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COMPENDIUM OF THE FY1990 & FY1992 RESEARCH REVIEWS FOR THE RESEARCH METHODS BRANCH

U.S. Army Biomedical Research & Development Laboratory
Fort Detrick
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Since 1983, the U.S. Army Biomedical Research and Development Laboratory (USABRDL) has conducted a research program focused on the development of new methods for assessing the potential hazards resulting from exposure to chemicals in the workplace or the environment. This research has been conducted both in-house and extramurally and has involved scientists from other Federal agencies, academic institutions, and the private sector. The need to enhance the integration of the research became increasingly evident as the program grew in the late 1980's. This growth came principally as a result of the U.S. Army Corps of Engineers sponsorship of research to develop environmental applications of new, in vivo hazard assessment models for use in the burgeoning Department of Defense environmental remediation program. One of the many uses of these models is to answer the question, "How clean is clean?" and to assist in the establishment of more realistic clean-up goals for hazardous waste sites.

An annual research review meeting was undertaken to facilitate the integration and technology transfer of this research program. This publication is the second

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compendium of these meetings. The papers contained in this document are meant to provide the program management staff with an additional means of reviewing the status and progress of extramural and selected in-house studies in the area of new toxicity assessment models. The papers are, therefore, the principal investigators' (PIs') best efforts to characterize the research at the time of the meeting. Some papers cover the results of several years of research and others outline only the planned research of a new project. The data presented and conclusions of the authors represent the best professional judgment of the respective PI at the time of the workshop but are not equivalent to a peer-reviewed report of completed research.

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Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals, and experiments involving animals adhered to the standards stated in the Guide for the Care and Use of Laboratory Animals, NIH Publication 85-23, 1985 edition.

CONTENTS

FOREWORD

Since 1983, the U.S. Army Biomedical Research and Development Laboratory (USABRDL) has conducted a research program focused on the development of new methods for assessing the potential hazards resulting from exposure to chemicals in the workplace or the environment. This research has been conducted both in-house and extramurally and has involved scientists from other Federal agencies, academic institutions, and the private sector. The need to enhance the integration of the research became increasingly evident as the program grew in the late 1980's. This growth came principally as a result of the U.S. Army Corps of Engineers sponsorship of research to develop environmental applications of new, *in vivo* hazard assessment models for use in the burgeoning Department of Defense environmental remediation program. An annual research review meeting was undertaken to facilitate the integration and technology transfer of this research program. This publication is the second compendium of these meetings.

The papers contained in this document are meant to provide the program management staff with an additional means of reviewing the status and progress of extramural and selected in-house studies in the area of new toxicity assessment models. The papers are, therefore, the principal investigators' (PIs') best efforts to characterize the research at the time of the meeting. Some papers cover the results of several years of research and others outline only the planned research of a new project. The data presented and conclusions of the authors represent the best professional judgment of the respective PI at the time of the workshop but are not equivalent to a peer-reviewed report of completed research.

The scientific review of the program and interaction among investigators accomplished as a result of these workshops were extremely beneficial to the USABRDL staff and participants. I wish to express my gratitude to Ms. Jeannine Von Loewe and Ms. Bev Smith for the administrative coordination of these workshops. Their efforts were of inestimable value to the success of this project. I am keenly aware that the success of this research program is due to the innovative, conscientious, and unselfish research of the scientists and technicians at USABRDL and our extramural laboratories. I continue to learn from them all and am grateful for their efforts. This research has been supported by the U.S. Army Medical Research, Development, Acquisition and Logistics Command and the U.S. Army Corps of Engineers. Their continued encouragement has been critical to the success of this program.

Henry S. Gardner, USABRDL

1990 RESEARCH REVIEW MEETING

14-15 August 1990

IMMUNOTOXICOLOGY IN THE MEDAKA: CHEMICAL MODULATION OF MACROPHAGE CHEMILUMINESCENCE

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INTRODUCTION

Part of the process of establishing safety standards for chemical exposure involves the evaluation of immunotoxic and immunomodulatory effects of xenobiotics under consideration. Macrophages are important effector components of the immune system, and the immunomodulatory effects of many classes of chemicals on mammalian macrophages have been documented (Kutz et al., 1980; Schwartz et al., 1980; Tam and Hinsdill, 1984; Hilbertz et al., 1986; Snoeij et al., 1987). Macrophages destroy infectious agents and neoplastic cells by producing cytotoxic oxygen radicals as a result of contact with, and subsequent phagocytosis of, these agents. During this process, various oxygen intermediates are released by the macrophages, which can be quantified as luminol-augmented chemiluminescence (CL) activity. It is generally accepted that the CL represents a sensitive measure of the protective cidal capacity of these cells and that modulation of CL can be taken as an indication of immunological compromise. The aim of this study was to quantify the effects of representative xenobiotics on the CL response of medaka macrophages. The results were compared to data from studies on the effects of the same chemicals on mammalian macrophages in order to estimate the potential for intraspecies extrapolation. This *in vitro* assay has application as a preliminary screen for immunotoxic chemicals using primary cultures of fish cells as an alternative to the more costly and time-consuming use of mammalian experimental animals. Inhibition of macrophage CL also can be used to indicate the presence of immunotoxic agents in environmental samples.

BACKGROUND

Bick (1982) has proposed a tier system for immunctoxicological assays. Tier one assays study phenomena based on complex cellular responses or interactions, whose alteration could have broad consequences to the host. Such a system is CL, which accompanies the respiratory burst and release of reactive oxygen intermediates (ROI) associated with the interaction of macrophages and external stimuli such as microorganisms.

During the first year of this grant, several ROI assays were developed in this laboratory using oyster (*Crassostrea virginica*) hemocytes to model medaka macrophages. The rationale for those studies and the detailed protocols developed were presented in the *Compendium of the FY1988 and FY1989 Research Reviews for the Research Methods Branch.* As predicted, these methods were easily adapted for use with fish macrophages, and were used successfully with cells from goldfish, *Fundulus*, and medaka. Briefly, the assays measured (1) the superoxide anion (O_2^-) by quantitative nitroblue tetrazolium (NBT) reduction and by the reduction of ferricytochrome c, (2) hydrogen peroxide (H_2O_2) by phenol red oxidation, and (3) CL.

Involvement of ROI in cellular defense mechanisms seems to be a highly conserved mechanism present at all phyletic levels. The ultimate source of the microbicidal oxidants is O_2^-, which is produced in relatively large quantities after stimulation (Babior et al., 1988). The production of O_2^- is accomplished by the one-electron reduction of O_2, at the expense of NADPH. The dismutation of O_2^- to H_2O_2 and O_2 occurs spontaneously or is facilitated by the enzyme superoxide dismutase (SOD). H_2O_2 is highly toxic in its own right; in the presence of chloride ions and myeloperoxidase, it forms a potent antimicrobial system in phagocytic cells (Klebanoff, 1968). However, there is also abundant evidence that O_2^- exerts numerous direct toxic effects (Fridovich, 1988). Other oxidants associated with the respiratory burst include singlet oxygen and the hydroxyl radical (Britigan et al., 1986).

APPROACH

Chemiluminescence was selected for thorough examination as a primary *in vitro* screening assay to identify immunomodulatory chemicals as part of these studies of medaka *immunotoxicity*. In mammalian studies, CL has proven to be less laborious and more sensitive than measuring effects of chemicals on other macrophage functions such as phagocytosis and killing (Tam and Hinsdill, 1990). Furthermore, CL measures a suite of ROI activities simultaneously. Singlet oxygen produced by macrophages rapidly loses energy in the form of photons as it drops back to the ground state of oxygen; however, CL is inhibited by the presence of either SOD or catalase, indicating the concomitant involvement of O_2^- and H_2O_2 in the phenomenon.

The CL response can be rapidly and accurately quantified and provides many advantages over other ROI methods. In the procedure used here, medaka head kidney macrophages are exposed to xenobiotics for 1 hour and 20 hours prior to stimulation with yeast particles, resting CL levels are obtained prior to stimulation, and the kinetics of CL are followed for 2 hours poststimulation. The resultant data are analyzed to provide information on (1) the speed and intensity of the cells' response to phagocytic stimulation (peak height and time), (2) the total CL

response to stimulation (the area under the CL curve), and (3) the kinetics of the CL response as influenced by xenobiotics. Chemically induced CL impairment indicates a reduction of the ability of medaka macrophages to release cytotoxic ROI and suggests immunosuppression. Xenobiotic exposure can also induce macrophage activation under certain conditions; this condition can also be readily detected by CL. In addition to information on immunological capacity inducible by phagocytic stimulation after short-term (1 hour) and 20-hour exposure to test chemicals, the effects of chemical exposure on resting CL levels at these times were also measured. In this way, a relatively simple, short-term CL assay can be used to quantify the impact of xenobiotics on several important parameters of host defense capabilities.

GENERAL METHODOLOGY

Head kidneys, major hematopoietic organs in fish, were removed from medaka and pooled in phosphate-buffered saline (PBS). The head kidneys were disrupted mechanically and the macrophages either used directly or further purified using a Histopaque 1119 density gradient centrifugation step. Medaka macrophages were often used for CL without further separation to minimize cell losses and because they were minimally contaminated with erythrocytes, which do not participate in CL activity. The macrophage pool was adjusted to 2×10^6 cells/ml in L15 medium containing antibiotics, and at least four identical 1-ml aliquots were prepared. The xenobiotic in question was added to two of the vials (dark-acclimated scintillation counter pony tubes); the other two vials served as controls. All components of the system, including vessels and solutions, must be kept in the absence of light for at least 24 hours prior to running the assay. After 1 hour of incubation, the baseline CL level was measured; then the phagocytic stimulus (heat-killed, standardized yeast suspension) and luminol (to enhance the CL signal) were added to one of the control vials and one of the experimental vials. The samples were placed in a scintillation counter and adjusted to the out-of-coincidence mode for single-photon counting by use of a specific computer software package; the CL response was followed at 0.5-minute intervals for 2 hours. All incubations, transfers, and counting procedures were done in the dark or under red light (darkroom) illumination to eliminate spurious non-macrophage-related CL. After 20 hours of incubation at 20°C, basal CL was measured; then yeast and luminol were added to the remaining control and experimental vials, and the CL response measured as described above. Both 1-hour and 20-hour samples were checked for bacterial contamination by microscopic examination and for cell viability by trypan blue exclusion. Data from samples that were contaminated with bacteria or with <90% viability were considered invalid. Xenobiotics were run

at sublethal concentrations, based on previously determined 20-hour median lethal concentration (LC_{50}) levels.

RESULTS

1. The Typical Medaka CL Response

Figure 1 shows the CL response of medaka macrophages in the absence of xenobiotics. It is typical for the absolute value (cpm) of the CL response to vary considerably between individual medakas and/or different macrophage pools. However, the variation of values between aliquots of the same pool was small, and the modulation induced by xenobiotic exposure of a given pool was highly reproducible. Intrapool comparisons were made possible by determining relative percent inhibitions or stimulations and by calculating EC_{50} levels. If medaka macrophages were stimulated after 1 hour of incubation, the CL response developed gradually to a peak value of 10-50K cpm at about 40 to 60 minutes, after which time CL activity remained level or gradually dropped off. Unexpectedly, the response to stimulation at 20 hours of primary culture maintenance was consistently different in speed and intensity. In 20-hour cultures, the peak values were generally 200-400K cpm and the peak times were at 20 to 30 minutes. This augmented response was probably due to macrophage activation produced by cell contact and spreading on the internal surfaces of the plastic scintillation vials. Similar *in vitro* activation has also been observed with mammalian macrophages.

2. Modulation of CL by Heavy Metals

Inhibition of medaka macrophage CL was produced by sublethal concentrations of both cadmium (Cd) and lead (Pb) after 20 hours of exposure (Figures 2 and 3). None of the Cd concentrations tested (25-200 μg/ml) produced significant effects after 1 hour of incubation. However, a dose-dependent decrease in 20-hour peak height and total CL (area under the curve) was recorded. Figure 2 shows the data for 100 and 200 μg/ml; 25 and 50 μg/ml (data not shown) gave intermediate levels of inhibition at 20 hours, but no peak displacement with regard to time. Unlike Cd, Pb (75-150 μg/ml) exposure inhibited CL in the 1-hour samples, as well as in the 20-hour samples (Figure 3). The reduction in peak value and total CL was produced by 50 μg/ml in the 20-hour samples and was dose-dependent in all concentrations tested. No peak displacement was seen. In addition to metal-induced inhibition of phagocytically stimulated CL, both Pb and Cd exposure for 20 hours produced a dose-dependent reduction in unstimulated (resting) CL. Taken together, the data indicate that these metals are immunotoxic to medaka macrophages.

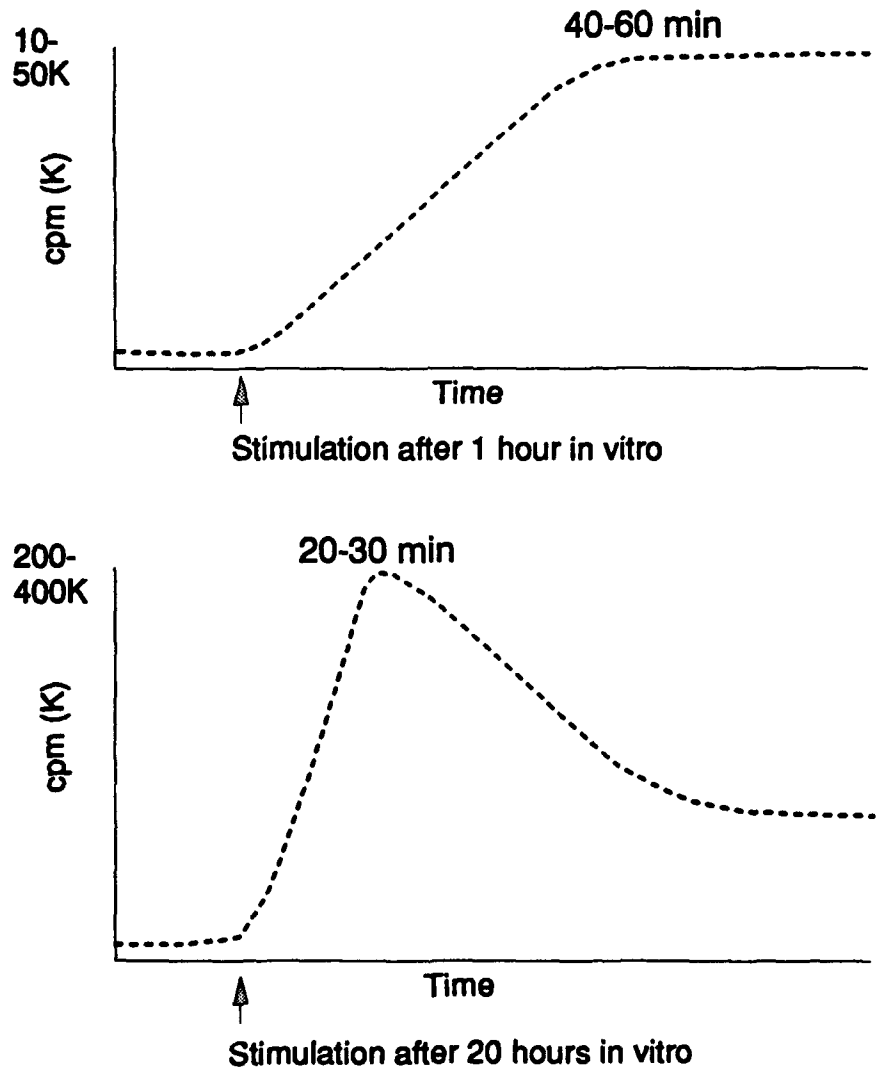

Figure 1. Normal medaka chemiluminescence (CL) response.

