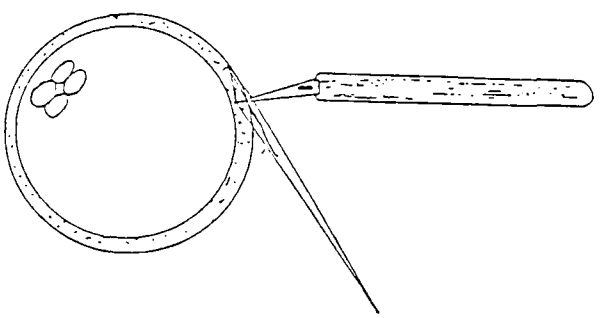


Figure 3

Figure 1. Diagramed penetration of the chorion by the tip of a pair of Dumont forceps. Note the chorion (C), perivitelline space (PS), yolk (Y), and blastomeres (B).

Figure 2. Diagramed rotation and closure of the forceps.

Figure 3. Splitting the chorion with a scalpel blade.

glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. In the second method developed by Winstead et al. (9), the caudal peduncle was severed and the live fish was placed in a centrifuge tube with the caudal end down. The fish was secured with a Kimwipe which was wrapped about the body of the fish. Masking tape was used to secure the upper portion of the Kimwipe, which extended beyond the head of the fish to the outer side of the centrifuge tube. The posterior portion of the fish hung free over a solution of 2% cacodylate buffered glutaraldehyde. The fish was centrifuged at the lowest speed on the clinical centrifuge. No anticoagulant was needed, as blood from the severed aorta dripped directly into the glutaraldehyde fixative during centrifugation.

Light Microscopy: Blood to be used for light microscopy was taken from heparinized capillary tubes and placed on slides or coverslips for viewing fresh preparations or stained smears. The blood was viewed on an Olympus Vanox light microscope, using either bright field or phase contrast microscopy.

Blood smears were made by placing a small drop of blood from a heparinized capillary tube on a #1 thickness coverslip. Another identical coverslip was placed on the original coverslip and the blood between the coverslips was allowed to spread evenly to the margins. The coverslips were then rapidly pulled apart in a manner which caused the coverslips to slide across one another in the plane of the coverslips. The smears were subsequently air dried and stained with either Wrights, Wrights-Giemsa or May-Gruenwald blood stains for two to three minutes before washing in distilled water. Care was taken not to use an excess of blood as this necessitated a longer drying time in air which allowed some breakdown of cells and caused morphological changes in thrombocytes.

Living cells were observed by placing a drop of blood on a slide, covering it with a #1 thickness coverslip, and rapidly sealing it around the edges with stopcock grease. Blood kept in this manner could be viewed at room temperature for as long as two hours and would remain viable in the refrigerator for up to eight hours. Congo red was used for *in vitro* observations of the uptake properties of the blood cells.

Mean red blood cell counts were made with an improved Neubauer's hemacytometer. A Hayem's solution was used which was made with 0.1 ml ammonium salt heparin (1000 USP units/ml) in 20 ml of Hayem's diluting solution. Both red and white blood cell counts were taken. An average of twenty counts was used for total red and white blood cell counts.

Electron Microscopy: Blood from either of the collection methods was centrifuged in a clinical centrifuge for three minutes at maximum speed. After twenty minutes in the 2% buffered glutaraldehyde solution, the blood was washed three times at ten minutes per wash in the cacodylate buffer at room temperature. The glutaraldehyde was then replaced by a 1% osmium tetroxide in cacodylate buffer. After twenty minutes in this fixative at room temperature, the tissue was washed three times at ten minutes per wash in cacodylate buffer at room temperature and dehydrated through a graded ethanol series to absolute ethanol. The pelleted blood

sample was then removed and the buffy coat was cut from the top of the pellet and sectioned into approximate 1 mm cubes. These were infiltrated with Spurr's embedding medium and placed in Beem capsules. Thin sections were cut with a diamond knife using a Sorvall Porter-Blum ultramicrotome and examined on a Siemens Elmiskop 1A transmission electron microscope at 80 Kv.

Aseptic Embryo Preparation

Egg development was induced by the HCG injection method. During the harvest of eggs, the females were sacrificed or the eggs were milked into a Petri dish containing artificial sea water by applying pressure along the lateral surfaces of the gravid female. Fertilization was accomplished by placing macerated testes into a Petri dish containing the eggs.

The fertilized eggs were maintained in Petri dishes with two daily changes of artificial sea water. On the third or fourth day, the eggs were washed with several changes of artificial sea water and, under a laminar flow hood, placed in Petri dishes containing sterile Hank's Balanced Salt Solution (HBSS) and 5% antibiotics (penicillin-streptomycin-fungizone, Gibco). The HBSS was changed three times before the eggs were transferred to a fresh Petri dish containing sterile medium. The medium consisted of 9 ml of Leibovitz's L-15 medium plus 1 ml of antibiotics. With sterile forceps or sterile wide-mouthed Pasteur pipettes, the eggs were transferred to a second Petri dish containing sterile medium, and incubated overnight at 37°C. The eggs were then placed in a fresh dish of sterile medium and incubated until all were dechorionated.

Aseptic Embryo Technique

Five to fifteen embryos prepared according to the aseptic embryo procedure were placed into 150 cm^2 plastic flasks (Corning) containing 50 ml of sterile medium. The embryos were maintained at 25°C, and monitored daily for viability. Experiments were conducted in which embryos were maintained in basal L-15 medium plus various combinations of supplements. In some experiments, the embryos were maintained in sterile artificial sea water. Pilot LD50 experiments were performed in which the medium was contaminated with various concentrations of BEN.

Embryo-Primary Cell Culture Technique

One embryo prepared according to the aseptic embryo procedure was placed in each well of a 24-well Linbro plate with a sterile wide-mouthed Pasteur pipette, and four drops of growth medium were added to each well. This low volume of medium is important to the attachment process. The developing embryos were incubated at 25°C, and observed daily until they were attached to the wells (usually 2-3 days). The growth medium consisted of 10% fetal calf serum in L-15 medium plus 0.02% antibiotics and 0.01% L-glutamine. When the embryos became securely attached, 1 ml of growth

medium was added to each well. Throughout the experimental period, the growth medium should be changed when necessary, but no more often than once every 2-3 days.

Primary Hepatocyte Cell Culture Technique

Livers were excised from 2-4 large adult C. variegatus and placed in a Petri dish containing sterile HBSS plus 5% antibiotics at 25 °C. The livers were transferred to a second Petri dish containing the same solution and a small gauge hypodermic needle was used to inject them with digestion buffer. The digestion buffer was the HBSS plus 0.05 % collagenase (Sigma, Type I). The procedure was a modification of that of Berry and Friend (10). After 5 minutes, the livers were transferred to fresh digestion buffer and minced into 2-3 mm pieces with a razor blade. The minced tissue was transferred to a digestion flask (Bellco) containing fresh digestion buffer and gently swirled for 20 minutes. Approximately 7 ml fractions were collected in centrifuge tubes containing 3 ml of tissue culture medium precooled to 4 °C. The tissue culture medium consisted of 100 ml L-15, 2.5 ml HEPES (Gibco), plus 15% fetal calf serum and 1% antibiotics at pH 7.4. Following centrifugation at 600 x g for 2-3 minutes, the resulting cellular pellet was resuspended in fresh tissue culture medium at 4 °C. The suspended cells were plated out into 25 cm^2 T-flasks which had been coated with a collagen substrate (11). A minimum of 500,000 cells per ml of medium was desired, and about 3 ml of cell suspension was placed into each flask. The cultures were maintained at 25 °C, and the cells can be expected to become attached to the substrate in about 24-48 hours.

Immunological Studies

Serum collection: Blood was obtained initially by completely severing the caudal fin, making a cut from the dorsal side to the ventral. Modifications to the procedure included the combined use of an anticoagulant followed by an anaesthetic and by a modification of the cutting procedure. Fish were exposed to 1% sodium citrate (Sigma) in water for 10 minutes. They were then exposed to 0.001% tricaine methane sulfonate (MS222) (Sigma) in water for 5 minutes. A cut was made (using a scalpel blade) on the ventral side of the caudal peduncle, but the caudal fin was not removed entirely. Rather the cut was terminated at the backbone, so that the caudal artery and vein were severed, but not the spinal cord. Blood was collected in heparinized capillary tubes, then centrifuged, and the serum separated and frozen at -20 °C pending use.

Collection of leucocytes: Two techniques were used to separate and collect leucocytes from whole blood of C. variegatus. In the simpler technique, blood was collected in heparinized capillary tubes, then centrifuged to sediment the cells. The tubes were then broken just above the cells and the buffy coat (containing the leucocytes) removed with a capillary syringe. If desired, collected leucocytes from several fish were pooled in a capillary tube and again centrifuged to reduce contamination with erythrocytes. In the second technique, 20 µl of collected blood was layered

onto 20 µl of Ficoll-Paque solution in a capillary tube and centrifuged at 400 X g for 30 minutes. Erythrocytes were sedimented at the bottom of the Ficoll-Paque layer, whereas leucocytes formed a loose layer at the interface between the serum and Ficoll-Paque layer. Cells collected by either method were placed on microscope slides, stained by Wright's stain, and examined to determine cell types present. Cell viability was determined by exposing cell suspensions to trypan blue and observing dye exclusion by viable cells.

Serum electrophoresis: Serum was collected from fish exposed to 1 ppm BEN for seven weeks and from normal control fish. Serum was electrophoresed, using the Corning Agarose Electrophoresis system which requires a total serum volume per fish of 0.6 µl. Electrophoresis was carried out for 30 minutes at a pH of 8.6, using barbital buffer. Bands were stained using amido-black 10B for 15 minutes and scanned on a Corning Densitometer at 500 nm.

Immune rosette formation: Human type 0, Rh+ cells were used as the immunogen. Cells were collected, washed three times in phosphate-buffered saline (PBS), pH 7.2 and adjusted to a 20% suspension according to the method of Ingram and Alexander (12). Fish were injected intraperitoneally with 0.1 ml of the cell suspension. Control fish received only PBS. Fish spleens were collected after 30 days and disrupted, using a conical tube cell homogenizer with a loosely fitting pestle to minimize cell rupture. Cells from the resulting pulp were resuspended in tissue culture medium (L-15, Gibco) and passed through fiber mesh to remove large masses. The resulting suspension, containing predominantly lymphocytes, was collected in a 0.4 ml microcentrifuge tube. Lymphocytes were gently sedimented by centrifugation at 10 X g and washed three times with L-15 medium. After the last wash, the cells were resuspended in 0.2 ml of a 5% suspension of human type 0, Rh+ cells This mixture was centrifuged for three minutes at 10 X g and allowed to incubate for 1 hour at room temperature or overnight at 4°C. The cell pellet was then gently resuspended in PBS and a drop added to a microscope slide. The smear was allowed to dry, and examined microscopically for the presence of rosette-forming cells, then stained with Wright's stain and re-examined.

Bacteriophage neutralization assay: The semi-micro bacteriophage neutralization assay of O'Neill (13) was employed. The neutralization titer of fish sera is defined as the amount of serum required to produce 50% inactivation of the bacteriophage (SD_{50}) (14). Two-fold serial dilutions of C. variegatus serum were prepared, using 25 µl amounts per dilution in Falcon microtest wells. MS2 phage, obtained from American Type Culture Collection and grown by the soft agar overlay method of Eisenstark (15), was diluted in R medium for phage lysates (Bacto tryptone, 1.0%; Bacto yeast extract, 0.1%; NaCl, 0.8%; $CaCl_2$, 0.02%; glucose, 4.0%) to contain approximately 150 plaque-forming units (PFU)/25 µl. Equal volumes of dilutions of serum (or non-serum controls) and phage suspensions were mixed and incubated at 25°C for 30 minutes. The dilutions were then added to 0.75 ml aliquots of R medium containing 0.8% agar (Difco) maintained at 40°C. Next, 0.05 ml of an overnight culture of E. coli K12 was added and the mixture was then transferred to overlay an R medium agar (1.5%) base in 60 mm plastic petri dishes (Falcon). After an incubation of 6 hours at 37°C,

the numbers of PFU/plate were counted.