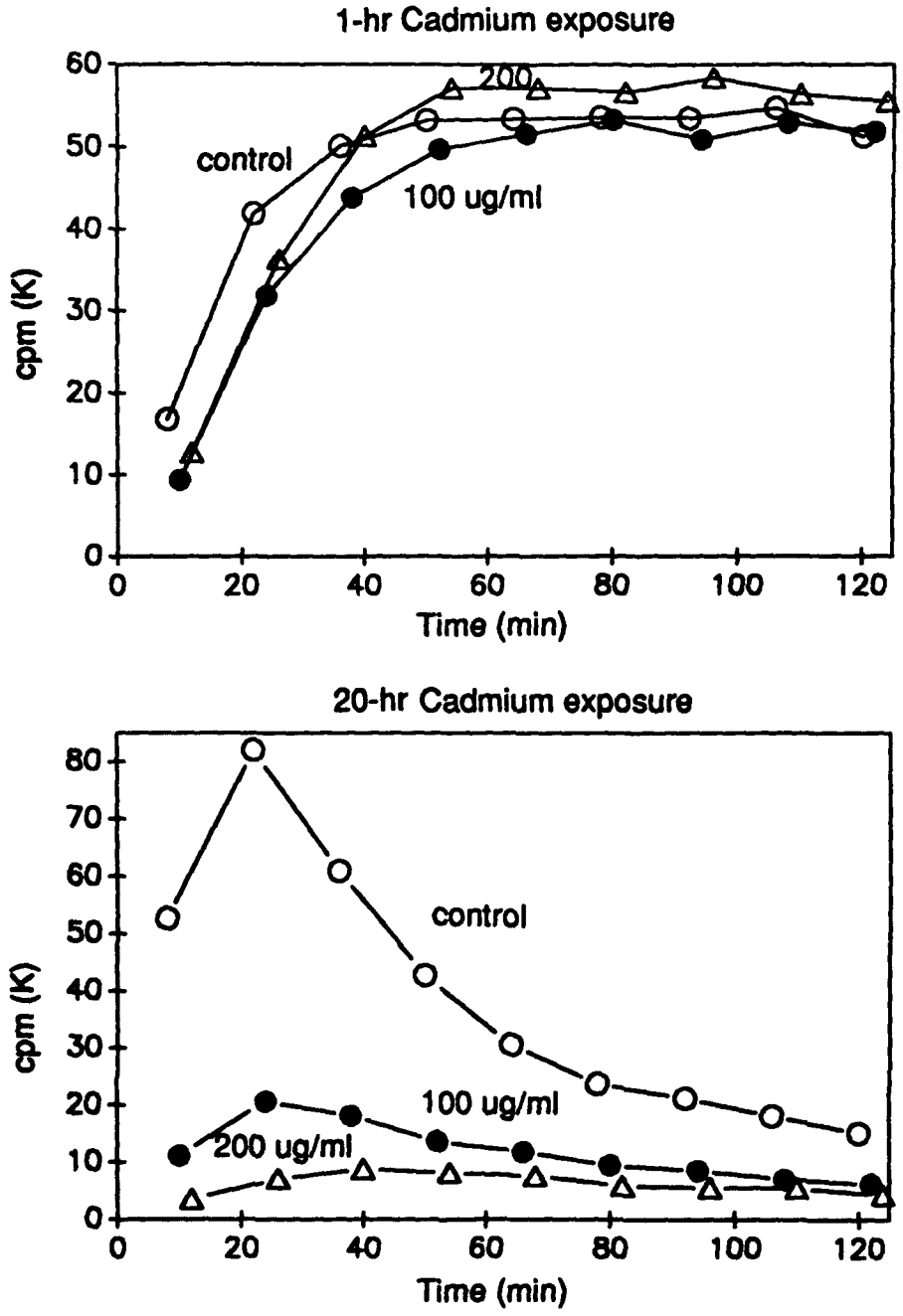

Figure 2. Inhibition of medaka CL response by exposure to cadmium at 100 and 200 μg/ml.

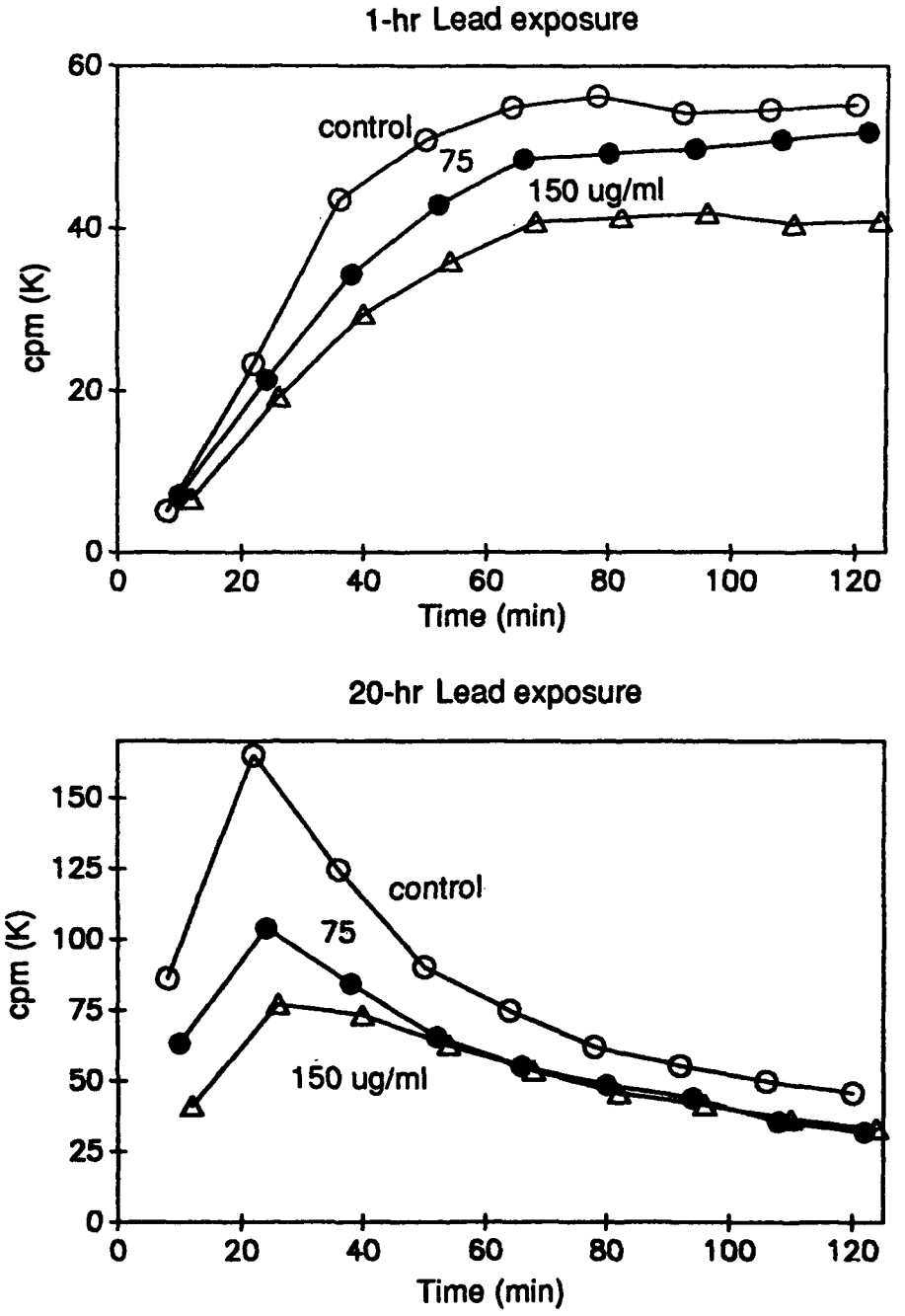

Figure 3. Inhibition of medaka CL response by exposure to lead at 75 and 150 μg/ml.

3. Inhibition of CL by Propyl Gallate

The phenolic food additive *N*-propyl gallate produced marked CL inhibition at 5-100 μg/ml in both 1-hour and 20-hour samples, both prior to and following phagocytic stimulation. Typical results for 5 and 20 μg/ml are shown in Figure 4. Peak values and total CL response were reduced >50% by 5 μg/ml and virtually eliminated by 20 μg/ml and above.

4. Inhibition of CL by Pentachlorophenol

Pentachlorophenol (PCP) is found in herbicides, microbicides, and wood preservatives; it is a common marine and estuarine contaminate that is toxic, persistent, and bioconcentrated. It is also immunotoxic in these medaka CL assays (Figures 5 and 6). At 5 and 10 μg/ml, PCP had little effect on CL after 1 or 20 hours of exposure; however, 15 μg/ml produced inhibition of peak values and total CL. Raising the concentration of PCP to 20 μg/ml (Figure 6) essentially abolished the CL response after both 1-hour and 20-hour exposures. There was no temporal displacement of the peak produced by 15 μg/ml.

5. Effect of Hydrocortisone on Medaka Macrophage CL

Hydrocortisone (HC) is an important immunomodulatory drug that has pronounced inhibitory action on mouse macrophages; its effects on fish CL were also suppressive at 1 μg/ml and higher concentrations after 20 hours of exposure. Interestingly, at 0.05 and 0.1 μg/ml, HC consistently induced CL after 20 hours (Figure 7). However, changes in peak values or total CL were never seen after only 1-hour exposure to any HC concentration tested (0.05-100 μg/ml). Furthermore, at 1 and 10 μg of HC per milliliter, the 20-hour peak CL values were reduced and delayed, but the total 20-hour CL response (over the first 2 hours of postphagocytic stimulation) was slightly induced or unchanged. After 20 hours, higher HC concentrations (Figure 8) strongly inhibited both CL peak values and total CL, as well as delayed the peak response. As was the case for the other chemicals tested, HC effects on the phagocytically stimulated CL response were dose-dependent. This was also true for the resting CL levels after 20 hours of HC exposure, prior to stimulation with yeast (Figure 9).

CONCLUSIONS

Medaka head kidney macrophages produce cytotoxic oxygen intermediates such as singlet oxygen, superoxide anion, and hydrogen peroxide, which function in immunological defense mechanisms underlying antimicrobial activity and resistance to disease. During phagocytosis, these cells generate chemiluminescence, a respiratory burst, and concomitant production of

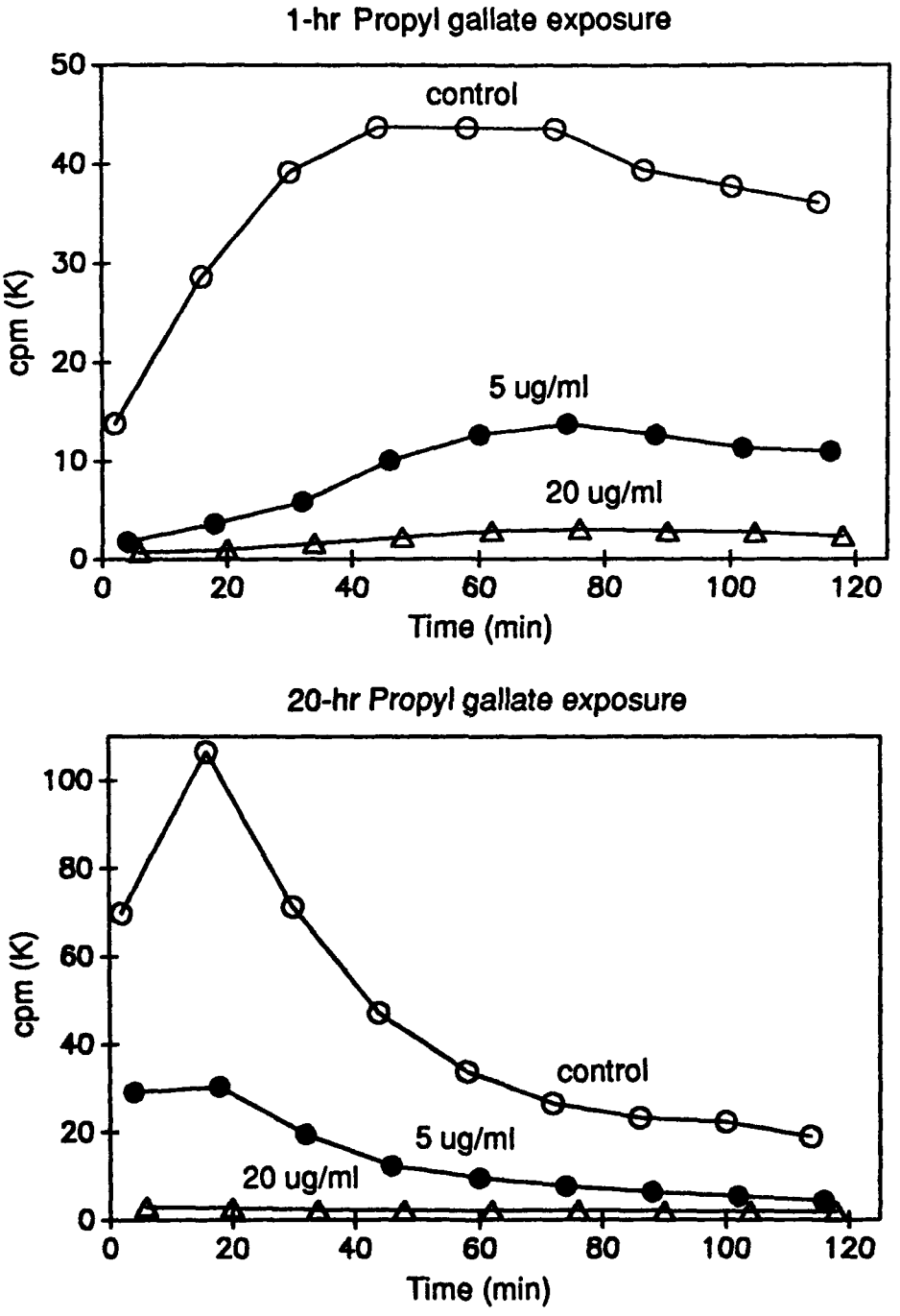

Figure 4. Inhibition of medaka CL response by exposure to propyl gallate at 5 and 20 μg/ml.

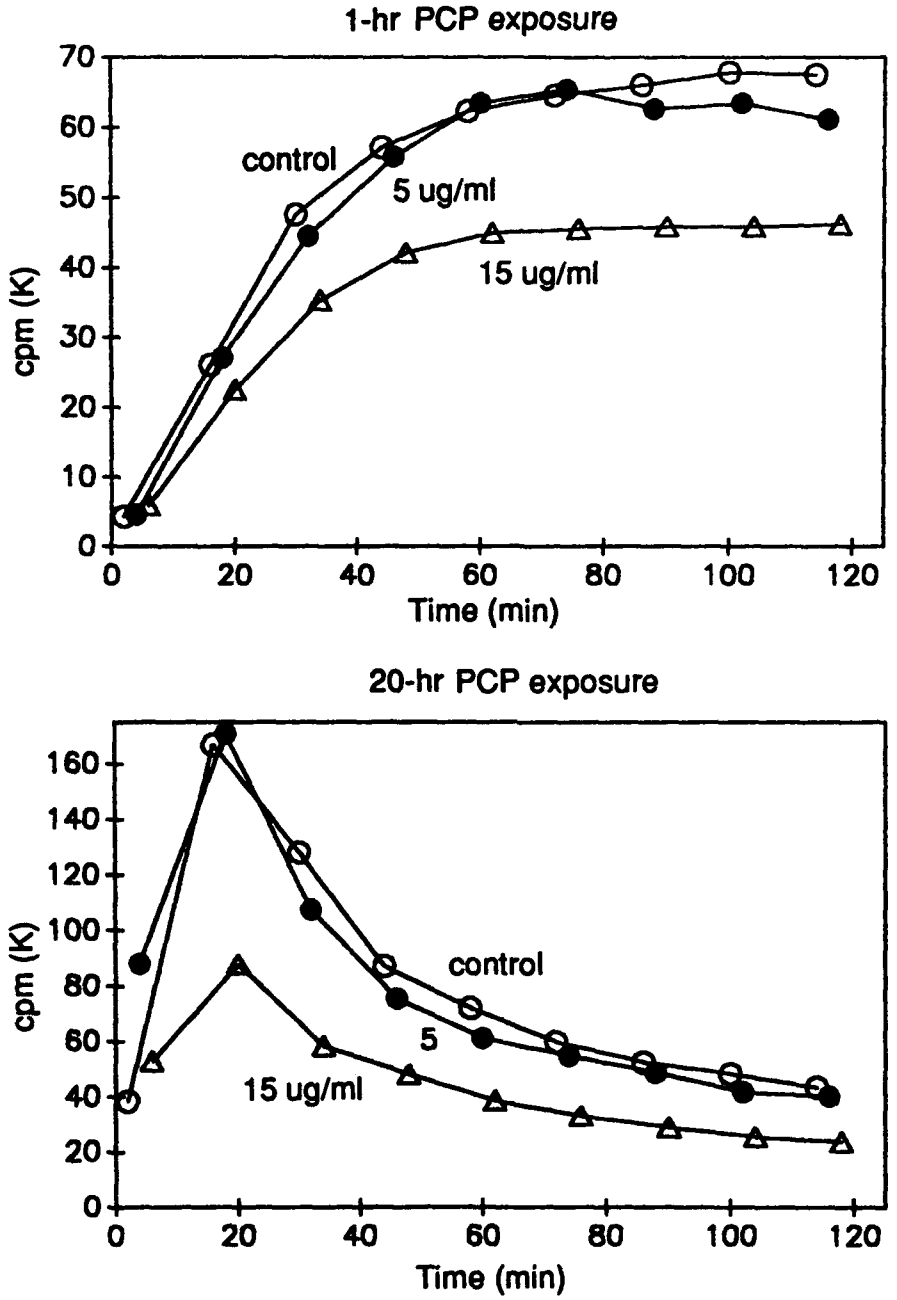

Figure 5. Inhibition of medaka CL response by exposure to pentachlorophenol (PCP) at 5 and 15 μg/ml.