Effects of Salinity, DOC, and Light on DENA Extractability

Extraction of DENA at trace levels from the various water matrices was accomplished by Method 607 (16) approved by the Environmental Protection Agency. The technique involves a simple extraction of a pre-cooled 25 ml water sample with 2 x 20 ml volume of cold dichloromethane (DCM). The DCM extract was collected and rinsed with 10 ml of 10% HCl. (All distilled, deionized water and HCl were previously extracted with DCM before use.) Subsequent filtration through anhydrous Na_2SO_4 removed residual water. The volume was then reduced in a Kuderna-Danish concentration apparatus until approximately 5 ml of extract remained. Further concentration was performed in a Synder microcolumn apparatus to a volume of 0.5 ml. Residual DCM was removed by addition of hexane and repeated concentration to 0.5 ml. The concentrated hexane sample was immediately analyzed by gas chromatography to minimize degradation effects during storage. Triplicate analyses were performed on all samples.

Due to the inefficient recoveries at 1 mg/l DEN by the EPA method, a revised method for extraction of 1 mg/l was developed. The method consisted of extraction of a 5 ml water sample with 3 x 10 ml volumes of hexane. Both the water samples and hexane used were kept at room temperature. The hexane extracts were combined and immediately analyzed by gas chromatography. Triplicate analyses were performed on all samples.

Gas chromatographic analysis was performed on a Perkin-Elmer Sigma-3B Gas Chromatograph equipped with a ^{63}Ni electron capture detector interfaced with a Sigma-10B Analyzer. The column used to separate DENA was the Supelco 10% SP-1000 on 100/120 Chromosorb W AW. The injector, oven, and detector temperatures were 200°C, 110°C, and 350°C, respectively. The nitrogen gas flow rate was 30 ml/minute for both the carrier and detector make-up gas. An attenuation of 3 was used.

The recovery efficiencies for both the EPA and revised methods in distilled, deionized water were determined. The water was spiked with DENA at less then 100 µg/l or 1 mg/l. Since DENA is a liquid at room temperature, the water samples were spiked using a 10 µl or 50 µl syringe. The weight delivered was calculated by using the density of DENA (0.9422). There was difficulty in obtaining 2.5 µl consistently under red light (and minimize personal exposure at the same time) for trace level spiking, so actual concentrations of DENA ranged from 55-85 µg/l.

Immediately after addition of DENA, each extraction volume was collected and taken through the corresponding extraction and clean-up procedures. Subsequently, the individual solvent extracts were analyzed by gas chromatography. Percentage recoveries of DENA partitioned in the individual extracts were calculated and compared.

The effect of salinity on DENA extractability was obtained by using various dilutions (S parts per thousand = 0.5-24) of I.A.P.S.O. Standard

seawater of known chlorinity, spiked with DENA at 1 mg/l or less than 100 µl/l. Immediate extraction by the appropriate method and gas chromatographic analysis were carried out as described above. Percentage recoveries of DENA at different salinities were then compared to recovery from distilled, deionized water.

To determine the possible effects of DOC (dissolved organic carbon) on DENA extractability, serial dilutions (1-22 mg/l) of humic acid (Aldrich Chemical Company) were made in standard seawater with a salinity of 10 parts per thousand. [Humic acid is 50% elemental carbon (17), thus DOC was 0.5-11 mg/l.] Each water sample was spiked with DENA at trace levels and 1 mg/l followed by immediate extraction and gas chromatographic analysis as before. The percentage recoveries of DENA from these water matrices were compared to recovery from standard seawater.

The loss of DENA due to photodegradation was monitored in brackish water (DOC = 6 mg/l, S parts per thousand = 10). Water samples spiked with DENA at 1 mg/l were irradiated by two fluorescent lights (General Electric, F04CW, Cool White, 40 watts each) at room temperature. The photoperiod was 12 hr/day for one week. In addition, blanks and control samples constantly kept in the dark were monitored to determine the direct effect of light and DOC on DENA loss. All experimental conditions were reproduced in triplicate, except for the blanks where only one sample was monitored. Each water sample was contained in a screw cap Erlenmeyer flask to eliminate water loss and for safety reasons. To reduce liquid waste, the total volume of each water sample was kept to a minimum so that little or no sample was left after the one-week period of extractions. Extraction of the water samples and immediate gas chromatographic analysis were performed every other day, as described above.

An attempt was made to extrapolate the laboratory data to the natural environment at trace levels of DENA and to chronic fish exposure experiments where high concentrations of DENA were used. A surface water sample was taken from a local stream, Crane Creek, near its entrance to the Indian River (Melbourne, Florida). The entire water sample was suction-filtered through a 0.45 µm Millipore glass fiber filter to remove particulates prior to the addition of trace levels of DENA and subsequent extraction. Salinity was determined by optical refractometry. The percentage recovery of DENA was compared to previous laboratory data.

A control sample from a chronic exposure study was taken from an aquarium in which a population of C. variegatus had been maintained for two months. This artificial saltwater sample was a mixture of dechlorinated tapwater and Rila salts. No DENA had been added to this water at any time. The aliquots of the water sample were spiked to 1 mg DENA/l, extracted, and analyzed by gas chromatography. There was no filtration prior to DENA addition as performed on the above creek water sample. Both the experimental samples and control water blanks were carried out in triplicate. DENA recovery was compared to the recovery of DENA in standard seawater.

SECTION 4

RESULTS AND DISCUSSION

Toxicity Tests

Prior to the conduction of long-term exposures in water contaminated chronically with BEN, the LC-50 (96 hrs) was determined for BEN with respect to C. variegatus. Based on these tests, the LC-50 for BEN was determined to be ca. 60 ppm (Table 1). The data are the cumulative results of four experiments, and the numbers represent cumulative mortality at the times indicated.

TABLE 1. The toxicity of BEN with respect to C. variegatus.

	6HR	12HR	24HR	48HR	72HR	96HR	TOTAL ORGANISMS	MORTALITY PERCENTAGE
102 PPM		4	7	27	36	40	40	100
64 PPM			1	8	13	21	40	53
40 PPM					2	7	40	18
25 PPM					1	3	40	8
16 PPM						6	40	15
CONTROL					1	2	40	5

Benzidine Exposures, C. variegatus

Twelve experiments were conducted in which approximately 400 C. variegatus were maintained in water contaminated weekly with BEN at 1 ppm. Five of these experiments were terminated after brief periods of time because of mechanical failures, disease, or other problems believed to be unrelated to the BEN contamination. In the remaining experiments, exposures were conducted for periods ranging from 8 to 11 months. A relatively high mortality occurred in some of these experiments, such that the total number of individuals surviving to the termination of the experiment was somewhat reduced.

In one of the more successful experiments, in which the fish were exposed for approximately eleven months, four individuals developed histopathologically similar lesions within a one-month period.

Nine individuals were sacrificed and examined histologically during the first 22 weeks of the exposure and no unusual lesions were observed. However, at 25 weeks, one individual became moribund, was sacrificed, and a liver lesion was discovered. During the next 4 weeks of this experiment, 3 of the remaining 20 fish developed what appeared to be the same lesion. The experiment was eventually terminated after 18 more weeks when only one individual remained. Thirteen fish died during this period and were so deteriorated that they could not be observed histologically. Four individuals were sacrificed and observed histologically, but none had developed the lesion.

The livers from the fish with the lesion contained large regions in which the typical parenchyma had been displaced by regions of randomly arranged collagenous tissue within which there were numerous zones that contained a proliferation of different sized and types of tubules (Figures 4 through 7). The time of occurrence of these lesions suggested a 5 to 6 month latency period. We are in the process of repeating these exposures and have already reproduced the lesion in a considerable number of BEN-exposed fish.

Three experiments were attempted in which C. variegatus were exposed to weekly contaminations at 10 ppm; however, the fish did not survive beyond 24 to 30 days. Thus, this level of exposure, although significantly less than the LC-50 for BEN with respect to C. variegatus, seems to produce a toxic effect. Even a 5 ppm chronic BEN exposure seemed to be toxic since in two experiments in which the weekly contamination was reduced to 5 ppm, most of the fish did not survive beyond two months.

Although the basic chemical parameters normally used to indicate water quality remained within acceptable ranges during our chronic exposures, our concern about the mortality occurring during the exposures caused us to install bottom filters (oyster shell) in most of the exposure systems within the last few months. This change seems to have brought about a significant reduction in mortality. In fact, in 4 experiments involving over 75 fish in which these bottom filters have been installed, no deaths have occurred in over 4 months.

Pulse Benzidine Exposures, C. variegatus

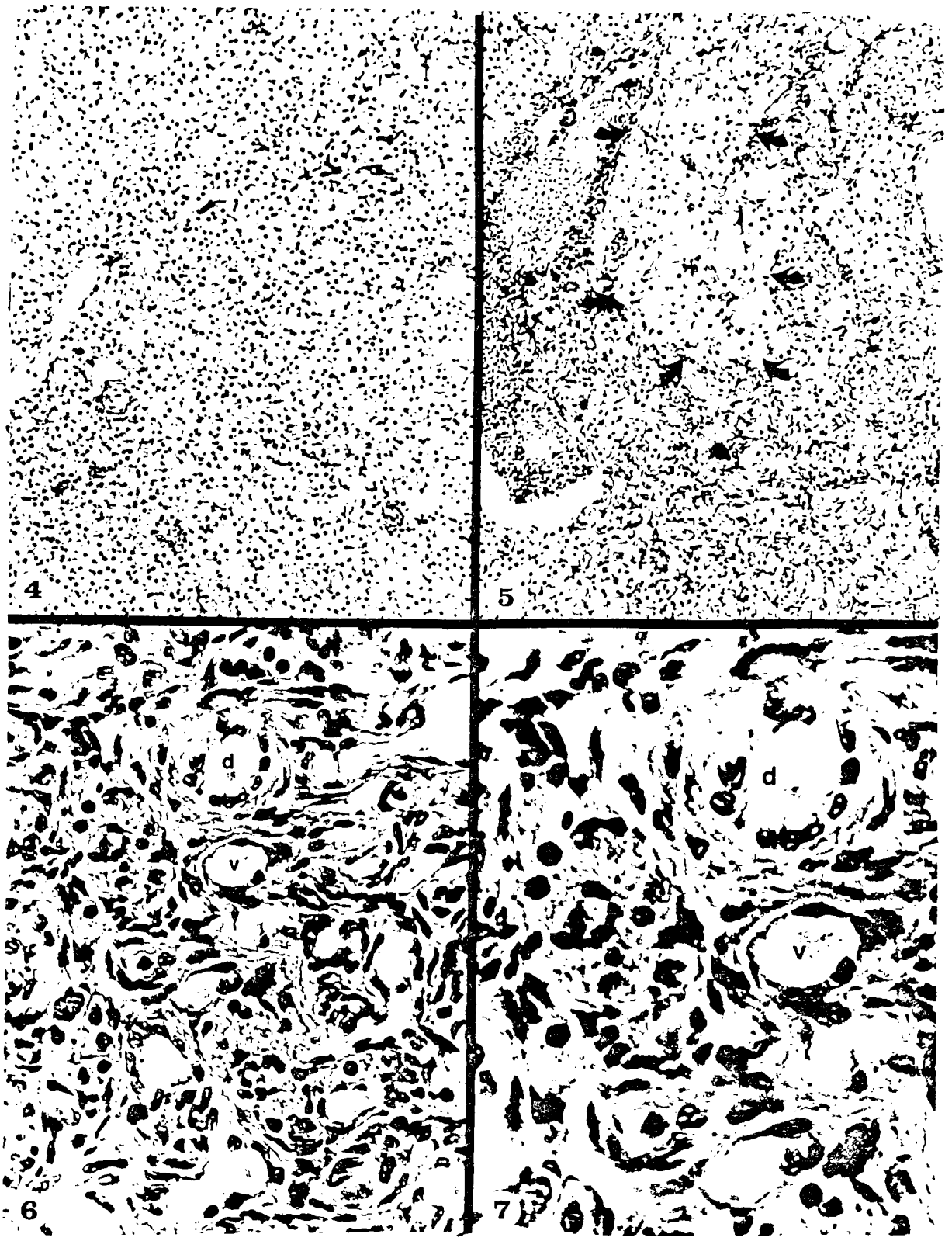
A pulse (one time) exposure of 27 C. variegatus to 50 ppm BEN resulted in 70% mortality in 5 days. A 40 ppm concentration of exposure seemed less toxic, since the exposure of 30 C. variegatus at this concentration did not begin to cause deaths until about ten days. However, the deaths continued until 50% had died by 60 days. Six of the remaining individuals survived the exposure and were alive and appeared to be in good health over one year after the exposure date. Similar results were obtained when C. variegatus were pulse-exposed at 25 ppm and 11.5 ppm. In each of these experiments, over 50% of the fish had died by 4 months, while some individuals were maintained for 7 to 12 months with no apparent ill effects.