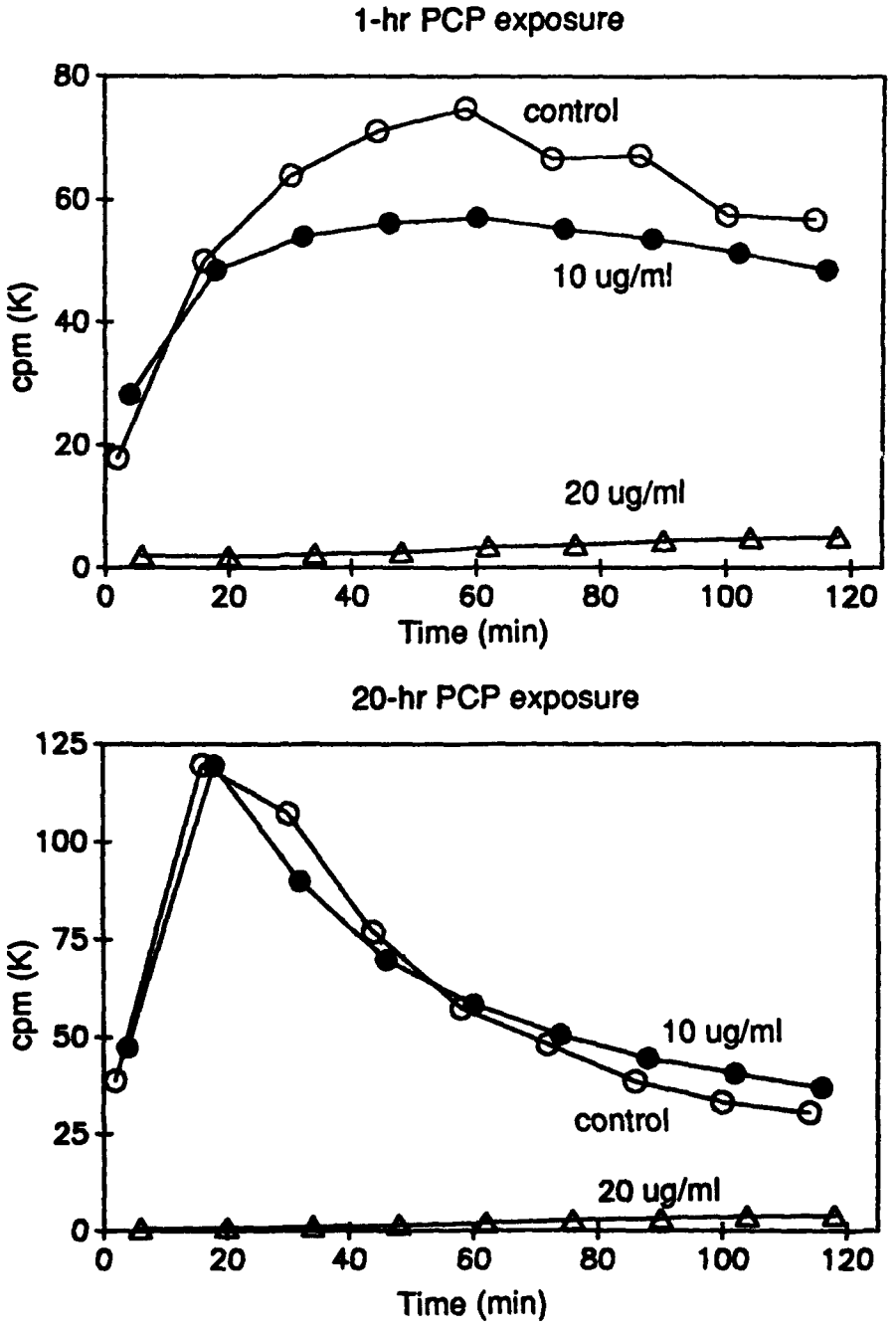

Figure 6. Inhibition of medaka CL response by exposure to pentachlorophenol (PCP) at 10 and 20 μg/ml.

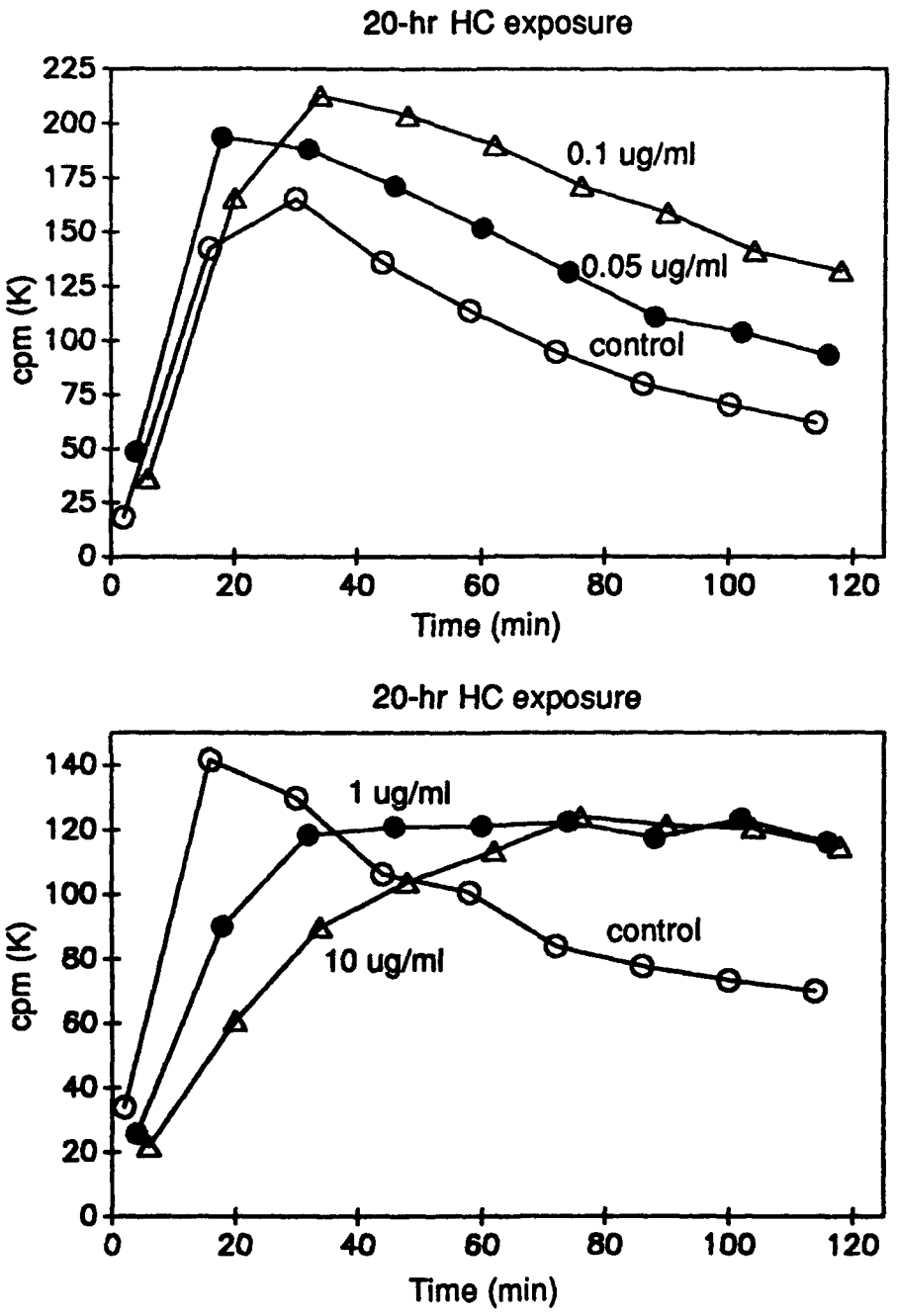

Figure 7. Effect of hydrocortisone (HC) from 0.1 to 10 μg/ml on medaka macrophage CL.

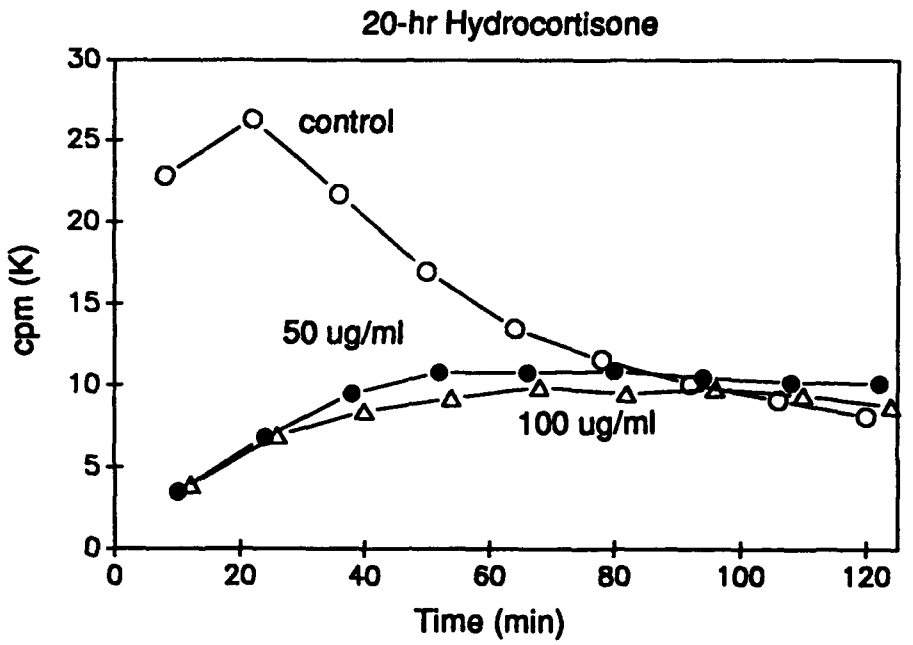

Figure 8. Effect of hydrocortisone (HC) at 50 and 100 μg/ml on medaka macrophage CL.

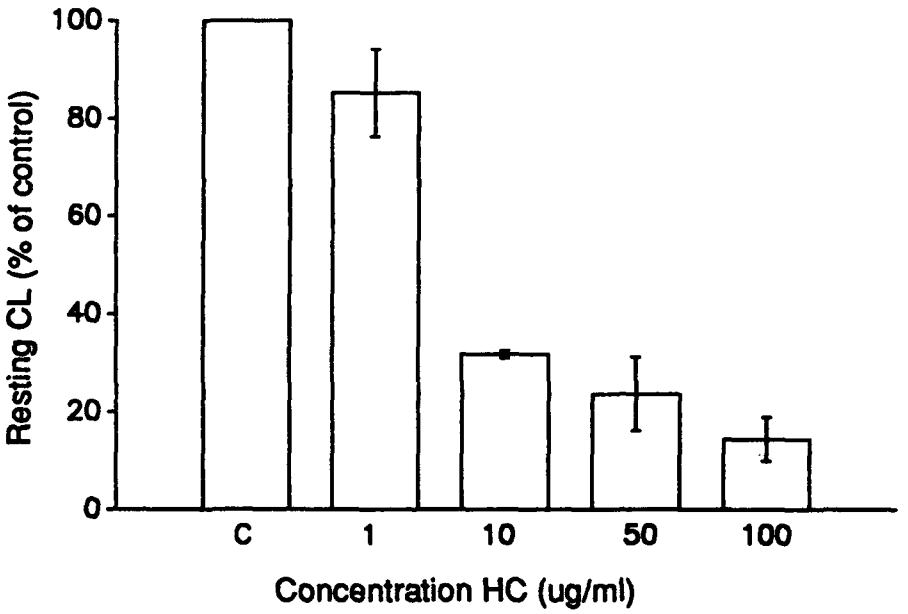

Figure 9. Resting level CL in medaka macrophages after 20-hour HC exposure.

reactive oxygen intermediates. The CL response of medaka macrophages was shown to be quite sensitive to known chemical modulators of mammalian immune functions such as cadmium, lead, hydrocortisone, and propyl gallate, as well as to the marine pollutant pentachlorophenol. The total 20-hour chemiluminescent response, peak 20-hour-induced CL response, and prestimulation (resting) CL after 20-hour exposure were all inhibited by these chemicals at various sublethal concentrations, in a dose-dependent manner. The 20-hour EC_{50} values with regard to total CL for these xenobiotics were calculated or estimated (Table 1) and compared with those recorded with female swiss mice (Tam and Hinsdill, 1990). Generally, these values are similar for the macrophages from these two species, with the fish cells' CL response slightly less sensitive to Cd, considerably less sensitive to hydrocortisone, and slightly more sensitive to propyl gallate.

The general effects of the chemicals studied to date are summarized in Table 2. Clearly, these agents can be immunosuppressive as manifested by reduced CL peak height and total CL, but the time of the peak CL activity was rarely displaced. Only higher doses of hydrocortisone displaced (delayed) peak time; lower doses of HC induced higher levels of total CL and peak CL values. This stimulatory effect, possibly via macrophage activation, was not seen with the other chemicals at any concentration. Three of the five chemical immunomodulators tested produced an altered CL response after only 1 hour, but all were effective at 20 hours, usually at lower concentrations than those effective at 1 hour. The 20-hour regimen is suggested for future work because the response of the macrophages is more rapid and intense than at 1 hour, but more sensitive to the effects of low xenobiotic concentrations.

Thus, CL is a simple, quantitative, and sensitive immunotoxicologic screening assay capable of identifying many known and putative immunomodulatory agents. It will be important to compare its sensitivity to those of other immunoassays of medaka macrophage activity such as the ROI tests already described, phagocytosis, and cell-mediated antimicrobial activity and also to study the CL activity of medaka macrophages collected from fish exposed to immunomodulators *in vivo*.