Figure 4. Typical morphology of the livers of non-exposed control fish. Mag. 180X.

Figure 5. A region of transition from normal liver tissue to the BEN-induced fibrous lesions. Arrows outline an extension of normal tissue down into the area of the lesion. Mag. 180X.

Figure 6. High magnification of the lesion illustrating its fibrous nature with numerous tubular profiles. Some of these profiles have a duct-like morphology (d) and others are more vascular (v) in appearance. Mag. 660X.

Figure 7. A higher magnification of an area from Figure 6 demonstrating the characteristic random arrangement of fibroblasts and collagen fibers. Note the duct cell morphology of the cells of the duct-like (d) profiles and the endothelial morphology of cells forming the vascular type profiles (v). Mag. 1056X.


4
5
d
v
6
d
v
7

Diethylnitrosamine Exposures

Weekly contaminations at 100 ppm DENA proved toxic to I. punctatus, and all 15 of the fish in the experiment were dead by 14 weeks. Exposure of C. variegatus at 20 ppm DENA was quite toxic and all 21 fish were dead by 8 days. Two experiments were then conducted in which C. variegatus were exposed to 10 ppm DENA. In one of these experiments involving 15 individuals, only 13% were alive at 40 days and similarly in the other experiment involving 75 C. variegatus, only ca. 15% were alive at 60 days. When the concentration of exposure was reduced to 1.2 ppm in an exposure of 75 C. variegatus, 50% were dead by 4 months and the remaining individuals had died by 6 months. Thus, DENA at these concentrations was toxic to both of these species. Because of this factor, further attempts to conduct long-term exposure experiments with DENA were terminated.

Benzo(a)pyrene Exposures

These experiments were conducted early in the project, according to methods previously published (4). In one experiment, 65 C. variegatus were maintained in a system that was contaminated weekly with BaP at a concentration of 50 ppb. The fish died rather continuously throughout the course of the experiment due to unknown causes, and when the experiment was terminated after 8 months, only 7 individuals remained. No tumors or other lesions were observed when these specimens were necropsied. In another experiment, 20 I. punctatus were exposed to weekly contaminations of BaP at 1 ppb. After one year, this experiment was terminated and all 20 specimens were necropsied and no lesions were observed. While these experiments were in progress, it became obvious that, because of the low solubility of BaP in sea water and its tendency to absorb to solid surfaces (18), it was not possible to conduct valid experiments in this manner. No further attempts were made to expose fish to BaP.

Benzidine Feeding Experiments

In three experiments involving ca. 125 C. variegatus, no lesions were observed that could be associated with BEN contamination of the food. The mortality seemed to be higher in these experiments than in control fish or fish maintained in BEN-contaminated water. Thus, BEN contamination of the food seemed to provide a stress factor. It is interesting in this respect that in BEN feeding experiments which have been in progress for over 3 months using the systems with oyster shell bottom filters, no deaths have occurred. Therefore, it seems likely that the bottom filters may provide a less stressful environment in which this type of experiment could be conducted with increased probability of success.

Three experiments involving a total of 105 fish were conducted in which I. punctatus were fed BEN-contaminated food. One of these experiments involving 30 fish was terminated at 12 days when a mechanical malfunction resulted in the death of the fish due to oxygen deficiency. In the two remaining experiments, the fish began to die in significant numbers during

the first month, and none of them survived beyond the second month. It was concluded that this feeding regime was toxic to I. punctatus, and no further experiments of this type were attempted with the species.

Diethylnitrosamine Feeding Experiments

In an experiment in which 20 C. variegatus were fed DENA-contaminated food, 50% died within 1 month, and only 25% were alive at 6 months when the experiment was terminated. However, when I. punctatus were fed this same regime, the fish exhibited no ill effects and remained in a good state of health for one year.

Benzidine Injection Experiments

In an experiment in which 8 I. punctatus were injected with 2.5% BEN, initially none of the fish displayed any ill effects from the injection. They continued to feed and looked well for about two weeks; however, at this point feeding stopped and on day 16, three individuals died and the remaining fish were moribund at the bottom of the aquarium.

In a similar experiment in which 7 C. variegatus were injected with 2.5% BEN, 6 individuals died within 24 hours. When the concentration of the injection fluid was reduced to 1% BEN and 15 C. variegatus were injected, 3 individuals were dead at 48 hours, and 4 more died at 10 days. However, in this experiment the surviving fish remained healthy for 12 months, at which time the experiment was terminated. Necropsies of these fish disclosed no lesions.

Diethylnitrosamine Injection Experiments

When 50 I. punctatus were injected with DENA, 18 died within 24 hours and all were dead by 48 hours postinjection. In a subsequent experiment, the concentration of DENA was reduced to 10% DENA in distilled water and 10 I. punctatus were injected. In this experiment, the first deaths occurred in 2 days and continued to occur until all were dead by 60 days. Thus, DENA appeared to be toxic to I. punctatus, even at a concentration of 10%. An experiment was attempted in which 85 I. punctatus were injected with 1% DENA. At this concentration, no toxic effects were observed and there were no deaths for one month; however, at this point, all the fish were killed due to accidental chlorine poisioning.

Injection of C. variegatus with 20% DENA proved to be toxic, resulting in the death of all 18 individuals within 24 hours. Ten percent DENA produced essentially the same effect. Five percent DENA was less toxic, but still resulted in 50% mortality within three months. Attempts to inject C. variegatus with 1% DENA produced essentially the same effect.

Benzidine Exposures, C. variegatus Embryos

Exposures of early C. variegatus embryos to BEN conducted at concentractions ranging 5 - 500 ppm produced abnormalities only at BEN concentrations of 50 ppm and higher. Experiments are in progress to establish the effective dose (ED-50), and initial results suggest that this value is likely to be ca. 35 ppm. During this project, about 600 C. variegatus early embryos have been exposed to BEN at 50 ppm or higher. Only about 13% of these embryos developed normally beyond the hatching stage. Thus, about 85% of the embryos exhibited some type of developmental anomaly, and only 13% of the 600 were capable of development beyond the hatching stage.

When these data are compared to the results of the control experiments in which 70% of the 300 embryos observed developed normally beyond the hatching stage, the detrimental effects of exposure to BEN at these concentrations are quite obvious.

Anomalies that were observed in embryos exposed to BEN at 50 ppm or higher were: 1) Tubed heart syndrome with distended pericardia, 2) Poor circulation, 3) Sparse distribution of melanophores around yolk, 4) Inability to hatch, 5) Abnormal head morphology, 6) Scoliosis, and 7) Faint RBC pigmentation.

Anomalies 1 through 4 occurred most frequently and anomalies 2, 3, and 4 occurred only if embryos were exposed after somite and lense development. Interestingly, some of the exposed embryos survived for up to 30 days, and yet, did not hatch. Histologic examination of the anomalous embryos is in progress, and scanning electron microscopic examination of the surface of the chorion is being attempted. Efforts are also in progress to quantitate the penetration of the chorion by BEN and determine its distribution in the developing embryonic tissues by use of radioactively labeled BEN and autoradiographic techniques.

SHF-1 Cell Culture Exposures

Benzo(a)pyrene, benzidine, and diethylnitrosamine were all found to be acutely toxic to SHF-1 cells. BaP was acutely toxic at 2.0 µg per ml of growth medium (7.9×10^{-6}M), BEN at 0.1-0.2 mg per ml (4×10^{-4} to 7.9×10^{-4}M), and DENA at 2.0 mg per ml (7.9×10^{-3}M). In cells exposed to subacute concentrations, toxicity was evidenced by cell vacuolization and a general stress response of SHF-1 cells. BaP, even at low concentrations, produced this response. SHF-1 cells were chronically exposed to subacute concentrations of each compound through as many as five subcultivations. After subcultivation, the time required for the cells to become confluent gradually increased with each pass. Foci of multilayered cells (approximately 1 mm in diameter) exhibiting a lack of contact inhibition were observed in several cell cultures exposed to low concentrations of BaP and BEN.

Exposure to BaP in the amounts of 100 ng per ml (4×10^{-7}M), 50 ng per ml (2×10^{-7}M), 40 ng per ml (1.6×10^{-7}M), 30 ng per ml (1.2×10^{-7}M), and 20 ng per ml (7.9×10^{-8}M) were carried through three passages (28-30)

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Exposure to BaP in the amounts of 100 ng per ml (4×10^{-7}M), 50 ng per ml (2×10^{-7}M), 40 ng per ml (1.6×10^{-7}M), 30 ng per ml (1.2×10^{-7}M), and 20 ng per ml (7.9×10^{-8}M) were carried through three passages (28-30)

TABLE 2. Exposure of SHF-1 cells to benzo(a)pyrene.