Table 1. Total CL after 20 Hours of Chemical Exposure

Chemical	Medaka EC50 (ug/ml)	Female Swiss Mice * EC50 (ug/ml)
Cadmium	~18.0	>3.2
Lead	181.1	137.0
Pentachlorophenol	15.4	?
Hydrocortisone	47.7	0.05
Propyl Gallate	~2.0	13.1

* Tam and Hinsdill (1990)

Table 2. CL Responses of Medaka Macrophages to Chemical Modulators

Chemical	Peak Height	Displaced Peak	Total CL	1-hr Modulation	20-hr Modulation	Dose-Dependent Modulation
Cd	↓	no	↓	no	yes	yes
Pb	↓	no	↓	yes	yes	yes
PCP	↓	no	↓	yes	yes	yes
HC low dose	↑	no	↑	no	yes	yes
high dose	↓	delayed	↓	no	yes	yes
Prop. Gal.	↓	no	↓	yes	yes	yes

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DNA BIOMARKERS FOR IDENTIFYING GENOTOXIC CHANGES IN ANIMAL STUDIES

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(Presented by Russom Haimanot)

INTRODUCTION

The work conducted under U.S. Army Medical Research and Development Grant No. DAMD17-88-Z-8043 is directed toward the development of methods for elucidating oxidative changes in DNA of medaka (*Oryzias latipes*) exposed to chemically contaminated groundwater. The approach taken involves the isolation and subsequent hydrolysis of DNA from liver tissue, followed by the formation of trimethylsilyl derivatives that are analyzed by gas chromatography-mass spectrometry with selected ion monitoring (GC-MS/SIM) (Dizdaroglu and Bergtold, 1986; Malins et al., 1990).

After approximately 2 years of effort, the investigators have been able to develop a method that appears to be very effective for the analysis of oxidative modifications taking place in DNA of medaka exposed to trichloroethylene. Moreover, they have also been successful in demonstrating that the livers of tumor-bearing English sole from contaminated areas of Puget Sound show substantial elevations in oxidatively modified purine nucleotides, compared to fish from essentially uncontaminated waters (Malins and Haimanot, 1990; Malins et al., 1990).

The methods developed are very sensitive and applicable to the analysis of DNA from virtually any tissue. In fact, the high sensitivity of the method has allowed the investigators to structurally elucidate and quantify trace amounts of oxidative modifications in nucleotides present in normal DNA (Malins and Haimanot, 1990; Malins et al., 1990).

Two papers on the findings have been published in 1990 and a third is in preparation. Details are described below.

BACKGROUND

The reduction of molecular oxygen in aerobic eukaryotic cells results in the formation of intermediates that are highly toxic. These include the superoxide ion, hydrogen peroxide, and the hydroxyl radical. The superoxide ion and hydrogen peroxide individually may not be particularly injurious; however, their combined action leads to the formation of the highly reactive and

damaging hydroxyl radical (Lindahl, 1982). A number of recent studies have described the interactions of the hydroxyl radical with DNA and the nature of the modifications in nucleotide derivatives. To give one example, 8-hydroxyguanosine (8-OH-dG) residues were misread in a DNA synthesis system *in vitro* with *Escherichia coli* (Kuchino et al., 1987). This finding gave rise to the hypothesis that the introduction of oxygen into the nucleotide structure, via oxygen radicals, is a critical step in carcinogenesis. Moreover, it is recognized that increased concentrations of hydroxyl radicals occur in cells as a result of a variety of chemical exposures (Lindahl, 1982). Accordingly, there is a keen interest in obtaining sensitive methods for the detection and structural elucidation of DNA modifications resulting from the attack of the reactive oxygen species on DNA.

MATERIALS AND METHODS

The approach was to develop a highly sensitive method for the determination and structural elucidation of DNA modifications in tissues of medaka. The method involves the isolation of DNA from 50 to 100 mg of tissue, the isolation of the DNA and its hydrolysis, and the preparation of trimethylsilyl derivatives for analysis by GC-MS/SIM. This approach necessitates obtaining pure nucleotide bases from commercial sources or synthesizing them from commercially available precursors. Of necessity, considerable time had to be devoted to the synthesis of oxidized nucleotide bases [e.g., 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua)] in order to carry out the required analyses. Moreover, the sensitivity of the method had to allow for the identification of trace oxidative modifications present in normal DNA so that appropriate comparisons could be made with DNA from exposed organisms. All of the above requirements have been met so that analyses are now being performed with considerable reliability.

As indicated, the methodology involves the isolation of DNA, followed by hydrolysis and the preparation of trimethylsilyl derivatives. These derivatives are then analyzed by GC-MS/SIM. Details of the procedure have been published (Malins et al., 1990) and will thus not be presented here.

RESULTS

To test the method, it was decided to take advantage of the availability of two groups of English sole from Puget Sound that were either healthy or had tumor-bearing livers that were linked to exposure to environmental chemicals (Malins et al., 1985). These fish served as an excellent model in that considerable data had been collected previously on their exposure to environmental chemicals and development of neoplasia (Malins et al., 1985). Subsequent to this

work, two groups of medaka were received from the U.S. Army Biomedical Research and Development Laboratory, one of which was normal (unexposed) and the other exposed to trichloroethylene. These samples were analyzed using the same methods employed for the analysis of the Puget Sound fish.

It was found that the DNA from the environmentally exposed fish from Puget Sound contained over 200 times the concentration of the guanine derivative FapyGua than normal fish from essentially nonpolluted environments. The nucleotide modification in DNA appeared to arise from the attack of the hydroxyl radical at the C-8 of guanine and subsequent cleavage of the purine ring. In this case, FapyGua was not detected in the DNA of the normal livers (detection limit: 0.01 nm/mg DNA). The findings demonstrated that the method was capable of readily discriminating alterations in guanine present in the DNA of neoplastic tissues. The findings also supported the view that reactive oxygen species damage DNA in living systems and thus play a major role in the initiation and/or promotion of tumors. This work was published in *Carcinogenesis* (Malins et al., 1990).

In subsequent studies with both English sole and medaka, the range of modified nucleotides analyzed was extended to include 5-hydroxyguanine, 8-hydroxyadenine, and 4,6-diamino-5-formamidopyrimidine (FapyAde). The oxidized purine derivatives were significantly elevated in DNA from the livers of English sole with tumors and the medaka that were exposed to trichloroethylene. Findings on the English sole are presented in Malins and Haimanot (1990).

It is noteworthy that none of the observed DNA lesions have been found previously in neoplastic tissue, and to the investigators' knowledge, only 8-hydroxyguanine has been previously identified in a living system.

CONCLUSIONS AND FUTURE PLANS

In the approximately 2 years of work on this grant, the investigators have essentially reached their goal of developing a sensitive method for detecting and structurally elucidating lesions in DNA bases that result from exposure to environmental chemicals. As indicated, the DNA modifications appear to result from the attack of the hydroxyl radical at the C-8 of the purine structure. Two types of products are formed: (1) hydroxylated derivatives of guanine and adenine, and (2) ring cleavage products of these purine nucleotides. The high sensitivity of the method is demonstrated by the fact that the observed oxidative modifications of the purine ring can be assessed in normal DNA as well as in DNA from chemically exposed organisms. The assumption of these investigators is that elevated levels of hydroxyl radicals would result from a variety of chemical exposures because of the positive findings with English sole (which were

primarily exposed to aromatic hydrocarbons) and similarly positive results with the medaka (which were exposed to the trichloroethylene). However, the types of exposures that give rise to elevated levels of the oxidized purine nucleotides are not adequately understood.

Overall, the investigators believe that the method employed for the analysis of oxidatively modified DNA bases in tissues has wide application to the analysis of DNA from medaka and virtually any other organism—all that is needed is the ability to isolate sufficient DNA in pure form for chemical analysis.

For the next year, it is planned to continue to perfect the method and apply it to DNA from medaka that have been exposed to different chemicals at the U.S. Army Biomedical Research and Development Laboratory. It is also planned to further explore the possibility that oxidative modifications of the pyrimidine bases also occur as a consequence of chemical exposures. Assuming that future results are as promising as those presently obtained, the investigators believe that it would be beneficial to explore the possibility of substantially reducing the required sample size, thus expanding the scope and usefulness of the procedure.

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DEVELOPMENT OF FISH *IN VITRO* HEPATOCYTE SYSTEM FOR EVALUATION OF EPIGENETIC CARCINOGENS

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ABSTRACT

Primary cultures of hepatocytes were established from rainbow trout and medaka for the short-term evaluation of epigenetic carcinogens by measuring peroxisome proliferation potential. Activation of cytochrome P-450 by acetaminophen was used as an indicator of retention of hepatocyte function for 72 hours in trout. Preliminary experiments with trout hepatocytes exposed to the hypolipidemic drugs gemfibrozil, clofibric acid, or ciprofibrate showed induction of peroxisomal beta-oxidation at 48 hours. Qualitative response of acyl-CoA oxidase activity correlated with previous trout *in vivo* studies conducted by these investigators. This system supports the use of primary cultures of fish hepatocytes as a predictive model of metabolic events *in vivo.*

INTRODUCTION

Although the rodent *in vitro* system is well characterized and used as a standard mammalian model, fish cell culture is in the early stages of development. Isolated fish hepatocytes in suspension cultures have been utilized for certain short-term (<24 hours) metabolic studies (Sheridan, 1988; Flett and Leatherland, 1989), but are limited by cellular aggregation and rapid loss of viability and function. Klaunig et al. (1985), although able to maintain viable trout hepatocytes in suspension culture for 14 days, were unable to maintain cytochrome P-450 activity after the first 2 days of culture. Their efforts to enhance attachment and spreading of hepatocytes on various substances failed (Klaunig, 1984).

Several attempts have been made to maintain differentiated characteristics in primary monolayer cultures of teleost hepatocytes. Primary cultures of eel hepatocytes grown on fibronectin-coated dishes retained the ability to synthesize glycogen and glucose for 6 days (Hayashi and Ooshiro, 1985). Koban (1986), using secretion of albumin as a test of differentiation status, was able to maintain differentiated catfish hepatocytes for 10 days on a biomatrix substrate derived from catfish liver. Maitre et al. (1986) demonstrated vitellogenin synthesis for 6 days in a

primary culture of rainbow trout hepatocytes attached to plastic dishes in serum-free medium. The use of a commercially available extracellular matrix as a substrate enabled Lipsky et al. (1986) to maintain tyrosine aminotransferase activity over a 2-day period in trout hepatocyte monolayers. Kocal et al. (1988) were able to enhance attachment and spreading of trout hepatocytes on collagen-coated dishes, by supplementing the medium with trout serum, and demonstrate cytochrome P-450 activity for 2 days.

The potential for fish *in vitro* models is great and the paucity of such systems to date emphasizes the need for further investigation. The development of this *in vitro* system is not limited in its application to peroxisome proliferators; the system provides a necessary complement to mammalian cell cultures.

MATERIALS AND METHODS

Animals

Rainbow trout (*Salmo gairdneri*) weighing 300-350 grams were obtained from the Massachusetts Division of Fisheries and Wildlife fish hatchery in Sunderland, Massachusetts. Medaka (*Oryzias latipes*) 6 months in age were obtained from Carolina Biological Supply Company, Burlington, North Carolina.

Hepatocyte Isolation

Rainbow Trout

The procedure used by these investigators was based on the two-step hepatic portal perfusion for rodents (Seglen, 1973) and modified for fish (Moon et al., 1985; Lipsky et al., 1986; Maitre et al., 1986; Kocal et al., 1988). In order to facilitate the perfusion technique, the perfusion was not done *in situ*. Trout were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Company, St. Louis, MO), placed on a surgical board, and incised from the urogenital pore to the gills. The bile was carefully removed from the gall bladder with a 1-cc tuberculin syringe. The liver was then separated from the gall bladder and transferred to a sterile petri dish. The sinus venosus was cannulated using a blunt 18-gauge needle and ligated. Initially, the liver was perfused with 10 mM HEPES buffer, pH 7.4, containing 136.9 mM NaCl, 3.4 mM KCl, 0.4 mM KH_2PO_4, 0.4 mM Na_2HPO_4, 4.1 mM $NaHCO_3$, 5.5 mM glucose, 0.1 mM EGTA, followed by perfusion with the same buffer without EGTA but containing 1 mg/ml collagenase (Type II, Worthington Biochemical Corp., Freehold, NJ) and 5.1 mM $CaCl_2$. During perfusion with the dissociating solutions, the liver was gently massaged to increase both blood clearance and hepatocyte yield (Moon et al., 1985). At the end of the perfusion, the liver was washed with

HEPES buffer and the hepatocytes were dissociated, by using a sterile pipet and forceps. The hepatocytes were passed sequentially through 500, 250, 150, and 75 gauge Nitex screening (Tetko, Inc., Elmsford, NY). The cell suspension was then centrifuged for 2 minutes at 35 × g. The pellet was resuspended in culture medium and centrifuged for 2 minutes at 35 × g. The final pellet was resuspended in culture medium. Viability was determined by a trypan blue exclusion test.

Medaka

Medaka were anesthetized with MS-222, transferred to a dissecting tray, and incised from the urogenital pore to the gills. The liver was removed, separated from the gall bladder, and transferred to a sterile beaker containing 3 ml of 0.5% trypsin, 5.3 mM EDTA (Gibco Laboratories, Grand Island, NY) reconstituted in the HEPES buffer described above, and stirred for 10 minutes at room temperature. At the end of this time, most of the liver was dissociated. The cell suspension was transferred to microcentrifuge tubes and centrifuged for 2 minutes at 35 × g. The pellets were resuspended in culture medium and centrifuged for 2 minutes at 35 × g. The final pellet was resuspended in culture medium. Viability was determined by a trypan blue exclusion test.

Culture Conditions

Rainbow Trout

Chemicals were obtained from Gibco Laboratories unless otherwise stated. The culture medium, based on previous trout studies (Lipsky et al., 1986; Maitre et al., 1986; Kocal et al., 1988), consisted of Leibowitz L-15, 10 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg), amphotericin B (2.5 μg/ml, Sigma), insulin (1 μg/ml, pH 7.6), with or without fetal bovine serum. Two and a half million cells were plated on 35 × 10 mm (Nunc/Vanguard) or 4.6 million cells on 60 × 15 mm (Primaria, Beckton-Dickinson, Co.) plastic dishes with or without adsorbed collagen (Type VII, Sigma) as substrate. During preliminary studies to determine the effect of fetal bovine serum and substrate on attachment and viability, cultures were incubated at room temperature in 100% air. All subsequent studies utilized cultures incubated at 15°C in 100% air. The medium was changed every 24 hours. For evaluation of epigenetic carcinogens, 10 μM hydrocortisone (Sigma) was added to the medium to ensure maximum peroxisome proliferation (Mitchell et al., 1984). Stock solutions of agents were prepared in dimethyl sulfoxide (DMSO) and added to the medium to give a final DMSO concentration of 0.1%. DMSO control cultures were exposed to medium containing 0.1% DMSO without the agent. Treatment was initiated 5 to 6 hours after

plating. Medium and test compounds were changed every 24 hours. Viability was checked daily by a trypan blue exclusion test.

Medaka

The culture medium was the same as that used for trout without 10% fetal bovine serum. The cells isolated from one liver were resuspended in 1 ml of culture medium, and 0.5-ml aliquots were distributed to two wells of a 24-well culture dish (Corning, Fisher Scientific, Pittsburgh, PA). Cultures were incubated at ambient temperature in 100% air. The medium was changed every 24 hours. Viability was checked daily by a trypan blue exclusion test.

Cytochrome P-450 Activation

Beginning 4 hours after plating, cells were exposed to 16 mM acetaminophen (Sigma) as described by Kocal et al. (1988). Lactate dehydrogenase (LDH) activity in the acetaminophen-treated cultures was compared with that in control cultures as an indication of retention of hepatocyte function. LDH activity was measured with a Sigma LDH assay kit based on the method of Wroblewski and LaDue (1955).

Preparation of Cells for Acyl-CoA Oxidase Assay

The procedure used is a modification of Small et al. (1985). Hepatocytes were detached from the culture dish by gently pipetting the medium. Cell suspension was transferred to a plastic centrifuge tube, which was centrifuged 2 minutes at 35 × g. Supernatant was removed and cells were resuspended in 10% sucrose, 3 mM imidazole (SI) buffer. Triton X-100 was added to give a final concentration of 1%. The suspension was set on ice for 10 minutes, followed by centrifugation for 10 minutes at 6,000 × g. Supernatant was saved for analysis. Protein was determined by the BCA Protein Assay (Pierce Chemical Co., Rockford, IL).

Acyl-CoA Oxidase Activity

The activity of peroxisomal acyl-CoA oxidase was measured according to the method of Small et al. (1985).

RESULTS

Hepatocyte Isolation

Increased viability, attachment, and spreading of cells were observed when time and handling during isolation and plating procedures were minimized for both rainbow trout and medaka.