REPEATED EXPOSURE					EXPOSURE FOR P-28 ONLY				
P-28		P-29		P-30	P-28		P-29		P-30
100 ng[2]	--	100 ng*[1]	--	100 ng* 100 ng* 100 ng*	100 ng	--	0 ng	--	0 ng* 0 ng* 0 ng*
		100 ng*	--	100 ng* 100 ng* 100 ng*			0 ng	--	0 ng* 0 ng* 0 ng*
		100 ng*	--	100 ng* 100 ng* 100 ng*			0 ng	--	0 ng* 0 ng* 0 ng*
50 ng	--	50 ng	--	50 ng* 50 ng* 50 ng*	50 ng	--	0 ng	--	0 ng* 0 ng* 0 ng*
		50 ng	--	50 ng* 50 ng* 50 ng*			0 ng	--	0 ng* 0 ng* 0 ng*
		50 ng	--	50 ng* 50 ng* 50 ng*			0 ng	--	0 ng* 0 ng* 0 ng*
40 ng	--	40 ng*	--	40 ng* 40 ng* 40 ng*	40 ng	--	0 ng*	--	0 ng 0 ng 0 ng
		40 ng*	--	40 ng* 40 ng* 40 ng*			0 ng*	--	0 ng 0 ng 0 ng
		40 ng*	--	40 ng* 40 ng* 40 ng*			0 ng*	--	0 ng 0 ng 0 ng
30 ng	--	30 ng	--	30 ng 30 ng 30 ng	0 ng	--	0 ng	--	0 ng 0 ng 0 ng
		30 ng	--	30 ng 30 ng 30 ng			0 ng	--	0 ng o ng 0 ng
		30 ng	--	30 ng 30 ng 30 ng			0 ng	--	0 ng 0 ng 0 ng
20 ng	--	20 ng	--	20 ng* 20 ng* 20 ng*	0 ng	--	0 ng	--	0 ng* 0 ng* 0 ng*
		20 ng	--	20 ng* 20 ng* 20 ng*			0 ng	--	0 ng* 0 ng* 0 ng*
		20 ng	--	20 ng* 20 ng* 20 ng*			0 ng	--	0 ng* 0 ng* 0 ng*

1* Indicates foci of multilayered cells appeared in the culture.

2 Concentrations given are ng per ml of growth medium in tissue culture flasks.

(Table 2). During passage 28, the cells appeared unaffected and were subcultured within the normal seven day period. In passage 28, exposure was continued in some cultures and discontinued in others. During passage 29, multilayered foci appeared in a few cultures: those exposed to 100 ηg BaP per ml in both passages 28 and 29, those exposed to 40 ηg BaP per ml in both passages 28 and 29, and those exposed to 40 ηg per ml in passage 28 only. Passage 29 reached confluency in the normal amount of time (7 days). In passage 30, multilayered foci appeared in essentially all cultures except those exposed to 40 ηg BaP per ml and 20 ηg BaP per ml in passage 28 only. All of the experimental cultures of passage 30 failed to reach confluency.

Cultures at passage 30 were exposed to 0.6 μg BEN per ml (2.4×10^{-6}M), 0.5 μg BEN per ml (2×10^{-6}M), 0.4 μg BEN per ml (1.6×10^{-6}M), 0.3 μg BEN per ml (1.2×10^{-6}M), 0.2 μg BEN per ml (7.9×10^{-7}M), and 0.1 μg BEN per ml (4×10^{-7}M). These cells were subcultured through passages 31 and 32, using the same scheme as outlined for BaP exposures in Table 2. Multilayered foci appeared only in the cultures of passage 32 which had been exposed to 0.4 μg BEN per ml in all three passages (30, 31, and 32).

Foci of multilayered cells have not been observed in any DENA exposures, and have not appeared with BaP or BEN exposures after passage 33.

In any cellular assay system, certain criteria or events indicate a "transformation" of cells to a neoplastic state. Transformation is defined in terms of hereditary morphological changes, accelerated growth rate, abnormal karyotypic shifts, and tumor promotion in vivo (19, 20). Thus far, not all of the above listed criteria for transformation have been observed in SHF-1 cells exposed to benzo(a)pyrene, benzidine, and diethylnitrosamine. However, the occurrence of multilayered cell foci in cultures with an initial exposure to BaP, but no exposure in two subsequent passages, suggests a mutagenic effect. The explanation for the appearance of multilayered foci in cultures before passage 33, but not after, is not apparent at present. It is possible that the sensitivity of the cells may undergo variations throughout the life of the cell line.

Early Embryogenesis, C. variegatus

A search of the literature revealed that knowledge of the early embryogenesis of C. variegatus did not exist in the cellular detail needed as baseline information for our planned studies concerning teratogenesis. Consequently, a study of early embryonic development of C. variegatus was conducted (21).

Because of the nature of the chorion, it was necessary to develop a technique for observation of excised blastoderms in order to observe the details of cellular and subcellular activities that occur during embryogenesis.

The following are observations resulting from the use of this technique that extend our knowledge of teleost embryology.

Fertilization: The mechanism or mechanisms that initiate the polar concentration of protoplasm has been in question for many years (22). In the current study, this concentration of protoplasm was never observed in an egg in which subsequent cellular cleavage did not occur. Therefore, it appears that the penetration of the micropyle by a spermatozoon is at least partially responsible for initiating this phenomenon in C. variegatus.

Blastulation: Wedge-like structures were observed on two adjacent cells of the four-cell stage. These wedges likely function in the rapid elongation of the cells in one plane, causing the eight-cell stage to be oval in shape. The wedges are no longer apparent by the rounded thirty-two-cell stage.

Gastrulation: Early in epiboly, superficial blastoderm cells move onto the yolk, establish a leading edge which surrounds the yolk, and eventually the entire yolk becomes covered by superficial ectodermal cells of the blastoderm. The exact source of these cells has been a long-standing question in teleost embryology. The technique of excision and inversion of blastoderms used in this study made possible observations that provide evidence that the ectodermal cells covering the yolk come from the superficial blastoderm. Observation of an inverted blastoderm reveals a channel passing along the floor of the blastoderm providing a cellular pathway along the blastoderm floor leading to its edge. Our observations indicate that somatic ectoderm, endoderm, and mesoderm cells likely pass out of this channel and along the pathway mentioned. This pathway leads to the edge of the blastoderm where a divergence of its walls occur. Observations made over a period of time clearly revealed individual cells moving along this route to the edge of the blastoderm. These gastrular activities strongly support the hypothesis of Ballard (23), i.e., an outward movement of deep internal blastodermal cells.

Scanning electron microscope observation of intact eggs at this stage reveals numerous pores on the floor of the subgerminal cavity. These pores seem to lead to the yolk proper and may function in the conduction of nutrients to the blastoderm.

Attempts were made to study the earlier stages of neurulation with this procedure; however, pigmentation and growth begins to obscure the fine details of development from this point to hatching, and it appears that it will be necessary to prepare serial histological sections to study the details of histogenesis and organogenesis of C. variegatus.

Gross and Histological Anatomy of the Post-Pharynageal Digestive Tract, C. variegatus

This study was accomplished because a review of the literature indicated that a histological study of the structure of the normal digestive tract of C. variegatus adequate for our needs had not been accomplished. Although

the histology of the digestive tract of some species of cyprinodontid fishes had been studied, it was felt that a thorough study of the digestive tract of this particular species was needed as baseline data, since C. variegatus was being employed extensively in our long-term exposures (24).

Gross Anatomy:

The digestive tract of C. variegatus is similar to other cyprinodontid fishes grossly, being composed of an esophagus, intestine and rectum. The RLG (relative length of the gut) of this species is 2.8, which classifies it as an onmivore; according to the method of Al-Hussaini (25).

Histological Anatomy:

Esophagus -- The esophagus can be divided into three distinct histological regions; the anterior, middle, and posterior regions. All of the regions have a mucosa, submucosa, muscularis, and serosa.

The esophageal mucosa is composed of an epithelium, a basement membrane, and a stratum granulosum. The mucosa is thrown into prominent longitudinal folds which run the length of the esophagus. In the anterior esophagus, the folds are broad and flattened at their apices. The cross-sectional area of the lumen in the anterior esophagus is fairly small. In the middle esophagus, the mucosal folds are continuations of those folds of the anterior esophagus. The area of the lumen is slightly greater in this region. Posteriorly, the esophageal lumen reaches its maximum diameter as the longitudinal folds become thinner and the thickness of the mucosa decreases. The longitudinal arrangement of esophageal mucosal folds allows for optimum distensibility when large food items are encountered.

The mucosal folds of teleostean esophagi are typically covered by a stratified epithelium and mucus-secreting cells. The stratified cells are polyhedrally shaped and are 10-12 cells deep at the apices of the folds. The width of the mucosa thins to 2-3 cells at the bases of the folds. The epithelial cells measure 10 µm across. Each nucleus contains at least one darkly staining nucleolus and a thin chromatin network when stained with hematoxylin and eosin. Saccular mucus-secreting cells are present in all three regions of the esophagus. Most are on the luminal boundaries of the folds, but some are seen deeper in the mucosal lining. Mucous cells are present in the anterior esophagus, but are more numerous in the middle and posterior regions. The mucous cells measure 10 µm deep by 9 µm wide and are more numerous on the sides and at the bases of the folds. The apices of the mucous cells are covered by a single layer of squamous cells. In the posterior region of the esophagus, the stratified polyhedral cells are gradually replaced by simple columnar epithelium. Saccular mucus-secreting cells are still present and are numerous. The columnar cells that have replaced the polyhedral cells are slightly shorter than those lining the intestine, but are otherwise identical. They begin replacing the polyhedral cells on the sides of the folds, covering increasingly more of the mucosal folds posteriorly.

A stratum granulosum is present in the esophagus. It is composed of a more or less, continuous layer of granular cells that lies adjacent to the basement membrane. The stratum granulosum is seen as a band of darkly staining cells at the base of the epithelium.

The tunica (lamina) propria is a layer of fine connective tissue fibers that lies beneath the basement membrane of the mucosa and extends into the cores of the mucosal folds. A true tunica propria is absent from the esophagus of C. variegatus. The cores of the folds are formed by striated muscle. As there is no muscularis present in fish (26), the boundary between the tunica propria and the submucosa is indistinct.

The most obvious component of the esophageal submucosa is the irregularly arranged striated muscle that forms the cores of the mucosal folds. Copious amounts of adipose tissue are present in this area in the anterior esophagus. No blood vessels can be observed in the submucosa, although occasional red blood cells are seen.

The muscularis of the esophagus is composed solely of an unusually thick layer of circularly arranged striated muscle. Longitudinally arranged muscle underlying this circular layer is considered a part of the submucosa. At the junction of the esophagus and intestine, the muscularis triples in thickness, while internal muscle fibers are no longer seen. Granulocytes are commonly seen among the fibers of the muscularis.

The anterior esophagus is found in the cephalic region of C. variegatus and is bound to adjacent connective tissue by fibrous connective tissue. The serosa is continuous with the parietal peritoneum and is applied to the esophagus as it enters the visceral cavity. The serosa is composed of a layer of simple squamous cells that are darkly pigmented. Pigmentation disappears at the junction of the esophagus and intestine. Very little subserosal connective tissue is present.

Eosinophilic granular cells are numerous in the esophagus, especially in the area of the mucosa. At times, the granulocytes appear to have passed through the epithelium into the esophageal lumen. The granular cells form a stratum granulosum 2-3 cells thick adjacent to the basement membrane. Granulocytes measure 3 μm in diameter and contain obvious eosinophilic granules. Similar granulocytes may be observed throughout the digestive system and its accessory organs.

Intestine and Rectum -- The anatomy of the remainder of the digestive tract of C. variegatus, the intestinal swelling, intestine proper, and rectum, is basically the same histologically. Each region is composed of four layers: mucosa, submucosa, muscularis, and serosa.

The intestinal mucosa is composed of an epithelium of columnar absorptive cells, a basement membrane, a stratum granulosum, and a tunica propria. Neither a stratum compactum nor a muscularis mucosae is present. Secondary folding of the mucosal folds increases the absorptive surface area. In the intestinal swelling, mucosal folds are long and narrow. Secondary folds are obvious along the sides of the primary folds. Folds are broader and shorter

in the intestine proper, and near the rectum, the intestinal mucosal folds shorten and secondary folding is reduced. The columnar absorptive cells of the intestinal swelling and intestine proper have essentially the same structure and size, measuring 24 µm by 4 µm. Columnar cell nuclei average 6 µm by 4 µm. One to two (rarely three) nucleoli are present. Hematoxylin and eosin or Mallory's connective tissue stain reveals fine strands of chromatin in the columnar cell nuclei. Nuclei near the bases of the folds are sometimes compressed to 8 µm by 2 µm. Nuclei at the tips of the mucosal folds are mainly spherical and 4-5 µm in diameter.

The second type of cell common to the intestinal epithelium of C. variegatus is a mucus-secreting goblet-type cell. Goblet cells are generally most numerous at the bases of the mucosal folds, but do occur on the sides and at the tips. The "goblets" stain darkly with PAS. Also, PAS positive mucus may be seen in the lumen adjacent to the goblet cells. The dimensions of the "goblet" portion of the goblet cells average 12 µm by 8 µm. The nuclei of goblet cells are seen near the basement membrane below each "goblet". Often the enlarged part of a goblet cell is located deep in the epithelium and connects to the lumen by a duct 10-15 µm long.

The basement membrane is conspicuous when stained with PAS or Mallory's connective tissue stain. It appears as an unbroken line forming the basal border of the columnar absorptive cells.

A stratum granulosum is continuous throughout the intestine and rectum, running roughly parallel to the basement membrane in these regions.

A distinct tunica propria is seen in the intestine of C. variegatus, but is not clearly separate from the submucosa as no muscularis mucosae is present. The tunica propria is composed of very fine fibrous connective tissue that forms the cores of the mucosal folds and extends down into the submucosa. The connective tissue stains intensely with PAS. The tunica propria is well vascularized and contains fibroblasts, lymphocytes, red blood cells, and granulocytes.

The intestinal submucosa of teleosts is composed of loose areolar connective tissue that extends from the mucosa to the inner layer of the muscularis. In C. variegatus, collagen is the most conspicuous type of connective tissue, staining with both hematoxylin and eosin and PAS. Fibroblasts are the most prominent cell type present. Other cell types present include lymphocytes, granulocytes, and red blood cells. Blood vessels are usually seen in the triangles of submucosa formed when the basal portions of the mucosal folds approach the muscularis.

The intestinal muscularis is generally composed of two layers, an inner circular layer and an outer longitudinal layer. The muscle bundles are composed of smooth muscle, except at the junction of the esophagus and the intestine. In C. variegatus, the muscularis at the intersection of the esophagus and the intestine is composed exclusively of striated muscle arranged in a circular pattern. An external layer of longitudinally arranged striated muscle extends posteriorly from the junction, overlying the small amounts of circularly arranged striated muscle that

persists into the intestine. About 300 μm back from the junction an internal circular layer appears. Smooth muscle fibers gradually replace the striated muscle in the intestinal swelling. Isolated bundles of striated muscle are seen as far back as the junction between the intestine and the rectum. The muscularis is about 30 μm thick in the intestinal swelling and continues at that thickness posterior to the rectum. The layers of the muscularis are equally thick, about 15 μm.

The serosa of the intestine is continuous with that of the esophagus. It is composed of a single layer of squamous cells extending to the recto-anal junction. A layer of subserosal connective tissue (loose areolar) is present in the intestinal swelling but thins posteriorly.

Granular cells (granulocytes) are a common feature of teleostean alimentary canals. While they are present in the esophagus, they are more obvious in other regions of the digestive tract, including the accessory organs. Granular cells form a stratum granulosum in C. variegatus that is near, and parallel to, the basement membrane. Granular cells are often seen in the epithelium of the intestine, and some appear to have passed into the lumen. The majority of the granulocytes that are present in the epithelium are confined to the infranuclear zone of the columnar cells. The granular cells measure about 4 μm in diameter and contain several acidophilic granules. Secretory and storage functions (26) have been suggested for the granular cells, but no specific function has yet been conclusively demonstrated.

The rectum is a continuation of the intestine and is similar in structure. Histologically, several features distinguish the rectum from the intestine. The diameter of the digestive tube in the rectal region is slightly reduced from that of the intestine; however, the transition from the intestine to the rectum is gradual, and mucosal folds shorten and widen during the transition. The columnar cells of the rectum are the same size as those of the intestine. Rectal columnar cell nuclei are slightly smaller, 6 μm by 3 μm. Both the basement membrane and the stratum granulosum are continuous with those of the intestine. Goblet cells become numerous in the anterior rectum and increase in number posteriorly, staining intensely with PAS. The most striking feature of the rectum is the presence of PAS-positive granules in the sub-border region of the columnar cells that compose a continuous band across the epithelial lining. The granules stain more intensely in the middle and posterior regions and are located deep within in the columnar cells. The rectal tunica propria is more extensive than that of the intestine, forming the cores of short, wide mucosal folds.

The rectal submucosa is identical to that of the intestinal region, although there is a general reduction in submucosal tissue in the posterior portions of the rectum.

The rectal muscularis in C. variegatus continues from the intestine as a 30 μm thick layer. Both the outer longitudinal layer and the inner circular layer are composed of smooth muscle and are about 15 μm thick.

In the most posterior aspects of the rectum, the muscularis thins to 15-20 μm.

The rectal serosa is continuous with that of the intestine, and still composed of a layer of simple squamous cells. Subserosal connective tissue all but disappears in the posterior rectum.

Granular cells are most numerous in the rectal region of C. variegatus, forming the stratum granulosum that continues from the intestine. Also, many more granulocytes are seen in the rectal lumen than were present in the intestinal lumen. The morphology of the granular cells is identical to that of other regions of the digestive tract.

Rodlet cells are more numerous in the rectum of C. variegatus than in the intestine. They are visible when stained with Mallory's connective tissue stain, but are most obvious when stained with PAS and counter-stained with fast green. While most are located in the epithelial region, some are seen as deep as the submucosa. The rodlet cells average 8-10 μm in diameter, and each cell contains several rodlets that are 3-4 μm in diameter that stain intensely with PAS. The number of rodlets per cell ranges from 0 to 12. The precise function of the rodlet cells is yet to be determined; however, it has been suggested that these bodies may be sporocysts of a sporozoan parasite (27).

Peripheral Blood Cell Morphology, C. variegatus

This study was accomplished because of obvious importance of hematological data in the diagnosis of diseases, and the fact that data concerning teleost peripheral blood cell morphology is inadequate in the literature (28).

Light Microscopy:

Erythrocytes -- In fresh preparations, the definitive erythrocyte is biconvex and ellipsoid with a centrally located oval nucleus. The cytoplasm is somewhat opaque when a minimal exposure to air has occurred. Nuclei are visible in fresh preparations, but not prominent due to the partial masking effect of the cytoplasmic hemoglobin.

In Romanowsky-stained preparations, the homogeneous cytoplasm of erythrocytes is eosinophilic and opaque, staining a pale brownish pink. The staining properties of the cytoplasm are seen to be similar to those of mammalian red blood cells stained in the same manner. The nuclei of Romanowsky-stained erythrocytes are basophilic. The chromatin stains a deep magenta and appears as a network of cross hatched patches against the lighter nucleoplasm. There is often a light concentration of chromatin present at the periphery of the nucleus.

In both fixed and living in vitro preparations, one can often see erythrocytes which are teardrop-shaped due to a pointed pole at one end of the cell and a rounded pole at the other.

Erythrocytes that have been stained in fixed smears range from 8 x 6 μm to 11 x 8 μm, with a mean dimension of 10 x 6.3 μm. In fresh preparations, erythrocytes range from 10 x 6 μm, to 13 x 9 μm, with average dimensions of 12 x 7 μm.

The number of erythrocytes recorded in counts in females varies from 1.26 to 3.78 x 10^6/cu mm with a mean of 2.64 x 10^6/cu mm. In males, the counts ranged from 2.64 to 3.62 x 10^6/cu mm, with a mean of 3.12 x 10^6/cu mm.

In fresh preparations, the erythrocytic cytoplasm often clears when exposed to the air. This results from clumping of the cytoplasm into small globules which adhere to the nuclear and plasma membranes. The nuclear material also appears to clump at times. Usually nuclear clumping involves larger globules than cytoplasmic clumping.

In vitro preparations seem to indicate that the clearing and clumping are related since these two phenomena can almost always be observed to occur within a cell simultaneously. Often after a period of time in which clearing and clumping have occurred in a cell, globules can be observed outside of the cell, with some of them adhering to the exterior of the plasmalemma.

Erythroid Cells -- Several formed elements can be observed in the peripheral hematocrit which apparently either give rise to, or are derived from erythrocytes: 1) the erythroblast, an immature erythrocyte. This cell is larger than the definitive erythrocyte and has a more rounded nuclear and cellular shape. It is lightly basophilic and becomes pale blue with Romanowsky stain. 2) the erythroplastid, an anucleate, membrane-enclosed volume of cytoplasm derived from a definitive erythrocyte. Its elements possess the same eosinophilic, homogeneous cytoplasm as the mature erythrocyte. Erythroplastids are spherical, ovoid or teardrop-shaped. The diameters of these structures range from 3-5 μm. Definitive erythrocytes with pointed pseudopodia are often seen in areas where erythroplastids are found. 3) the senile erythrocyte, referred to in the literature as "basket" or "smudge" cells. It is seen in various stages of degeneration in fixed smears. The cytoplasm is more lightly stained, and the plasmalemma distended in comparison to younger definitive erythrocytes. The nuclei of this cell are larger than normal mature red blood cell nuclei. At times, the nuclear membrane ruptures, as chromatin is often found dispersed throughout some of the cells. Senile erythrocytes with cleared cytoplasm are often seen in fixed smears.

A certain amount of extracellular chromatin, presumably from disintegrating "smudge" cells, is almost always seen in fixed preparations. This extracellular chromatin is seen in increased amounts in thick smears, which require a longer drying period.

In fresh preparations, senile erythrocytes are not readily distinguishable due to cytoplasmic opaqueness of red blood cells. However, there is a degree of chromatin and other cellular debris present in these

in vitro preparations, which is more noticeable in older preparations a few hours following blood collection.

Acidophilic Granulocytes -- In stained smears, eosinophilic granulocytes are the only type of mature granulocytes found in the peripheral blood of C. variegatus. Immature cells vary according to the staining properties of the granules and the shape of the nuclei. Nondefinitive cells are seen to be basophilic, basophilic with some acidophilic properties, or completely acidophilic. The overwhelming majority of the granulocytes contain only eosinophilic granules. Indented or segmented nuclei are not observed in cells having basophilia. Truly lobed nuclei are not observed in any of the granulocytic cells. With the understanding that basophilia precedes acidophilia in blood cell ontogeny, it is reasonable to suspect that those granulocytic cells exhibiting basophilia are likely immature forms of eosinophilic granulocytes.

In fresh preparations, the granulocytes exhibit ameboid movement. Pseudopodia are formed which may be fingerlike or blunt projections. The fingerlike projections appear to initiate movement of these cells. These first projections do not contain granules. Following the initial movement, a large portion of the cytoplasm containing granules streams into the first, smaller projection, forming a second, large, blunt pseudopodium. During this movement, the nucleus usually has a peripheral location at a point most distant from the advancing end. Often a trailing nucleus can be seen at the end of a narrow, extended cytoplasmic isthmus at the end opposite from the advancing cytoplasm.

Most granulocytes move constantly among the formed elements of the blood and thrombocytic networks. These cells often attach to the slide or coverslip and move over a planar surface. Frequently, when free floating in plasma streams under the coverslip, the granulocytes are observed to round up into spheres. After coming to a stop, a fingerlike projection usually emerges from the sphere and the ameboid movement commences again.

The average diameter of the rounded form of eosinophilic granulocyte is 8 μm when stained and is slightly larger in fresh preparations. Extended living cells observed during times of motility attain a length many times the diameter of the spherical forms.