Culture Conditions

Rainbow Trout

The formation of hepatocyte monolayers was observed under all conditions tested. No difference in cell viability or longevity was observed between the two commercially tested plastic culture dishes. In this system, 10% fetal bovine serum did not facilitate attachment, viability, or longevity of cultures. Cultures grown at 15°C remained viable longer than those at room temperature. The investigators were able to maintain viable cells up to 7 days as measured by trypan blue exclusion.

Medaka

Hepatocytes attached and spread on plastic multiwell dishes at ambient temperature. Under conditions described above, the investigators were able to maintain viable cells up to 5 days as measured by trypan blue exclusion.

Cytochrome P-450 Activation

Table 1 shows the percentage of lactate dehydrogenase (%LDH) released over a 72-hour period. No difference was observed between control and DMSO control groups. Cultures exposed to 16 mM acetaminophen showed a greater percentage of LDH release compared to controls at all time points.

Acyl-CoA Oxidase Activity

Table 2 shows preliminary acyl-CoA oxidase activity data qualitatively expressed as a multiple of the increase over vehicle control. Cultures exposed to gemfibrozil, clofibric acid, or ciprofibrate for 48 hours showed induction of peroxisomal beta-oxidation.

Table 1. Acetaminophen Toxicity in Primary Cultures of Rainbow Trout Hepatocytes

Treatment	% LDH Release		
	24 hr	48 hr	72 hr
Control	3.7	10.7	19.5
DMSO Control	2.8	8.5	18.8
16 mM Acetaminophen	31.6	48.0	73.9

Table 2. Preliminary Acyl-CoA Oxidase Data Showing Qualitative Response of Trout Hepatocytes to Hypolipidemic Drugs

Treatment	Dose (mM)	*x*-Fold Increase Over Vehicle Control
Gemfibrozil	1.0	1.3
	1.5	1.0
	2.0	1.8
Clofibric acid	2.0	1.0
	3.0	2.1
	4.0	1.6
Ciprofibrate	0.5	1.9
	1.0	1.2
	1.5	1.6

DISCUSSION

The aim was to develop an *in vitro* fish hepatocyte system to serve as a predictive model for epigenetic carcinogens. The initial strategy was to culture cells in a serum-free medium to allow the study of cellular behavior under chemically defined and controlled conditions. However, since serum was used as a medium supplement in some trout studies (Lipsky et al., 1986; Kocal et al., 1988), the investigators tested the effect of 10% fetal bovine serum in their system. Based on their preliminary studies in which fetal bovine serum showed no effect on cell attachment,

spreading, and viability over time, these investigators have eliminated it from their medium components. A positive influence of lower temperature on longevity of trout cultures was expected. The results support previous *in vitro* fish studies that showed improved viability at lower temperatures (Klaunig et al., 1985) and enhanced cell attachment (Haschemeyer and Matthews, 1983; Kocal et al., 1988).

It has been shown that rainbow trout are sensitive to the cytocidal toxicity of acetaminophen, which requires cytochrome P-450 activation (Parker et al., 1981). Initial results with acetaminophen-treated cultures support the data of Kocal et al. (1988), which showed a 27.8% and 48.9% LDH release in trout hepatocyte cultures treated with 16 mM acetaminophen at 24 hours and 48 hours, respectively. Even though there was a decrease in viability, as evidenced by increasing %LDH release in control cultures over time, acetaminophen-treated cultures still showed greater %LDH release than controls at 72 hours, indicating retention of metabolic function. In contrast to Kocal et al. (1988), however, these investigators saw no difference in %LDH release between control and DMSO control cultures. Further experiments will be conducted to verify these results.

Preliminary experiments show induction of peroxisomal beta-oxidation as measured by acyl-CoA oxidase activity at 48 hours in cultures treated with the hypolipidemic drugs gemfibrozil, clofibric acid, or ciprofibrate. Interpretation of results at high doses is complicated by chemical toxicity, and further studies are needed to clarify the response. However, initial observations indicate good correlation with the investigators' previous *in vivo* studies with trout, which showed a 1.4-, 1.3-, and 1.8-fold increase in acyl-CoA oxidase activity over control following treatment with gemfibrozil, clofibric acid, or ciprofibrate, respectively (Yang et al., in press; L.J. Scarano et al., unpublished).

These investigators have developed isolation procedures and culture conditions for establishing primary cultures of rainbow trout and medaka hepatocytes. Under these conditions, they are able to maintain liver functions in trout hepatocytes for 72 hours and demonstrate induction of peroxisomal beta-oxidation at 48 hours. Currently, they are in the process of characterizing medaka cultures for metabolic activity and ability to respond to peroxisome proliferators. Their *in vitro* system supports the use of fish hepatocytes as a predictive model of metabolic events *in vivo* and provides an important tool to enhance understanding of the role of liver metabolism in the process of carcinogenesis.

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MOLECULAR AND CELLULAR MARKERS OF TOXICITY IN THE JAPANESE MEDAKA (*Oryzias latipes*)

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ABSTRACT

The Japanese medaka (*Oryzias latipes*) has been recommended for use as a model organism to detect carcinogenic, teratogenic, cytotoxic, and genotoxic compounds in aquatic systems. Studies with the medaka from other laboratories have focused on carcinogenesis, with particular emphasis on the histopathological identification of neoplastic lesions in the tissues of animals exposed to carcinogens. Because a long latent period often occurs between initial contact with deleterious chemicals and subsequent expression of the pathology, we are investigating early biologically relevant responses that can be used as genotoxicity markers of exposure and effect. This project focuses on the development of genotoxic bioassays and experimental protocols for exposing Japanese medaka to genotoxic compounds.

Biomarker responses in the Japanese medaka exposed to benzo[*a*]pyrene (BaP) were evaluated over a 16-day exposure period. Data were obtained on BaP uptake, DNA integrity (strand breaks, DNA distribution), detoxification enzymes, and reproductive and developmental parameters. Interpretation of the data obtained to date is consistent with the occurrence of a minor physiological response in the medaka to BaP shortly after exposure. This response was observed as an increase in fecundity and in the number of ovarian vitellogenic oocytes; however, we did not detect concomitant genotoxic effects as evidenced by DNA strand breaks.

INTRODUCTION

The Japanese medaka (*Oryzias latipes*) has been recommended as a model organism for detecting carcinogenic, teratogenic, cytotoxic, and genotoxic compounds in aquatic systems (Ishikawa et al., 1975; Donaldson and Scherer, 1983). Previous studies with the medaka have focused on carcinogenesis, with particular emphasis on the histopathological identification of neoplastic lesions in the tissues of animals exposed to carcinogens. Neoplastic histogenesis in medaka follows a course similar to that observed in rodents: tumor formation is preceded by preneoplastic lesions, which appear as both eosinophilic and basophilic foci (Hawkins et al., 1988).

A number of biological end points must be evaluated to measure biomarkers of toxicity in an organism and to determine the degree of exposure the organism has had to xenobiotic compounds. Biological markers can be used to measure both the short- and long-term effects of exposure to toxic substances (Shugart et al., 1987*a*). Early exposure to xenobiotic compounds may be detected via the interaction of such compounds with cellular macromolecules such as DNA, RNA, and proteins (Kurelec, 1990), changes in the activities and levels of detoxification enzymes, the induction of mutations, or repair of damaged DNA. Changes in DNA sequence can lead to both heritable mutations and carcinogenesis. The interactions of xenobiotics with cellular macromolecules can perturb metabolism and result in cytotoxic effects. Longer term responses to toxic substances can be evaluated by studying neoplastic transformation, developmental (teratogenic) abnormalities, survivability, fecundity, immunocompetence, or pathology.

In the medaka, the establishment of experimental protocols for exposure to xenobiotic compounds is particularly important to the development of bioassays for measuring toxicity in this fish. Three cytotoxic and genotoxic compounds were selected for study: diethylnitrosamine (DEN), benzo[*a*]pyrene (BaP), and acetylaminofluorene (AAF) (Taningher et al., 1990). Medaka are currently being exposed to these compounds either by injection or in water. This report will focus on recent experiments in which medaka were exposed to BaP dissolved in water.

Experiments and bioassays currently underway at this laboratory to assay exposure of the Japanese medaka to BaP are (1) determination of the uptake of radiolabeled BaP from water and accumulation of radiolabel in the whole organism; (2) measurement of DNA damage and/or modification by quantitating DNA strand breakage, 5-methyl deoxycytosine content, and DNA adducts; (3) toxicant-induced changes in reproductive physiology and fecundity; (4) teratogenic studies of the development and hatchability of embryos exposed to xenobiotics; (5) mixed-function oxidases and other detoxification enzyme assays; (6) flow cytometry and histopathology, and (7) evaluation and modification of current protocols and development of new bioassays. The sections that follow will discuss DNA damage (alkaline unwinding), reproductive and developmental effects of BaP exposure, and determining the rate of BaP accumulation in the medaka during aqueous exposure.

MATERIALS AND METHODS

Uptake and Accumulation of Radiolabeled BaP

Twenty-five fish (Japanese medaka; 15 female and 10 male) were maintained in one 20-liter aquarium. Water in the aquarium was amended with [^{14}C]BaP + unlabeled BaP in 1% Tween 80 (Kennedy et al., 1989) on days 0, 2, 4, and 6 of an 8-day exposure. The stock solution of

[^{14}C]BaP was prepared in toluene (specific activity 12.1 mCi/nmol; 1 μl stock solution = 1 μCi = 21 ng BaP). To prepare BaP + [^{14}C]BaP-saturated water, 30 μl of [^{14}C]BaP stock in toluene was dried and dissolved in 100% methanol; the [^{14}C]BaP was purified by high-performance liquid chromatography (HPLC) on a Varian 5000 Liquid Chromatograph equipped with a 250 x 4.6 mm C18 column. Fluorescence was detected with a Schoeffel FS-970 fluorometer with an excitation monochrometer set at 246 nm and a 370-nm emission filter. An isocratic flow system using 100% methanol as solvent was used to elute purified BaP. The [^{14}C]BaP eluted from the C18 column was dried and dissolved in 7.5 ml of 1% Tween 80 containing 40 μg of unlabeled BaP per milliliter; 250 μl of this solution was added per liter of aquarium water (each liter contains: 1 μCi [^{14}C]BaP/21 ng [^{14}C]BaP plus 10 μg unlabeled BaP).

Medaka were exposed for 8 days. Each day, 10 ml of water was removed and extracted twice with 1 ml of methylene chloride. The extract was centrifuged; the methylene chloride layer was separated, dried by evaporation, and resuspended in 1 ml of methanol. Replicate samples were analyzed by HPLC as previously described. On sampling days 0, 1, 2, 4, and 8, five fish (3 female and 2 male) were sacrificed by cervical scission. The liver, gonads, and carcass of the fish were analyzed separately for ^{14}C content. Frozen tissue was minced and homogenized in 5 volumes of 0.02 M Tris (pH 7.4) for 1 minute using a Virtis Model 45 macro homogenizer. Tissue was treated with pronase (77.5 U/mg in 10 ml buffer; 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM NaCl) by adding 1 ml of enzyme/500 mg tissue and incubating for 2 hours at 38°C. Two milliliters of the digest was dispersed in 10 ml of scintillation cocktail and analyzed for ^{14}C content by liquid scintillation spectroscopy.

BaP Exposure Experiment I

Fish were maintained at 25-26°C in 30-liter glass aquaria with a photoperiod of 16 hours of light and 8 hours of dark. Three static tanks each contained 70 fish; the female to male ratio was 3:2. Fish were fed both flake food (Stress Flakes) and freshly hatched brine shrimp daily. One-third of the water volume was changed every 48 hours by siphoning; the contaminated water was disposed of through a charcoal filter, and fresh water saturated with BaP was added to replace the water removed.

One aquarium contained 10 μg of BaP per liter and 1% Tween 80 (BaP tank); BaP was added to the water from a concentrated stock solution with 40 μg of BaP per milliliter in 1% Tween 80. The second aquarium was a carrier control, and contained only 1% Tween 80 (Tween tank). The third tank was a clean control (control tank). Ten fish (6 females and 4 males) were sampled from each tank on days 1, 2, 4, 8, and 16. A day 0 sample was also taken from the control tank. Before

fish were removed from the tanks, eggs were harvested from females. Water changes on sampling days were made after sample fish were removed.

Fish were sacrificed by cervical scission and the livers, gills, and gonads were removed for analysis. The livers were weighed and placed into small Eppendorf tubes; the tubes were immediately dropped into liquid nitrogen for subsequent DNA alkaline unwinding analysis using a miniprep procedure adopted from Shugart (1988). A pooled sample of 20 mg of tissue was homogenized in a 1.5-ml Eppendorf microcentrifuge tube in 100 μl of 1 N NH_4OH/0.2% Triton X-100 followed by addition of 500 μl of G-50 buffer (150 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 1 mM $MgCl_2$), pH 7.4. Proteins were removed by extracting the sample with 700 μl phenol-chloroform-isoamyl alcohol (CIP) followed by extraction with 700 μl of chloroform. The aqueous layer was removed after centrifugation, and G-50 buffer in 1 M NaCl was added to a final volume of 2 ml. The samples were then analyzed for DNA alkaline unwinding.

The ovaries were weighed and placed in L-15 culture medium. The carcass was placed in 1.5-ml Eppendorf tubes and stored in liquid nitrogen. Ovarian tissue was examined for oocyte size, fecundity (daily clutch size), numbers of vitellogenic oocytes, and oocyte atresia (Greeley et al., 1987; Lin et al., 1989).

Fertilized eggs were placed into hatching solution immediately after being removed from the females (medaka hatching solution: 0.017 M NaCl, 0.40 mM KCl, 0.27 mM $CaCl_2$ $2H_2O$, 0.66 M $MgSO_4$ $7H_2O$, and methylene blue at 1 mg/l). The eggs were separated with fine forceps under a dissection microscope, and each individual clutch was divided into equal numbers and placed into petri dishes (60 x 15 mm). Replicate dishes held between 9 and 25 eggs. The width of the perivitelline space was determined by subtracting the outer sphere diameter from the inner sphere diameter by measurement (in millimeters) under a dissection microscope for each individual sample. Eggs were then incubated at room temperature and allowed to develop. These were observed daily for embryonic death and developmental staging (Kirchen and West, 1976, Carolina Biological Supply, Inc.). Death of the embryo was determined by uptake of the methylene blue dye. Time to hatch and hatching success were also recorded. Fry that hatched were moved to small culture dishes and fed from cultures of mixed paramecium and mixed nematodes for 8 days. They were then fed a regular diet of brine shrimp and Stress Flakes.

BaP Exposure Experiment II

Adult medaka (male and female) were divided into four treatment groups with 5 fish per tank: (1) 15 fish, untreated control; (2) 15 fish treated with verapamil (1 μg/l); (3) 15 fish treated with BaP (4 μg/l); and (4) 15 fish treated with verapamil (Kurelec and Pivcevic, 1989), which

inhibits the p170 glycoprotein pump and subsequent removal of xenobiotic compounds from cells, (1 μg/l) and BaP (4 μg/l). Medaka from each group were maintained in three aquaria containing 8 liters of water each. BaP-saturated water was prepared by passage through a Generator column containing glass beads coated with BaP (Shiu et al., 1988). No additional BaP water was added to the BaP and the BaP plus verapamil tanks during the experiment. Tanks that contained verapamil were given a daily dose of verapamil dissolved in methanol; the doses were sufficient to deliver a final concentration of 1 μg/l of the drug. After 7 days of exposure, the medaka were sacrificed by spinal scission. The DNA from individual whole fish was extracted and analyzed using the alkaline unwinding assay as described by Shugart (1988).

BaP Exposure Experiment III

Adult medaka (male and female) were exposed for 42 hours to BaP and BaP plus verapamil in a small scale follow-up miniexposure experiment. Three treatment groups were (1) 6 fish, untreated control; (2) 6 fish, treated with BaP (4 μg/l); and (3) 6 fish treated with verapamil (1 μg/l) plus BaP (4 μg/l). The fish were maintained in small aquaria, each of which contained 5 liters of water. BaP concentrations were maintained at a constant level by continuous saturation with a BaP generator. Verapamil, dissolved in methanol, was added daily to a concentration of 1 μg/l.