Nongranular Leucocytes -- At the light level, three morphologically distinct nongranular leucocytes are observed which have been classified into two separate groups, thromboid cells and lymphoid cells.

The thromboid cells are subclassified into "lone nucleus" forms and "extended" forms. The nuclei of the "lone nucleus" cells are round to ovoid with little or no visible cytoplasm surrounding the nuclei. In stained smears, the nuclei in these cells are a deeply basophilic magenta to dark purplish blue. The cytoplasm, when observed, is pink. In fresh preparations, the nuclei are opaque with little or no visible cytoplasm. These cells are often found in clusters with long, thin pseudopodia-like structures forming extended branching

networks among the cells. These structures are actually long fibrin strands radiating from ruptured plasma membranes. They are often several times the length of the cells. The "lone nucleus" form of thromboid cells are numerous in fish that have been unduly stressed before or during blood collection, or if the blood is not quickly heparinized or fixed.

The "extended" forms of thromboid cells are either oval or they have one or two spiked poles. The nuclei in stained preparations are magenta, with a pointed or somewhat rounded indentation usually present in the nucleus. In both stained and fresh preparations, the nuclear chromatin has a crosshatched and somewhat patchworked appearance. These cells were designated "spindle cells" in the earlier literature because of their characteristically elongated shape.

Lymphoid cells are usually slightly larger than the "lone nucleus" forms of thromboid cells, but resemble them to the extent that reliable differential thromboid-lymphoid cell counts could not be made. A narrow rim of cytoplasm is seen surrounding the nucleus of these cells in stained and fresh preparations. In Romanowsky-stained smears, the nucleus is basophilic, having a magenta or violet coloration, but rarely containing the dark, purplish blue coloration of the "lone nucleus" forms. The eosinophilic cytoplasm is a light pink and short pseudopodia are often seen around the cell periphery. There are usually one or more deep indentations in the rounded nucleus. These cells resemble mammalian small lymphocytes morphologically.

Electron Microscopy:

Erythrocytes -- At the ultrastructural level, the cytoplasm of the erythrocytes is seen to possess a finely granular composition that does not appear to contain free or membrane-bound ribosomes. Scarce smooth endoplasmic reticulum and a few thin mitochondria are seen.

In the nucleus, dark chromatin and lighter interchromatin material may be observed. Lining the nuclear periphery are dense chromatin patches which often penetrate deep into the nuclear interior and sometimes extend to the opposite edge of the nucleus. The two perinuclear membranes are not contiguous, and a prominent perinuclear space is normally visible. The perinuclear membranes unite to form the nuclear pores. At these sites, the cytoplasm is continuous with the lighter nuclear matrix.

Lymphoid Cells -- This cell type normally appears unevenly circular to elliptical with a relatively large, irregularly round to oval nucleus. The nucleus usually contains one or more deep clefts and is centrally located within a surrounding, thin ring of cytoplasm. At times, deep nuclear indentations divide the nucleus providing the appearance of two nuclei in some sections. Areas of nucleoplasm and dense chromatin material provide a light and dark patchwork pattern in the nucleus. Nuclear pores are present, providing continuity between the cytoplasm and the lighter nuclear matrix.

The most conspicuous organelles in the cytoplasm are the large mitochondria. These are usually elongated and contain well-developed internal cristal structures. Extensive rough endoplasmic reticulum and many free ribosomes are visible in the cytoplasmic matrix, but smooth endoplasmic reticulum is not abundant. A Golgi apparatus is often seen. The cytoplasmic membrane is plicated and usually contains many small pseudopodia as well as areas of pinocytotic vesicle formation. These areas of pinocytosis are discerned by the presence of adjacent vacuoles in the cytoplasm. A few dense granules are apparent within the cytoplasm.

Thromboid Cells -- Electron microscopy reveals a superficial resemblance between thromboid and lymphoid cells. Both have a centrally located nucleus surrounded by a relatively thin ring of cytoplasm. The nuclei of both cells are composed of clearly defined areas of chromatin and interchromatin material. Both cells have a relative abundance of ribonucleoprotein.

Unlike the lympoid cells, however, the thromboid cells possess nuclei which are not deeply indented. In the cytoplasm, numerous, dark, medium-sized to large granules are usually present, and mitochondria are fewer and smaller in thromboid cells. Also, fewer small pseudopodia are present at the periphery of thromboid cells. The most prominent cytoplasmic feature of the thromboid cells are the numerous electron-lucent vesicles.

An important physiologic mechanism peculiar to thromboid cells is the clotting process. Groups of these cells seen in sections of clotted blood show long, thin fibrin strands. During clotting, these strands radiate from the interior of the cells through newly formed openings in the disintegrating plasmalemma. The strands form dense, interconnecting networks between the thromboid cells and attach to nearby erythrocytes and any other cells in the immediate vicinity. These radiant strands appear to be continuous with an extracellular flocculent material seen throughout the sections and may, in fact, contribute to it.

During the clotting process, the thromboid cell nuclei become pyknotic and the cells lyse. Many large electron-lucent vacuoles appear in the cytoplasm of the cells. Electron-opaque granules are often seen inside and immediately outside the ruptured cells. Cytoplasmic organelles in thromboid cells are difficult to recognize in sections in which fixation occurred during the process of coagulation. Partially enclosed, membrane-bound cytoplasmic portions of some of the cells participating in the clotting process become separated from their original cell mass.

Aseptic Embryo Technique

Our initial experiments indicated that C. variegatus embryos could be maintained for up to 18 days under a variety of aseptic conditions (Table 3). Fry that were fed sterile particulate food

tended to survive longer than those that were not fed. Cannibalistic behavior was observed when large numbers of individuals were placed in the same flask. It was determined that this behavior did not occur if the population density was kept below one individual per 10 ml of medium. The goal of experiments currently in progress is to establish the specific aseptic medium that will provide maximum survival time. Also, the morphology of the aseptic embryos is being compared with embryos maintained for the same period of time in a septic environment. The effort includes size and weight comparisons, light microscopy, and scanning and transmission electron microscopy. Preliminary observations of the external gross anatomy of the two types of embryos suggest that they are quite similar.

Experiments are currently in progress to determine BEN toxicity of both septically and aseptically maintained embryos. Once these experiments are completed, both types of embryos will be exposed chronically to subtoxic levels of BEN.

TABLE 3. -- Survival times of aseptic embryos under different culture conditions.

	Culture Conditions	Number of Experiments	Ave. No. embryos per experiment	Ave. Survival time (days)
A.	L-15 + 1% Abs[1]	1	7	7
B.	L-15 + 1% Abs + Supplements	4	9	13
C.	L-15 + 1% Abs + Supplements + TM[2]	1	10	18
D.	Sterile artificial seawater + sterile *Artemia* eggs	1	2	10
E.	Sterile artificial seawater + 1% Abs + TM	1	10	16

[1]Antibiotics are from Gibco. Cat. No. 6005240, 100X Penicillin 10,000 U/ml-Fungizone, 25 mcg/ml-Streptomycin, 10,000 mcg/ml.

[2]TetraMin Tropical Fish Food.

A major advantage of the aseptic embryo technique is that it provides an opportunity to study the effects of a contaminant on the organism during its highly susceptible embryonic period entirely free of any influence by bacteria or other organisms normally present. Thus, the system should be particularly valuable in carcinogen studies related to enzyme induction and the pathways by which procarcinogens are metabolized into active carcinogenic agents.

Although the survival times already attained are sufficient for many of the studies anticipated for this system, it is reasonable to assume that continued study of the system will lead to the development of a procedure that will allow normal development of the organisms to an adult state. Experiments designed to attain this goal are currently underway. They involve observing the effects of changes in basal media

and substrate supplements, determining food preference and optimum nutrition, understanding the effects of different population densities, and establishing the best photoperiod and level of aeration for embryonic development.

Laale and Lerner (29), in a recent review, have indicated a need for continued study of specific teleost teratogens and suggest that these studies must involve the determination of specific metabolic targets. They state that this will require detailed studies of the responding embryos at all levels of organization. Obviously, the present system, in which embryonic development in both contaminated and uncontaminated sterile environments can be carefully observed, provides an excellent system for such ichthyoteratological studies as well as general studies of teleost embryonic development.

Embryo-Primary Cell Culture Technique

With 75-80% efficiency, these embryos become attached to the surface of the Linbro wells within 2-3 days and a mixed population of cells begins migrating from the attached region of the embryo. As cell migration continues, the attached region of the embryo becomes progressively disorganized. Three morphological types of migrating cells are commonly observed: fibroblast-like cells, pigmented cells, and ovoid-shaped cells. The pigmented cells often develop interconnecting processes. Frequently, the pigmented cells form rather extensive interconnecting networks.

Since the embryo-primary cell culture technique blends the use of an aseptic embryo with cell cultures into one system, it is possible to observe simultaneously the effects of a specific carcinogen on a relatively intact organism, and primary cell cultures from that same organism. The technique provides numerous advantages. It provides better genetic continuity; i.e., the cells of the primary cell culture can be expected to be more genetically similar to the cells of the intact organism than cells of an established cell line. The major portion of the embryo remains intact with relatively normal organ arrangement and function, and the system can be removed for histological examination. Also, the carcinogens make direct contact with the various cells of the system in a chemically defined medium. Not only does the system provide for exposure of the organism during the highly susceptible embryonic development period, but the embryo likely "conditions" the medium, bringing about increased viability and differentiation in the cells of the primary culture. Such conditioning factors have been reported (30, 31), and a conditioning phenomenon is consistent with the observations in the present study that the cells in the primary cell culture region change their appearance and behavior after the intact portion of the embryo becomes disattached or is removed. Finally, the system bridges the gap between in vitro systems, such as established cell lines, and in vivo systems that are currently in use.

The technique may be used for a variety of tests and assays, such as: 1) tests for toxicity, teratogenicity, and cell transformation 2) karyologic alterations, 3) detection of antigenic or enzymatic changes, 4) metabolic activation/cocultivation, 5) effects on macromolecule synthesis (collagen, mRNA, etc.) and, 6) viral genome activation.

Factors that may be considered limitations are: 1) the embryos do not contain the normal microbial flora of their septically reared counterparts, 2) the feeding mechanisms of the cells, and to some degree, the intact embryos are likely different from normally reared embryos, and 3) the restricted embryo movement and partial disorganization may bring about metabolic differences as well as other subtle effects. However, even with these limitation, the model provides a useful system that should be reproducible, standardizable, and easily comparable to *in vivo* systems. Laale (31) has been able to achieve 40-day survival times with a similar system that employs blastoderm isolates from the the zebrafish, *Brachydanio rerio*, so it is reasonable to assume that equal or greater survival times can ultimately be obtained with the *C. variegatus* system. This should provide adequate time to perform assays. It is noteworthy here that Hillebert and Martin (32) were able to obtain multilayered foci in an established cell line from the sheepshead, *Archosargus probatocephalus*, exposed to benzo(o)pyrene after only three passes.

Primary Hepatocyte Cell Culture Technique

Data obtained from primary vertebrate hepatocyte cultures have demonstrated excellent correlation with bacterial mutagenicity assays (33), and it follows that vertebrate cells should be more relevant than the procaryote assay systems often used to determine the mutagenic and carcinogenic potential of suspect chemicals (34). Recent studies employing rat primary hepatocyte monolayers (35, 36) have demonstrated the usefulness of these methods in providing a better understanding of the metabolic processes responsible for the conversion of procarcinogens to the active molecules. Additionally, rat liver culture systems have proven useful in studying mechanisms of tumor promotion (37, 38).

Since primary hepatocyte cultures can be expected to be more genetically similar to the cells of the intact organism than an established cell line, data derived from *C. variegatus* primary hepatocyte cultures should be more directly comparable to the whole animal system. Once the methods have been optimized for maintaining these primary hepatocyte cultures and have been adequately standardized, it should be relatively easy to conduct assays using numerous duplicates with positive and negative controls. In addition to its value in extending the usefulness of *C. variegatus* as an assay system, the system may provide advantages over mammalian systems currently in use. For example, the systems can be operated more economically because the cultures can be maintained at room temperature, thus

avoiding the use of expensive incubators with gas-controlled environments.

Immunological Studies

The immune system of fish is known to be very complex involving many substances ranging from specific "acute phase" enzymes to interferon, to immunoglobulin production (39). The primary problem in studying the immune response of C. variegatus lies in its size. The immune response to specific antigens has been routinely studied in many larger species of fish, such as brown trout (Salmo trutta) (40) and sockeye salmon (Oncorhynchus nerka) (41), in attempts to increase production of economically important species. In studies of the phylogeny of the immune response, large species of shark have also been studied (42). In order to study the humoral immune response of any organism, serum must be easily obtainable in reasonable volumes, which is not a problem in larger fish where blood may be obtained in milliliter amounts via heart puncture or caudectomy. The blood volumes of the largest C. variegatus, however, is measured in microliters. It has been necessary therefore to miniaturize or modify techniques used to study the immunology of the fish and to develop a bleeding procedure that significantly enhances blood recovery from these organisms.

Serum collection -- Using fish averaging 5.4 cm in length, the volume of blood collected averaged 18.5 µl per fish when the caudal fin was completely severed and no treatment with anticoagulant or anaesthetic was used. When fish of similar size (averaging 5.5 cm) were treated with sodium citrate and MS222, then bled using the modified cut, the volume of blood collected averaged 33.7 µl/fish. Thus, the modifications introduced to the bleeding procedure significantly increased (almost doubled) the blood volume obtainable (Table 4). Omission of any of the additional steps in the modified procedure resulted in collection of smaller blood volumes.

Collection of leucocytes -- Both techniques employed were successful in separating leucocytes from whole blood. Collection of cells from the buffy coat resulted in relatively more contamination with erythrocytes than collection by the Ficoll-Paque procedure. Leucocyte preparations collected by either method contained cell types identified as normally present in fish blood, based on comparison of cells in Wright-stained suspensions with descriptions of previously described cell types (43). A high percentage of cells were viable.

TABLE 4. -- Comparison of Blood Volumes Collected By Different Bleeding Procedures.

BLEEDING PROCEDURE	AVERAGE LENGTH OF FISH	AVERAGE BLOOD VOLUME COLLECTED
NO PRETREATMENT, CAUDAL TOTALLY SEVERED.	5.4±0.6	18.5±3.4
PRETREATMENT WITH MS222 AND CITRATE, CAUDAL PARTIALLY CUT.	5.5±0.8	33.7±16.0

Serum electrophoresis -- Serum electrophoresis was performed to provide baseline data for serum immunoelectrophoresis as well as for its own intrinsic value for comparison of normal and carcinogen-exposed fish. The small volume of serum required for each electrophoresis run allowed replicate samples of individual fish sera to be tested. Densitometer scans of duplicate runs showed good reproducibility. Figure 8 compares the results of densitometer scans of serum from normal C. variegatus and from fish exposed to 1 ppm BEN for seven weeks. A scan of normal human serum is included as a procedural control, and shows a typical pattern. There are obvious differences between the serum profiles of the normal and BEN-exposed fish. The normal serum shows eight peaks, whereas the serum from the BEN-exposed fish shows only five peaks, with some components evidently present only in very weak concentrations or missing altogether. Sera from different normal individuals showed some variation but were always similar, producing seven to nine peaks in densitometer scans, with most producing eight. Sera from BEN-exposed fish, on the other hand, showed wide variations in serum profiles, both in size and number of peaks.

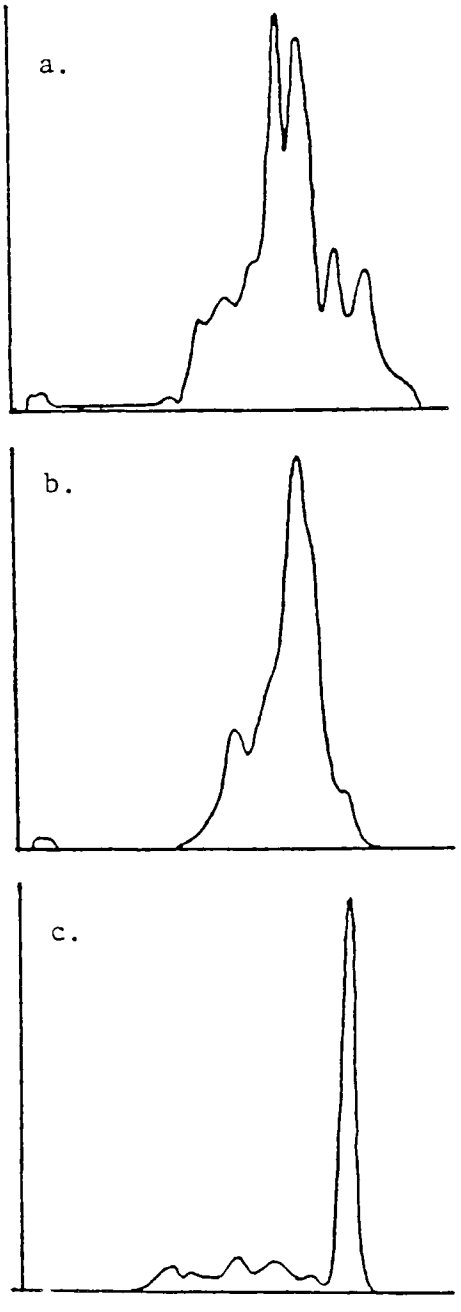

FIGURE 8. -- Comparison of serum electrophoresis profiles of (a) normal serum of C. variegatus, (b) serum of C. variegatus exposed to 1 ppm benzidine for 7 weeks and (c) normal human serum. Fastest migrating peak is at right of profiles.

Immune rosette formation -- Immune rosettes were successfully produced and identified in spleen cell suspensions from C. variegatus. Complete "halos" of erythrocytes were frequently observed with no leucocyte evident inside. Careful examination of these preparations led to the conclusion that the mounting or staining procedure often caused the destruction of the rosette-forming cells, which evidently are rather fragile. Destruction of such cells within a complete ring of erythrocytes left only the ring with little or no evidence of the rosette-forming cell itself. If only a few erythrocytes were bound to a rosette-forming cell which lysed, it is likely that the rosettes would not have been scored. It is necessary to eliminate this problem if the rosette-forming technique is to be of use in quantitating antibody-forming cells in spleen suspensions of C. variegatus. Use of a mild fixative prior to mounting the suspension and use of wet mount preparation are being explored as possible solutions.

Bacteriophage neutralization assay -- Prior to carrying out the bacteriophage neutralization assay with serum from fish immunized with MS2 phage, preliminary experiments were done with serum from normal non-immune fish and from non-immune fish exposed to 1 ppm BEN for seven weeks. These experiments were done to determine if any natural neutralizing substances were present. Table 5 presents partial results. Serum from normal fish had no significant effect on the virus titer, as may be seen by comparing number of plaque-forming units (PFU) in normal serum-exposed phage inoculum with the number in phage inoculum not exposed to serum. By contrast, significant reduction of PFU/inoculum was observed if the inoculum was exposed to serum from BEN-exposed fish. At a serum dilution of 1/32 to 1/256 or higher, the plaque reduction became less marked, but still significant. Further experiments are necessary to demonstrate the dilution necessary to eliminate plaque reduction completely. The results suggest that there are at least two components of serum from the benzidine-exposed fish which reduce plaque titer. One dropped below the minimal concentration for effect at a dilution of 1/16 to 1/32 and the other still exerted an effect at a dilution of at least 1/256. The results further suggest that the plaque reduction may not be dose-dependent. Additional studies are planned to determine the identity of the serum components.

The bacteriophage neutralization assay has thus been easily adapted to the C. variegatus system. Normal and carcinogen-exposed fish are currently being immunized with MS2 phage. Upon completion of the immunization process, the assay will be applied to comparison of neutralization titers, with non-immune serum from normal and exposed fish included as controls.

TABLE 5. -- Effects of serum from normal and benzidine-exposed Cyprinodon variegatus on the titer of MS2 bacteriophage.

Source of serum	Serum dilution								
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	No serum
Benzidine-treated fish	65[a]	65	62	89	113	106	110	117	---
Normal fish	153	159	132	158	154	152	153	169	---
Phage control (no serum used)	---	---	---	---	---	---	---	---	141

a. Numbers represent number of plaque-forming units (PFU). A dilution of phage stock was used which should have produced approximately 150 PFU/ inoculum volume.

Effects of Salinity, DOC, and Light on DENA Extractability

Nitrosamines, known chemical carcinogens, have been found in a variety of foods (44, 45), and recently there have been some efforts to detect these compounds in the environment (46, 47, 48).

However, studies that identify the factors affecting the chemical behavior of nitrosamines in the environment are limited. Tate and Alexander (49) monitored degradation of nitrosamines in soil, water, and sewage using extremely high concentration spikes of nitrosamines (100-2000 mg/l). Also, the chemical behavior of nitrosamines in super-acid solutions have been studied (50, 51). Extrapolations of data from such studies, however, may not reflect the behavior of nitrosamines at trace levels in natural aquatic systems. Thus, in order to further our ability to interpret data from experiments exposing fish to parts-per-million concentrations of nitrosamines (3, 4, 52, 53), this study was conducted in collaboration with Mr. Robert A. Fricke and Dr. Richard H. Pierce, Jr. at the Florida Institute of Technology (54).

In this study, the effects of salinity dissolved organic carbon (DOC), and light on the extraction of diethylnitrosamine (DENA) from water at trace concentrations (55-85 µg/l) and at 1 mg/l, were investigated.

Method 607 (16) approved by the U.S. Environmental Protection Agency, when used for trace concentration extraction of DENA, provided rather inefficient recoveries of DENA at 1 mg/l. For example,

concentration of the extraction solvent, dichloromethane, employed by the EPA method caused as much as 75% of the originally extracted DENA to be lost by evaporation. The revised extraction method that was developed, employing hexane as an extraction solvent, requires no concentration of extracting solvent when simple water extraction of 1 mg/l (or greater) of DENA is being performed, thus, avoiding the volatilization loss of the previous method.

Varying the salinity (0.5 - 24 parts per thousand) and DOC (0.5 - 11 mg/l) had no effect on the extractability of DENA at 1 mg/l (Figure 9). However, a significant decrease in extractability of DENA (at 55-85 μg/l) with increasing salinity was found (Figure 9). A decrease in trace level extractability was also found at high concentrations (11 mg/l) of DOC (Figure 10).