After 42 hours of exposure, the fish were sacrificed by spinal scission, and DNA was purified from whole individual fish by a modified procedure adopted from Maniatis et al. (1982). Whole fish were homogenized in a 10-ml glass homogenization tube with ten strokes of a Teflon pestle in 3 ml of extraction buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 250 mM NaCl). To the homogenate was added 250 μl of 10% Sarkosyl and 50 μl of RNase (2 mg/ml stock, DNase-free RNase). The mixture was incubated at 50°C for 30 minutes to digest RNA. Incubation continued for an additional 60 minutes after 6 μl of proteinase K solution (10 mg/ml stock solution) was added. The contents of the tubes were gently shaken during incubation to ensure complete digestion of RNA and protein.

After incubation, the digested samples were transferred to 6 ml of SST serum separation vacutainer tubes. An equal volume of CIP (phenol-chloroform-isoamyl alcohol; 25:24:1) was added and the tubes mixed by inversion for 5 minutes. They were then centrifuged at 3,000 × g for 20 minutes at room temperature, and the aqueous phase was reextracted in chloroform to remove traces of phenol. The aqueous phase was collected, and the DNA in this phase was precipitated by adding 2 volumes of cold 100% ethanol. DNA was pelleted by centrifugation at 3,000 × g for 20 minutes before being briefly dried in a vacuum and resuspended in 3.5 ml of

alkaline unwinding buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA and 250 mM NaCl). Alkaline unwinding analysis was performed according to the procedure described by Shugart (1988).

RESULTS

Uptake and Accumulation of Radiolabeled BaP

Benzo[*a*]pyrene in the water during the 8-day experiment declined following each water renewal event, and radiolabeled [^{14}C]BaP accumulated in the medaka (Figures 1 and 2). The initial rate of [^{14}C]BaP accumulation into whole body tissues of the fish was high; during the remainder of the exposure time, it was lower (Figure 2). BaP appeared to concentrate more extensively in the tissues of female medaka than in the tissues of the males (Figure 3).

BaP Exposure Experiment I

The reproductive parameters (Table 1; days 1 and 16 of exposure) indicate that the fecundity of BaP-treated fish increased by approximately 250% during the 16-day experiment. In fish treated with Tween 80 only, fecundity actually decreased by 70%. The number of immature vitellogenic oocytes in the ovaries of fish treated with BaP increased by about 130% during the 16-day experiment, whereas the number of vitellogenic oocytes in ovaries of fish treated with Tween 80 did not change. No apparent difference was seen in the incidence of oocyte atresia between the BaP- and Tween 80-treated fish.

The results from the developmental studies are summarized in Table 2. No significant difference (*t* test) was found in the overall size of the eggs, the inner diameters, or the difference between the two. These measurements were made under a dissection microscope within 6 hours of fertilization, and the data from each set of replicate plates were pooled for this analysis. Exposure to BaP and Tween 80 had no obvious effect on the morphology of fertilized eggs. The average number of days over which hatching occurred was obtained by monitoring replicate plates within each treatment group. For example, on day 0, there were three replicate plates of embryos that hatched over a 4½-day period. In contrast, controls on day 16 had two replicate plates, and the eggs on these plates required an average of 8 days from the first hatch to the last hatch. No significant difference (*t* test) was found between the groups when analyzed this way. All replicate plates were pooled for determination of the time of fertilization to the last hatch. No significant differences (*t* test) were seen between groups. The percent survival of successfully hatched fry in each group is given up to April 12, 1990 (about a 30-day period). Since all fry from individual petri dishes were maintained in the same culture, it was impossible to separate

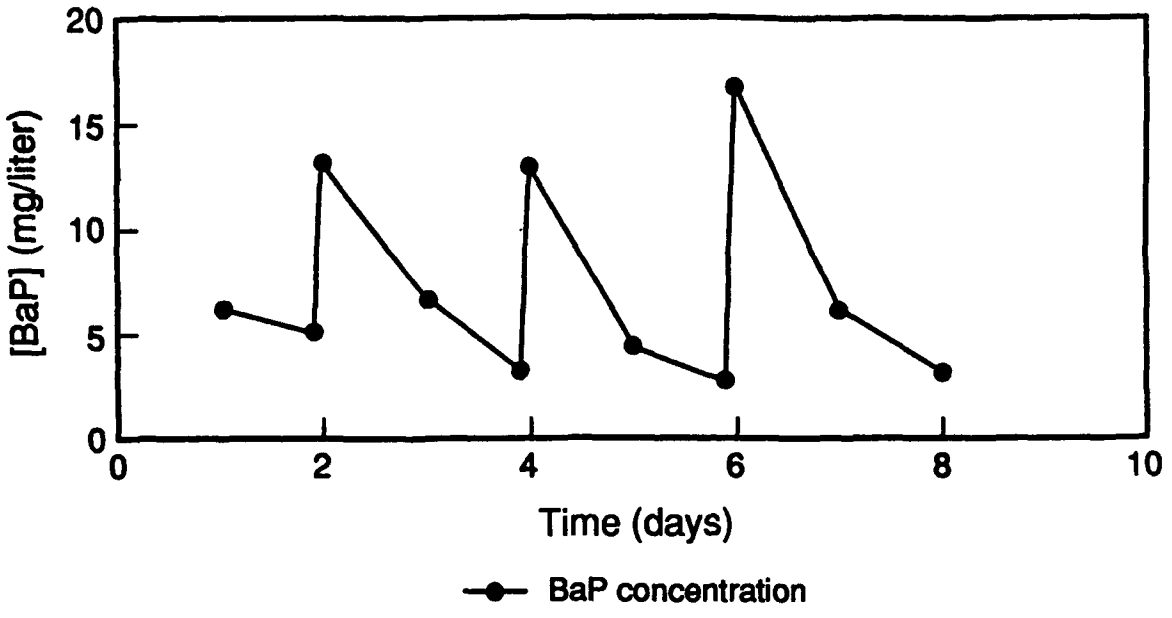

Figure 1. Concentration of BaP in water during medaka exposure (8 days).

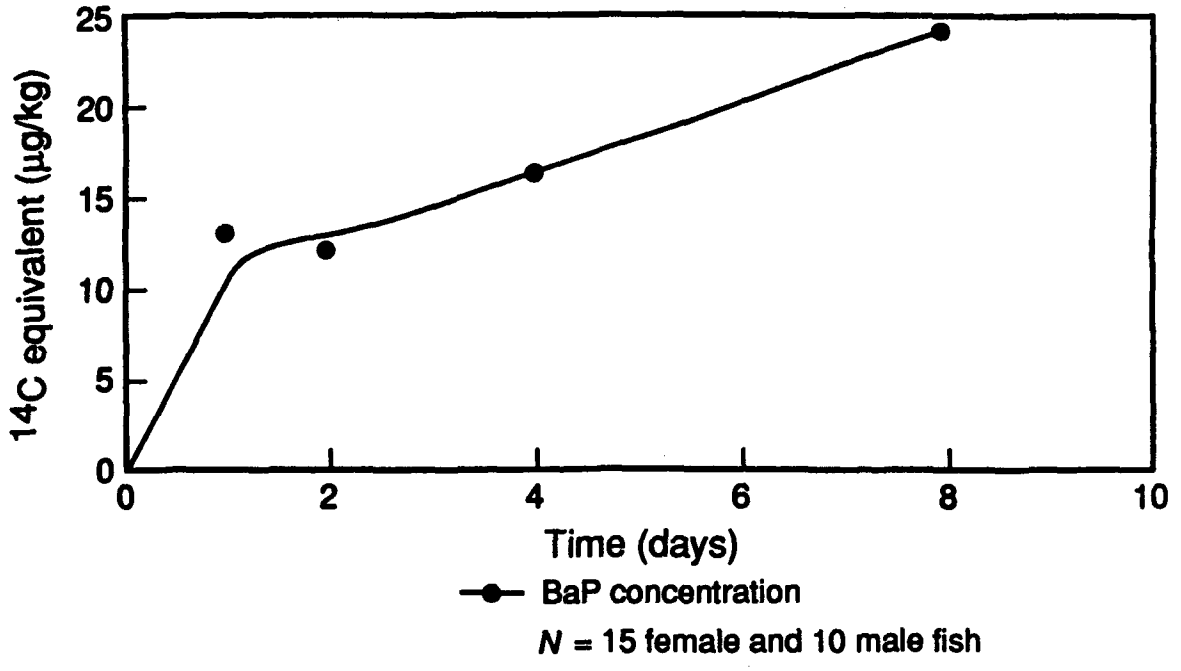

Figure 2. Accumulation of BaP in whole-body tissues of adult medaka.

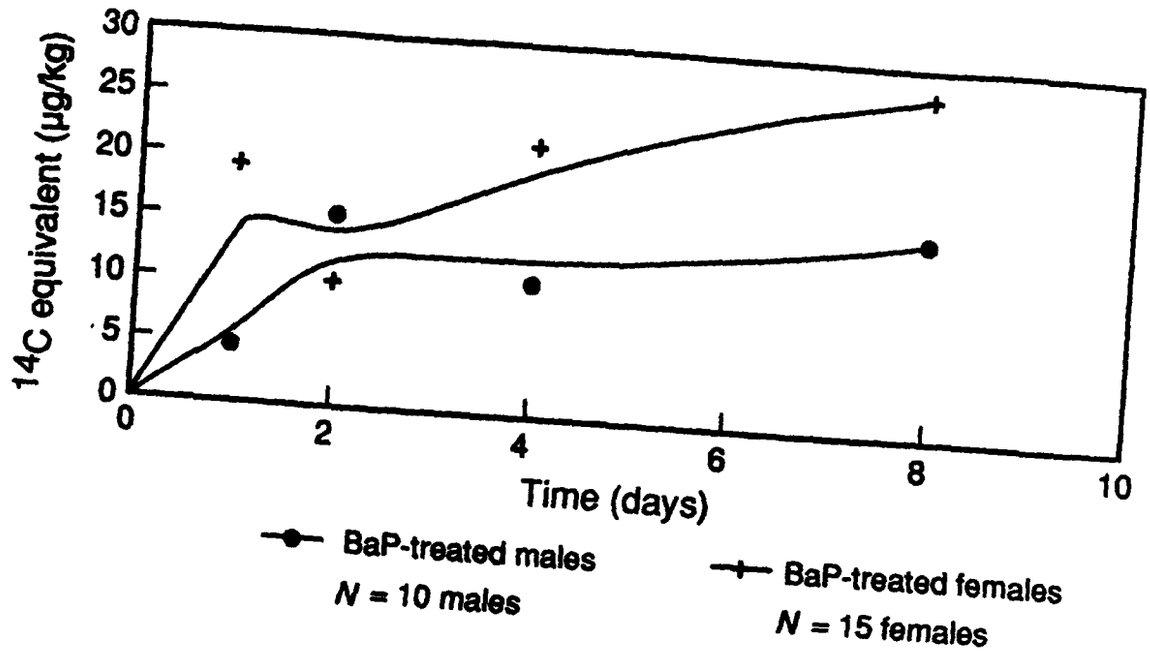

Figure 3. Comparison of accumulation of BaP in tissues of adult medaka, males versus females.

deaths that occurred as a result of transfer of fry with glass pipettes from any that might have been caused by exposure to BaP or Tween 80.

Alkaline unwinding analysis was performed on liver DNA from the medaka using the mini-prep method. The alkaline unwinding F values (% of the DNA in double-stranded form) are shown at days 0, 1, 2, 4, 8, and 16 (Figure 4). The means of the alkaline unwinding F values within the three treatment groups were quite variable and not significantly different on day 16 of exposure (ANOVA: $F = 0.774$; $F_{0.05},2,6 = 5.14$, $n = 3$, $N = 9$, $k = 3$; $p > 0.25$).

BaP Exposure Experiment II

The alkaline unwinding F values from the second BaP experiment are shown in Figure 5. The F values for these four treatment groups were essentially the same except that DNA from fish treated with both verapamil and BaP had higher F values at 38°C (ANOVA: $F = 152.3$; $F_{0.05},3,32 = 2.92$, $n = 9$, $N = 36$, $k = 4$; $p < 0.001$). Medaka DNA from all four exposure groups was quite resistant to alkaline unwinding even at a temperature of 50°C.

Figure 6 shows a comparison of the extent of alkaline unwinding (measured by the decrease in fluorescence of DNA + Hoechst dye #33258) of BaP-exposed medaka DNA with increasing

Table 1. Medaka Reproductic .

Day	Treatment	Female GSI[a]	Fecundity[b]	Atresia[c]	Vitellogenic Oocytes[d]
1	Control	5.5 ± 0.4[e]	6.1 ± 1.1	0.3 ± 0.2	68.2 ± 7.6
	Tween 80	4.5 ± 0.9	8.5 ± 2.8	0.7 ± 0.7	68.8 ± 18.5
	BaP	5.3 ± 0.3	5.5 ± 1.7	6.5 ± 5.5	85.8 ± 19.4
16	Control	5.7 ± 0.5	11.3 ± 2.4	1.3 ± 0.8	94.5 ± 16.1
	Tween 80	4.4 ± 0.5	5.8 ± 1.1	1.5 ± 0.6	70.2 ± 6.5
	BaP	6.0 ± 0.3	13.3 ± 1.4	0.8 ± 0.5	113.5 ± 10.8

[a]GSI: Gonadal Somatic Index; (ovary weight/body wt) × 100.
[b]Fecundity: the number of mature eggs released per ovary or daily clutch size.
[c]Atresia: the number of dead or damaged oocytes per ovary.
[d]Vitellogenic Oocytes: the number of yolk-containing oocytes per ovary.
[e]Values represented as means ± SEM; $n = 6$ in each case.

Table 2. Medaka Development

Parameter	Control $n = 21$	Tween 80 $n = 15$	BaP[a] $n = 36$
Outer diam. of fertilized eggs[b] (mm)	1.25 ± 0.06	1.20 ± 0.06	1.25 ± 0.05
Inner diam. of fertilized eggs[b] (mm)	1.10 ± 0.06	1.10 ± 0.06	1.10 ± 0.06
Size of the perivitelline space in fertilized eggs[b] (mm)	0.15 ± 0.06	0.15 ± 0.05	0.15 ± 0.05
Number of days over which hatching occurred[b]	8.00 ± 1.41	4.50 ± 3.53	7.33 ± 0.58
Days from fertilization to last hatch[b]	12.75 ± 2.43	12.73 ± 3.02	15.24 ± 2.61
Fry survival	83.3%	90.9%	67.3%

[a]10 ppb BaP in 1% Tween 80.
[b]Day 16 of exposure; mean ± SEM.

temperature. Medaka DNA was resistant to unwinding between 23°C and 50°C, but began to unwind at higher temperatures.

Medaka DNA from the three exposed groups (BaP, verapmil, and BaP + verapamil) had an increased resistance to alkaline unwinding (about a 25% decrease in fluorescence) in contrast to control and fresh, salt-extracted DNA (about a 45% decrease in fluorescence) after 60 minutes of incubation as shown in Figure 7 (ANOVA: $F = 39.25$; $F_{0.025},4,20 = 2.87$, $n = 9$, $N = 25$, $k = 5$; $p < 0.001$). The freshly extracted medaka DNA showed an increased rate of unwinding in the first 30 minutes of incubation, which was quite different from control and treated DNAs.

BaP Exposure Experiment III

Figure 8 shows the alkaline unwinding F values for medaka DNA from the third experiment. The yield of high-molecular-weight DNA was considerably higher than NH_4OH/Triton X-100 extraction, possibly due to more efficient removal of protein, which reduces loss of DNA to the aqueous-CIP interface during phenol extraction. The increased yield of DNA should make possible multiple DNA analyses from a single fish. There was no difference between the alkaline unwinding F values for DNA from controls, BaP- and BaP + verapamil-treated fish at alkaline unwinding incubation temperatures of 4°C, 38°C and 52°C (ANOVA: $F = 0.0123$, $p > 0.25$ at 38°C; $F = 1.88$, $p > 0.10$ at 52°C; $F_{0.05},2,12 = 3.89$; $n = 5$, $N = 15$, $k = 3$). In addition, there was considerable unwinding of the DNA at lower temperatures in contrast to DNA extracted by the procedure of Shugart (1988) in the previous exposure experiments.