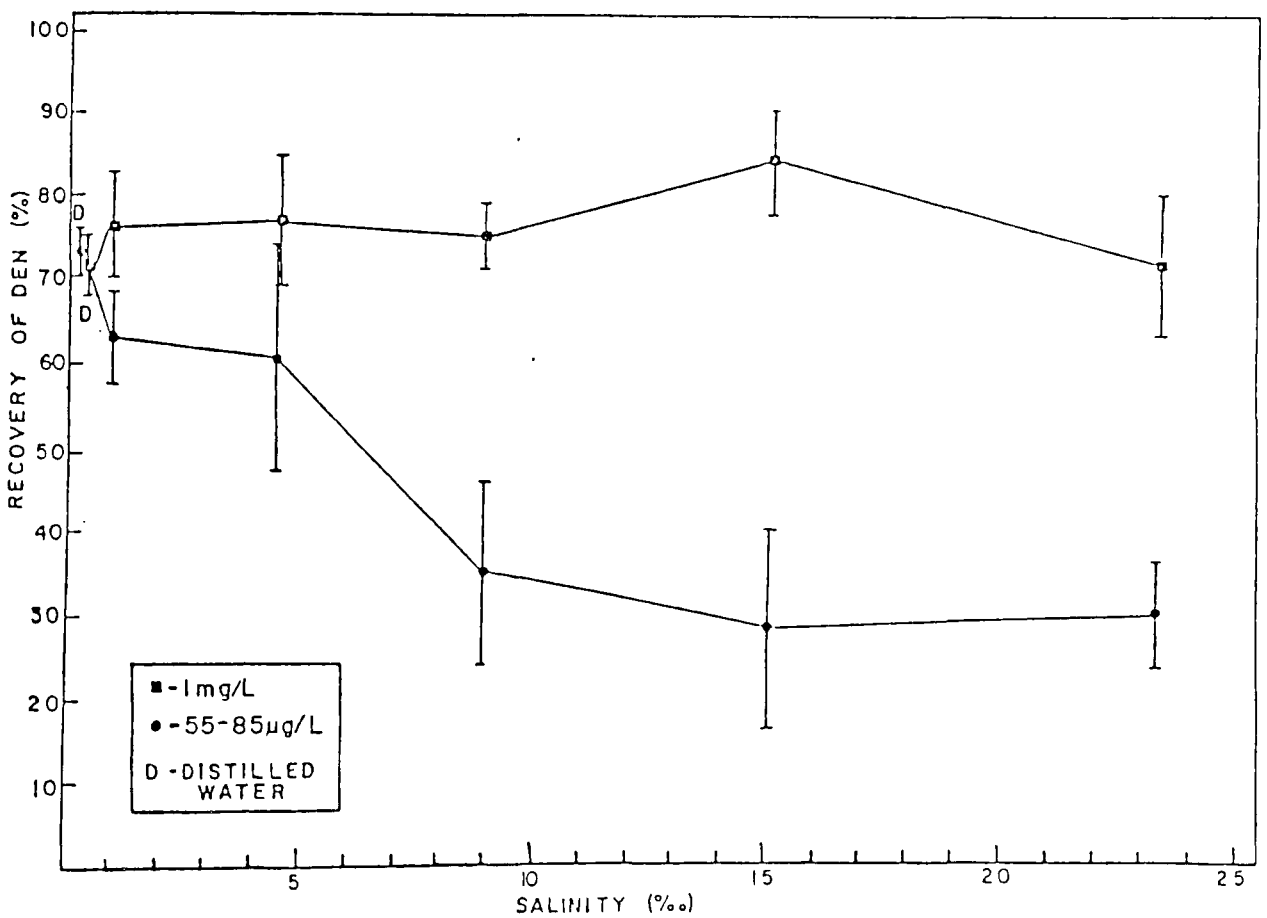

Figure 9. The effects of salinity on DENA extractability.

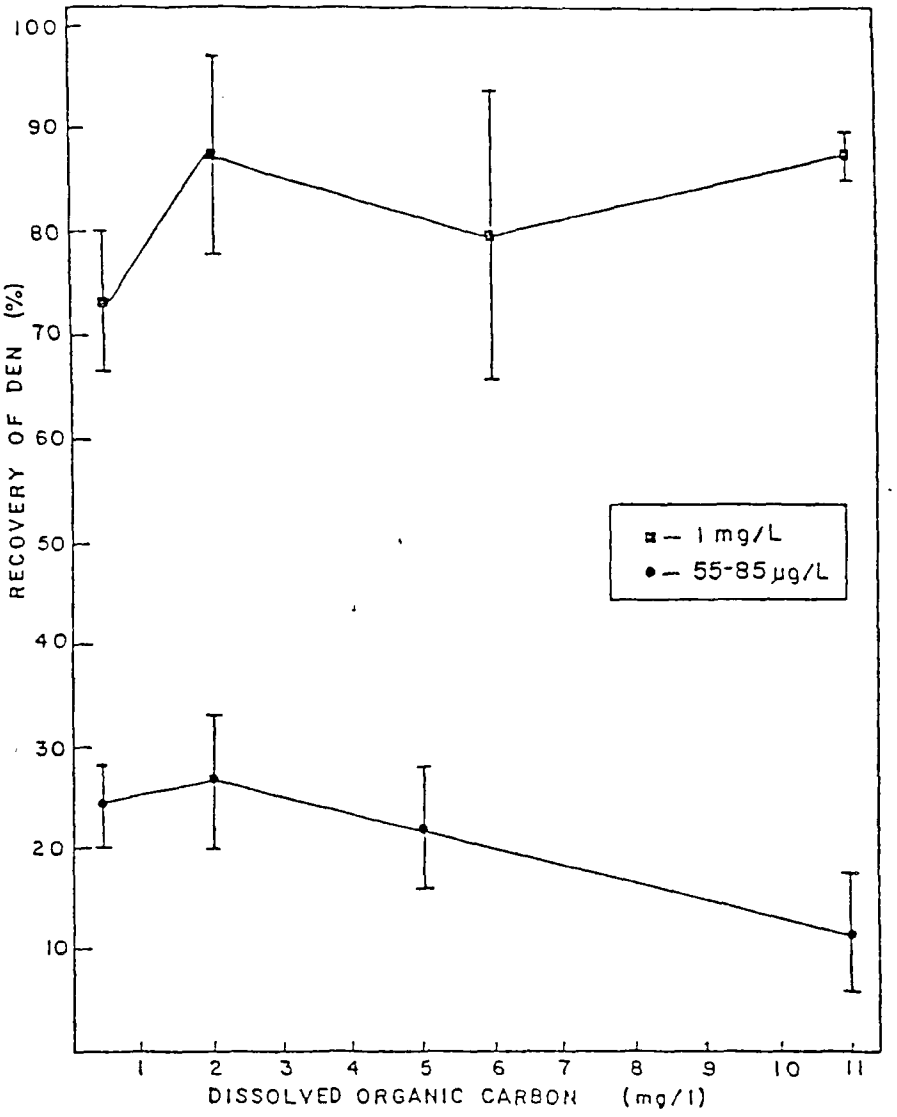

Figure 10. The effects of dissolved organic carbon on DENA extractability.

No significant loss of DENA in brackish water occurred over a 7-day period (Figure 11). However, the addition of DOC to the brackish water enhanced the photodegradation of DENA, resulting in a 30% loss in 7 days (Figure 11).

In order to determine the recovery of DENA in the 1 mg/l range from natural brackish water, samples were taken from a brackish creek (Salinity, 13 parts per thousand) and spiked with 1 mg/l DENA. Control water for these experiments was artificial seawater

taken from aquaria in which C. variegatus had been maintained for two months. These experiments indicate that the laboratory data from this study could be validly extrapolated to the natural environment. Finally, the revised method for DENA extraction devised in the present study is a feasible approach to monitoring DENA at the concentrations normally present in the chronic exposures of teleosts conducted in this project.

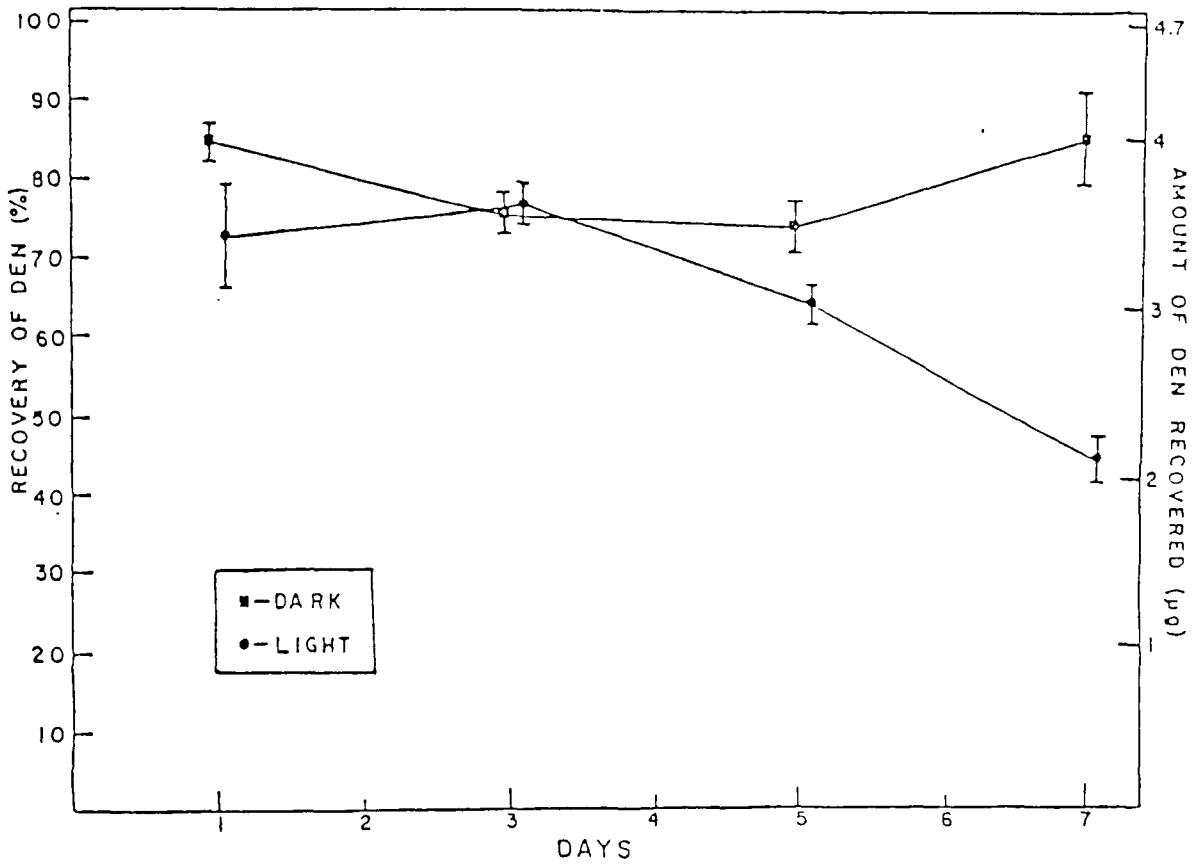

Figure 11. The effects of light on extractability of DENA from brackish water (Salinity = 10 ppt) with added DOC (6 mg/l) during a 7-day period.

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16. ABSTRACT

The objective of this project was the development of systems to assay the effects of chemical carcinogens on marine teleosts. It was determined that the LC-50 for benzidine with respect to Cyprinodon variegatus was ca. 64 ppm. Weekly contaminations of 1 ppm benzidine caused some individuals to develop proliferative liver lesions. Exposure of C. variegatus early embryos produced the following anomalies at concentrations of 50 ppm and above: tubed heart syndrome with distended pericardia, poor circulation, sparse distribution of melanophores, inability to hatch, abnormal head morphology, scoliosis, and faint RBC pigmentation. Chronic exposure of a cell line from Archosargus probatocephalus to benzidine and benzo(a)pyrene produced mutagenic effects. Two novel techniques were developed to study the effects of carcinogens on C. variegatus at the cellular level -- an aseptic embryo technique and an embryo-primary cell culture technique. Standard immunological techniques were miniaturized to study the immune system of C. variegatus. Serum electrophoresis disclosed that the serum proteins of benzidine-exposed fish differed from unexposed controls.

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