DISCUSSION

Japanese medaka were exposed to water in which BaP dispersal was facilitated by the presence of a detergent (Tween 80). Since the maximal solubility of BaP in water without detergent is about 4 μg/l, the presence of Tween 80 may promote temporarily higher concentrations of BaP in water, but this BaP may not have been available to the fish. The rapid decline in BaP levels seen in Figure 1 could be attributed to either uptake into the medaka, binding to particulate organic matter, or absorption to glass and plastic in the aquaria, which is characteristic of hydrophobic polyaromatic hydrocarbons.

During the time that BaP levels decreased during the exposure, there was a rise in BaP levels in the tissues of the medaka. The increase is more rapid during the first day of exposure and changes to a slower but steady rate of accumulation on subsequent days (Figure 2). The total amount of BaP accumulation in the 25 fish was less than 150 ng as compared with a decrease in BaP concentrations in water of greater than 30 μg; thus, most of the BaP may have been absorbed

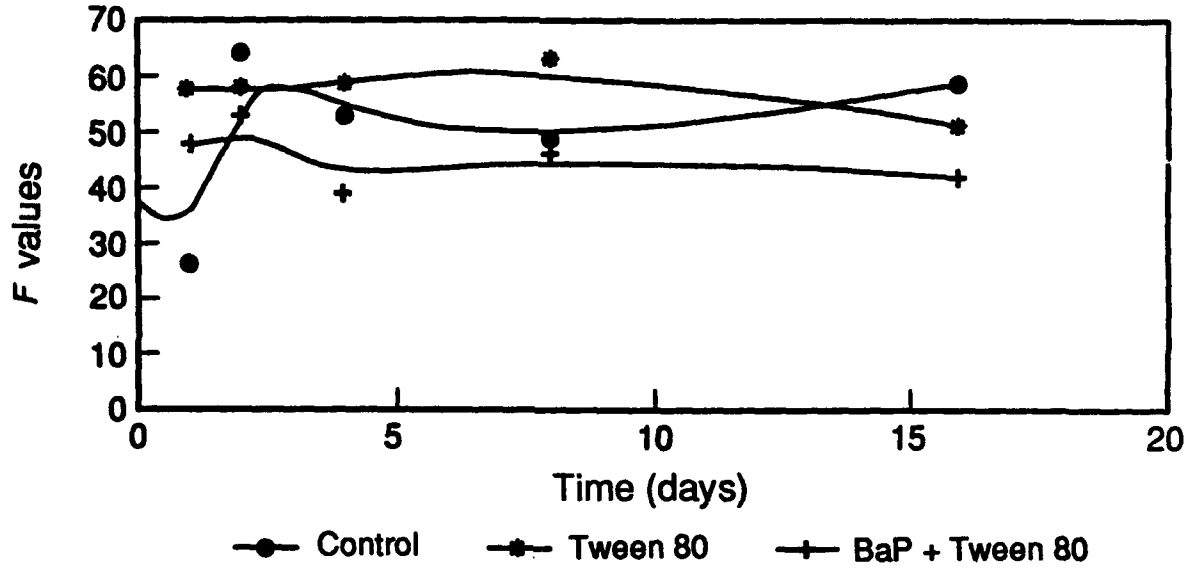

Figure 4. BaP exposure Experiment I. *F* values for percentage of double-stranded DNA in adult medaka livers.

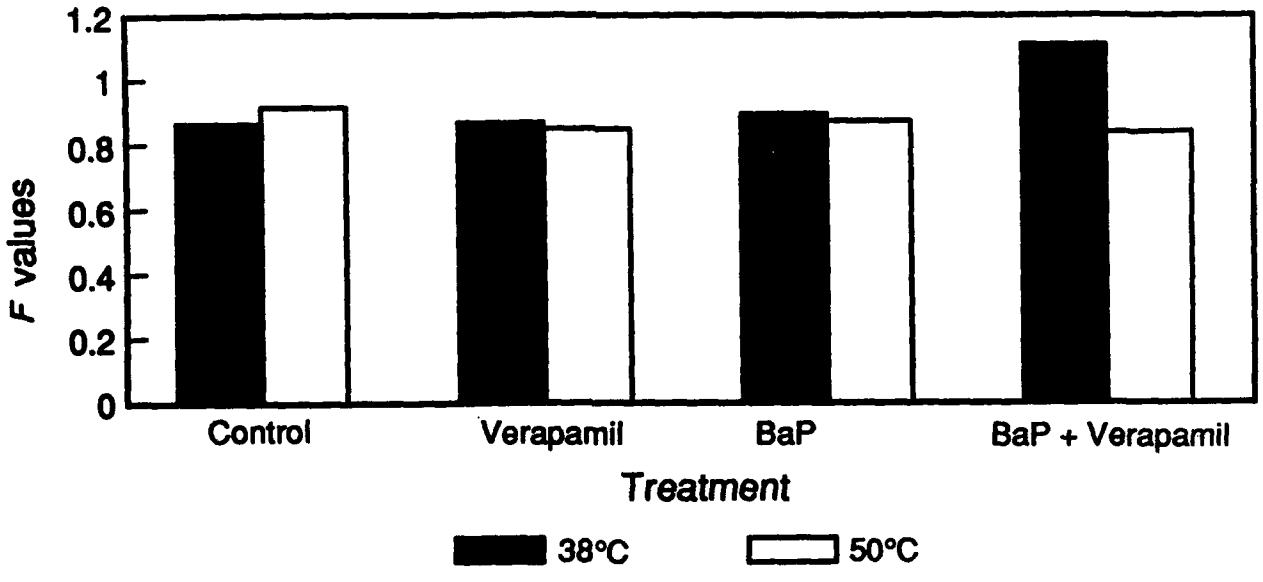

Figure 5. BaP exposure Experiment II. *F* values for percentage of double-stranded DNA in adult medaka livers (7-day exposure).

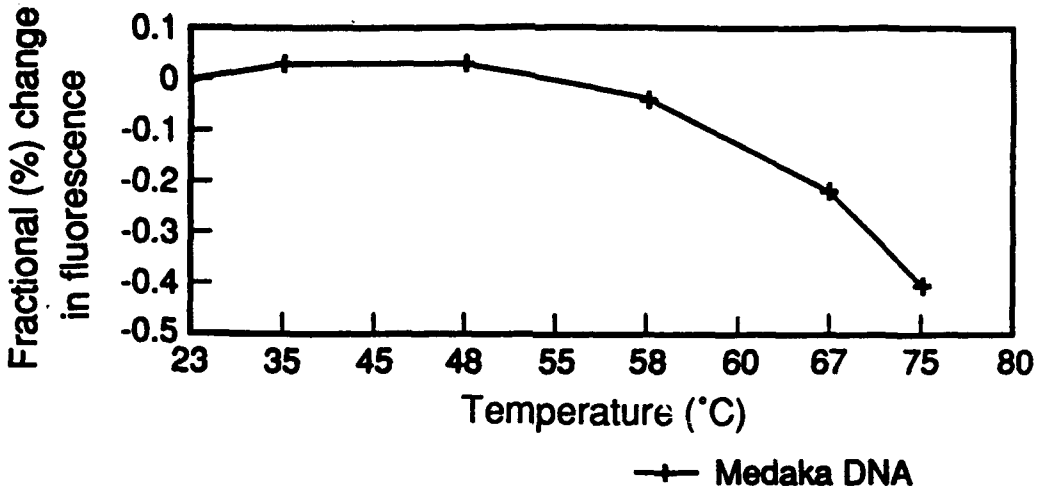

Figure 6. Thermal denaturation of BaP-treated medaka DNA under alkaline conditions (10-day exposure to BaP).

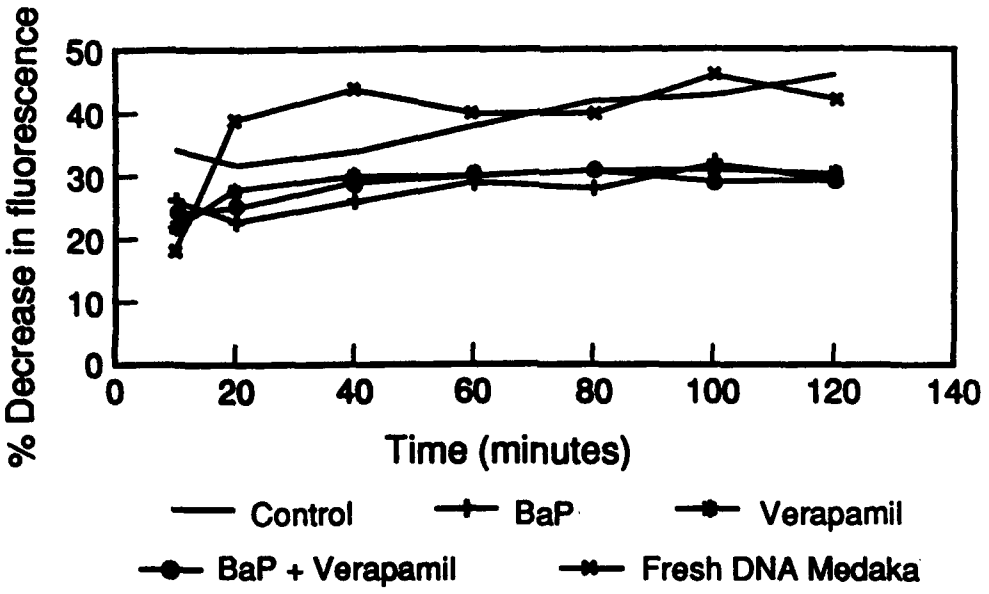

Figure 7. DNA fluorescence change after exposure to BaP, verapamil, or both (at 60°C).

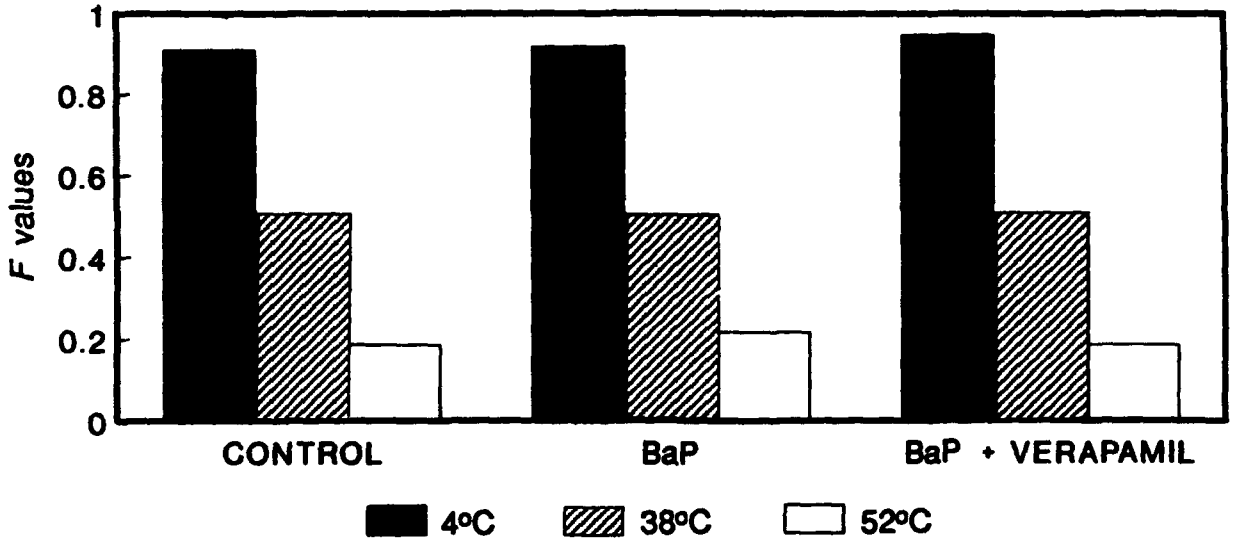

Figure 8. BaP exposure Experiment I. *F* values for percentage of double-stranded DNA in adult medaka livers (42-hour exposure).

to other materials in the aquaria or subjected to photodegradation. Female medaka also appeared to accumulate BaP at a higher level in their tissues than male fish. However, we do not yet know whether this is a result of metabolic or tissue-specific differences between male and female medaka.

Medaka eggs were exposed to BaP only briefly (1 hour after spawning). This exposure did not result in significant changes in the numbers of developmental abnormalities or in the morphology of fertilized eggs. The study of fecundity and ovarian oocyte atresia for BaP-treated fish did not indicate overt ovotoxicity. In medaka, BaP may have actually stimulated oocyte development and egg production, possibly via an estrogenic effect on the ovaries. BaP may inhibit ovarian function at higher effective concentrations. The lack of ovotoxicity in Experiment I may have been caused by the medaka detoxifying and excreting BaP at the levels present in the water, or perhaps BaP was not biologically available to reproductive tissues in concentrations sufficient to greatly affect reproduction and development. Tween 80 alone may have exhibited some deleterious effect on fecundity, although this effect was not seen in combination with BaP. The use of Tween 80 as a vehicle for BaP may have to be reconsidered.

The high variability in survival of embryos derived from BaP-exposed parents may have resulted from problems with glass pipette transfer techniques. Improved handling techniques for

embryo transfer have been developed, which should minimize damage to developing fish. Future studies will additionally focus on genotoxic and teratogenic effects of BaP exposure in medaka. The dominant lethal assay was developed as a measure of genotoxicity in mammals (mice) exposed to a large number of xenobiotic compounds (Bateman and Epstein, 1971). Previous studies using intraperitoneal injection of mitomycin C and ethyl methanesulfonate into medaka have demonstrated a considerable dominant lethal effect as measured by hatchability of fertilized eggs (Shimada and Egami, 1984). The medaka is currently being used for studies of teratogenesis by exposing developing fertile eggs to xenobiotic compounds (Cameron et al., 1985; Shi and Faustman, 1989). Fertilized medaka eggs can be easily monitored through a compound microscope during development. Future studies incorporating the dominant lethal assay, teratogenic analysis, and reproductive studies could help identify more specific genotoxic and cytotoxic effects. These studies would complement experiments described in this report in which parental medaka were exposed to BaP and in which the reproductive and developmental effects were analyzed and would be more characteristic of environmental exposure to toxicants.

Alkaline unwinding analyses of liver DNA indicated that BaP may not have caused DNA strand breaks in medaka (Figure 4). Considerable variability of alkaline unwinding *F* values within treatment groups, though, may make it necessary to modify the procedure to improve both the yield and integrity of DNA extracted. Flow cytometric analysis of liver and gill cells (which provides an estimate of the DNA content of cell nuclei) failed to detect an effect of BaP on nuclear DNA (data not shown).

Tween 80 was not used as a vehicle for dispersing BaP in the water in Experiments II and III. The water in the second exposure was saturated with BaP at day 0 and was not renewed thereafter. In Experiment II, the concentration of BaP was initially high but rapidly declined to undetectable levels in the water by day 7 (Shugart et al., 1987*a*). Verapamil, which inhibits the p170 glycoprotein xenobiotic pump (Kurelec and Pivcevic, 1989), was renewed daily in the verapamil and BaP + verapamil treatments.

The alkaline unwinding rates obtained from DNA extracted from whole medaka carcasses (Figures 5 and 6) showed that medaka DNA was very resistant to unwinding at incubation temperatures successfully used to measure DNA strand breaks in BaP-exposed bluegill sunfish and in other animal species. We found no significant difference in the DNA strand breakage of fish treated with BaP alone or those treated with verapamil. Also, resistance of medaka DNA to alkaline unwinding was greater for fish exposed to BaP, verapamil, and BaP + verapamil than it was for unexposed control fish (Figure 7). Thus, high concentrations of salt (>250 mM NaCl) and/or proteinase K - RNase digestion during DNA extraction may have removed an unidentified

unwinding inhibitor that (1) associates with medaka DNA and (2) may be induced by exposure of the fish to xenobiotic compounds.

In summary, we found no evidence of BaP- or verapamil-induced genotoxicity as measured by direct DNA damage (strand breaks) in the medaka. In addition, little evidence was found for physiological effects of these two chemicals upon reproductive or developmental parameters. The low solubility of BaP in water may necessitate longer exposure times to overcome the natural xenobiotic defense mechanisms in medaka.

Several hypotheses for the resistance of medaka to DNA damage from xenobiotic compounds can be formulated: (1) these fish have efficient metabolism and excretion of BaP from the gills; (2) medaka may be able to induce or increase the activity of a xenobiotic pump (the use of higher concentrations of verapamil or vanadate may effectively inhibit the p170 glycoprotein pump and permit toxicants to enter the cells at a higher rate); and (3) an as-yet unidentified factor naturally associated with medaka DNA and induced to higher levels by exposure to xenobiotics may protect DNA from damage or facilitate its repair.

A continuation of these studies on BaP exposure in medaka will focus on the nature of the salt-extractable factor, which apparently associates with the DNA. Proteins and polyamines (Bachrach, 1973) are potential candidates for such factors, though protein may be the more likely candidate, because protease treatment greatly facilitates alkaline unwinding of DNA. This phenomenon may in itself be a marker of DNA damage because DNA-associating protein (rec A) is induced in *Escherichia coli* after DNA damage has occurred (Hanawalt et al., 1979). The possibility of protein-protein or protein-DNA cross linking cannot be excluded.

The dominant lethal assay and teratogenesis analysis will be used as an additional measure of genetic damage in the medaka after exposure to xenobiotics (Shimada and Egami, 1984). Agarose gel electrophoresis would be another means to determine the amount of double-stranded DNA breaks (Maniatis et al., 1982). Two other assays to estimate DNA damage are (1) the quantitation of BaP adducts (Shugart et al., 1987*b*) and (2) 5-methyl deoxycytosine (Shugart, 1990) levels in the DNA of exposed organisms. Other areas of analysis would employ flow cytometry, DNA repair assays, stress protein synthesis, and detoxification enzyme activities.

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DIETARY REFINEMENTS IN A SENSITIVE FISH LIVER TUMOR MODEL

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INTRODUCTION

The development of new, faster eukaryotic models for bioassay to identify potential carcinogens and modulators of carcinogenesis is a pressing need for research. The Japanese medaka (*Oryzias latipes*), a small aquarium teleost species, develops tumors of the liver after exposure to proven mammalian genotoxic carcinogens (Ishikawa et al., 1975; Aoki and Matsudaira, 1977; Hinton et al., 1988). A brief latency period, sensitivity to a variety of procarcinogens, and decreased cost while maintaining large numbers of exposed individuals (Matsushima and Sugimura, 1976; Consensus Committee, 1984) argue for the further development of this *in vivo* model. However, the lack of a defined diet and absence of established tumor incidence data under standardized and reproducible conditions hinder the exploitation of this interesting species (Consensus Committee, 1984). Furthermore, recent studies on critical parameters in the quantitation of the stages of liver carcinogenesis indicate that considerable variation between bioassay occurs even with inbred rodent species. Further indication is that diet is a major source of this variation (Hendrich et al., 1988; Pitot et al., 1989).

Using a purified casein-based (PC) diet developed at the Bodega Marine Laboratory, University of California (DeKoven, 1990), the investigators have compared growth between medaka fed solely the PC diet and medaka maintained using a conventional feeding regime commonly used by other workers using this species for carcinogenesis studies (Ishikawa et al., 1975; Aoki and Matsudaira, 1977). This conventional regime includes the use of a commercial flake diet (Tetramin, Tetrawerke, West Germany) with twice weekly supplementation of brine shrimp (*Artemia salina*) nauplii.

The purpose of this report is to describe characteristics of medaka fed either PC diet or the flake/*Artemia* regimen from day 1 after hatch to 110 days. The investigators have compared wet and dry weights, and established quantitative features of external morphology, liver histology, and activity of one microsomal enzyme. Results indicate that the PC diet-fed fish, without *Artemia*

supplementation, perform comparably or superiorly to those fed the flake/*Artemia* regimen. With the PC diet, possible xenobiotic contamination associated with live *Artemia* supplementation may be avoided. Studies are ongoing to evaluate tumorigenic responses in groups of medaka fed the two diets and initiated with proven mammalian and medaka hepatocarcinogens.

MATERIALS AND METHODS

Diets

Two diets were used: a formulated PC diet and a commercially available flaked fish food (Tetramin, Tetrawerke, West Germany). Flake-fed fish were supplemented twice a week with newly hatched *Artemia salina* nauplii (San Francisco Bay Brand, San Francisco, CA). Aspects of composition of the PC diet are given in Table 1, which compares proximate analyses of the flake diet, newly hatched *Artemia* nauplii (<24 hours posthatch), and the PC diet.

Table 1. Proximate Composition of Diets

Diets	%W[a]	%CP	%P	%CR	%F	%L	%CH	%A
Artemia Nauplii[b]	90.1	58.0	-	[13.0]	-	19.0	-	10.0
Artemia Adults[c]	90.0	58.0	43.0	3.5	-	24.5	0.5	20.6
Tetra Min[d]	8.0	49.0	-	[29.0]	5.0	5.0	-	12.0
PC Diet[e]	4.8	-	50.0	27.2	3.6	12.2	-	3.0

[a]Measures: %W, percent water in original tissue or diet; %CP, percent crude protein; %P, percent protein; %CR, percent carbohydrate; %F, percent fiber; %L, percent lipid; %CH, percent cholesterol; %A, percent ash; all are expressed as percent of dry weight.
[b]Larvae of San Francisco Brand (Tokyo) (Watanabe et al., 1983, p. 119).
[c]Gallagher and Brown, 1975.
[d]Product label.
[e]Calculated from ingredient composition.

The formulated diet was prepared in 1-kg batches, pressed through a 1.5-mm stainless sieve (U.S. Standard Tyler Sieve #10), and stored at -80°C. The food was then freeze dried at -100°C

(LABCONCO freeze dryer) and passed through a series of sieves and separated by pellet size. Table 2 shows the pellet sizes fed during the study.

The flake diet was kept vacuum packed until ready for use. It was then sifted to ensure that both groups of fish were fed the same particle sizes. Newly hatched *Artemia* nauplii were fed twice a week to the flake-diet fish. Nauplii were separated from unhatched and empty cysts and rinsed; excess water was removed before weighing. Both diets, PC and flaked, and *Artemia* cysts were stored at -20°C.

The food ration was adjusted on the basis of biomass at 10-day intervals (Table 2). Daily allotments of food were preweighed for each 10-day interval. The fish were fed one-half of this preweighed amount twice daily (morning and afternoon) during the week and all of the amount once a day during weekends.

Water Conditions

Water in the recirculating system (Núñez and Hinton, in preparation) was treated with reverse osmosis after charcoal prefiltration. Reconstitution (EPA guidelines for moderately hard water) was in 1,892-liter batches, and resultant water was stored in a 1,987-liter container following EPA guidelines. Prior to use, each batch of reconstituted water was analyzed for hardness, conductivity, and pH, and a 24-hour bioassay was conducted using newly hatched medaka. Twice a week 25% of the water in the rearing system was replaced with newly reconstituted water.

Table 2. Percentage of Biomass and Size of Pellet Fed During Each 10-Day Period

Day	PC Diet (%)	Pellet Size (μm)	Flakes (%)	Pellet Size (μm)	*Artemia* (%)
1 - 10	25.0	<106	25.0	<106	4
11 - 20	25.0	106-250	25.0	106-250	4
21 - 30	25.0	106-355	25.0	250-355	4
31 - 40	25.0	250-355	25.0	250-355	4
41 - 50	20.0	250-355	10.0	250-500	4
51 - 60	20.0	250-355	10.0	355-500	4
61 - 70	15.0	355-500	10.0	355-850	4
71 - 80	15.0	355-500	7.5	500-850	3
81 - 90	15.0	355-500	7.5	500-850	3
91 - 100	10.0	355-500	5.0	500-850	2
101 - 110	7.5	355-500	5.0	500-850	2

Water in the recirculating system was stored in a sump, heated, and pumped through a 16-μm mechanical and an activated carbon filter. The water was then passed through two fluidized sand biological filters and prefiltered before ultraviolet sterilization and entry to the head tank. Water in the head tank was aerated and subsequently distributed to the aquaria by gravity flow. Overflow water was returned to the sump. Biological filters were established prior to the introduction of the fish. No metallic parts or toxic compounds and fittings were used in the system. Water quality was monitored weekly for nitrites, ammonia, nitrates, pH, dissolved oxygen, and conductivity. Temperature was monitored daily. Water quality parameters are shown in Table 3.

Table 3. Weekly Water Quality Parameters Measured

Date	Temp. Degree C	pH	Dissolved Oxygen (ppm)	Ammonia (ppm)	Nitrite (ppm)	Nitrate (ppm)	Hardness (mg/l)	Conductivity (μmho)
4/05/90	25.0	8.0	7.6	0	0	0.0	110	330
4/13/90	25.0	8.1	7.6	0	0	0.2	130	340
4/20/90	25.0	8.0	8.0	0	0	0.8	130	340
4/25/90	25.0	8.0	8.0	0	0	0.8	110	340
5/02/90	25.0	8.2	7.7	0	0	1.0	110	340
5/09/90	25.0	8.0	7.9	0	0	1.0	100	340
5/16/90	25.0	8.1	7.7	0	0	1.5	120	340
5/23/90	25.0	7.9	7.7	0	0	2.0	130	340
5/30/90	25.0	7.8	7.7	0	0	2.0	130	340
6/08/90	25.0	7.9	7.6	0	0	2.5	110	345
6/13/90	25.0	7.9	7.6	0	0	2.5	100	360
6/21/90	25.0	8.1	7.6	0	0	2.0	100	345
6/28/90	25.0	8.2	7.8	0	0	2.5	100	345
7/05/90	25.0	8.1	7.7	0	0	2.5	100	345
7/11/90	25.0	8.2	7.6	0	0	3.0	110	400
7/18/90	25.0	8.1	7.7	0	0	3.0	120	400
7/25/90	25.0	8.1	7.7	0	0	3.0	120	400

Fish Husbandry

The fish for this study were reared in ten 37.8-liter aquaria (five replicates for each of the two dietary regimes). Aquaria were randomly placed on system shelves. Water temperature was maintained at 25±0.5°C. Photoperiod was 16L:8D. Water quality tests performed weekly indicated no detectable levels of ammonia or nitrite.

Medaka eggs were collected from broodfish maintained at 25°C under a 16L:8D photoperiod, which stimulated continuous oogenesis and spermatogenesis. Eggs were collected by individually netting females and carefully removing extruded egg clusters with blunt forceps. Eggs from several females were pooled. To separate individual eggs from clusters, filaments surrounding the eggs were broken by gently rolling them between moistened finger tips. Eggs were then incubated in aerated embryo-rearing medium (Rugh, 1962; Kirchen and West, 1976) at 25±1°C. The embryo-rearing medium was changed, and dead eggs were removed daily. Larvae hatched 9 to 10 days after fertilization.

One day after hatch, 2,500 normal hatchlings were selected using a dissecting microscope. Selection criteria were normal external morphology and presence of an inflated swim bladder. Normal fish were placed in 25-ml plastic cups (10 fish in each) and randomly assigned to the experimental aquaria until a total of 250 newly hatched normal larvae per aquarium was reached. Plastic cups with larvae were floated in aquaria for 15 minutes before release to equilibrate temperatures. After completion of larval counting and selection, all fish were fed their respective diets.

Sampling

Each diet treatment consisted of five replicate aquaria. Frequency and number of samples for wet- and dry-weight measurements, histology, morphometry, and biochemical assay are indicated in Table 4. Fish were not fed for 24 hours prior to sampling. Prevalence of abnormal fish in each sample was recorded. To sample, approximately one-fourth of the total number of fish in each aquarium were concentrated in a net. From this pool, the appropriate number per sample was randomly collected.

For individual measurements, each fish was overdosed with tricaine methanesulfonate (Finquel, Argent Co.), carefully blotted dr placed in individual preweighed aluminum foil cups, and weighed on a microbalance (0.0001 g). For dry weights, 10 fish from each aquarium, in the same preweighed aluminum cup, were oven dried to a constant weight at 60°C. After drying for 24-48 hours, fish were removed from the oven, allowed to cool in a desiccator, and weighed. Morphometric measurements (length, depth, width) were recorded using a computer-assisted image analysis system and a dissecting microscope. Thirty newly hatched and unfed normal larvae (zero time sample) were measured as above.

Table 4. Number and Frequency of Parameters Measured During Diet Comparison

Parameter	*N*	Frequency
Wet weight/dry weight	10/tank	every 10 days
Histology	10/treatment	every 20 days
Morphometry (length, depth, width)	10/tank	every 30 days
Biochemical assays of key Phase I & Phase II liver enzymes	20/treatment	days 35, 70, 110

Instantaneous growth rates (IGRs) based on wet weight increase were determined for the replicates of each diet treatment by the formula:

$$IGR = \frac{(\text{Ln weight final} - \text{Ln weight initial}) \times 100}{\text{number of days}}$$

All aquaria were monitored daily, dead fish were removed, and numbers recorded. Uneaten food and feces were removed by vacuum daily. The aquaria were monitored for presence of eggs on the females, and occasional courtship behavior was observed. The time to first egg production from each diet treatment was recorded as well as the number of eggs produced in each aquarium.

Histology

General histology was compared at intervals of 20 days. Fish were anesthetized, fixed, and processed for paraffin embedment, and sections were stained with hematoxylin and eosin.

Biochemistry

At days 35, 70, and 110, visceral masses or livers were dissected free from anesthetized fish, pooled (20 from each diet treatment), and frozen at -80°C. Ethoxycoumarin *O*-deethylase (ECOD) was measured fluorimetrically by the production of 7-hydroxycoumarin. Samples were homogenized in cold sucrose buffer with a Potter/Elvehjem (Teflon/glass) homogenizer. The S9 fraction was prepared by centrifugation of the homogenate for 30 minutes at 9,000 × g. The supernatant was assayed fluorimetrically for ECOD activity as described by Greenlee and Poland (1978). Proteins were quantitated by the method of Bradford (1976).

RESULTS

Diets

The proximate analyses of diets are provided in Table 1. Protein content is similar in Tetramin and PC diet (49% and 50%, respectively). However, the protein content of nauplii is 58%. Percent lipid is 12.2 in PC, twice that of Tetramin (5%, see Discussion). Pellet sizes during study proved appropriate, since fish easily ingested the diet. Pellets were large enough for consumption and minimum leaching of nutrients. Active feeding was observed at the surface and at the bottom of aquaria. Both PC and flake diets floated immediately, but eventually sank, with flakes tending to float longer. The investigators observed no problems with palatability of either diet. The presence of *Artemia* nauplii was associated with enhanced feeding activity.

Water Conditions

Water quality was at optimal levels throughout the study. Neither ammonia nor nitrites were detected. Other water quality parameters (Table 3) were maintained at constant and optimal levels, which is consistent with the high survival and growth rate. Nitrate levels had increased to 3 ppm by the conclusion of the study, indicating proper functioning of the biological filters. This maximum value is commonly found in recirculating systems and is within the appropriate range for aquatic organisms.

Effects of the Diets

Growth

Flake/*Artemia*-fed fish grew faster than PC-fed fish during the first 100 days (Figure 1). A student *t* test shows that these differences are significant ($p < 0.01$). At day 110, no significant difference was found between the two groups, indicating similar growth rates at that last sampling date.

Percent body weight increase (%BWI) was significantly higher in flake/*Artemia*-fed fish during the first 30 days (Figure 2). However, at day 40, %BWI for PC-fed fish was significantly higher than %BWI for flake/*Artemia*-fed fish. The statistical analysis shows no significant difference for the remaining samples (days 50-110), but the PC-fed fish consistently showed a slightly higher %BWI during this period.

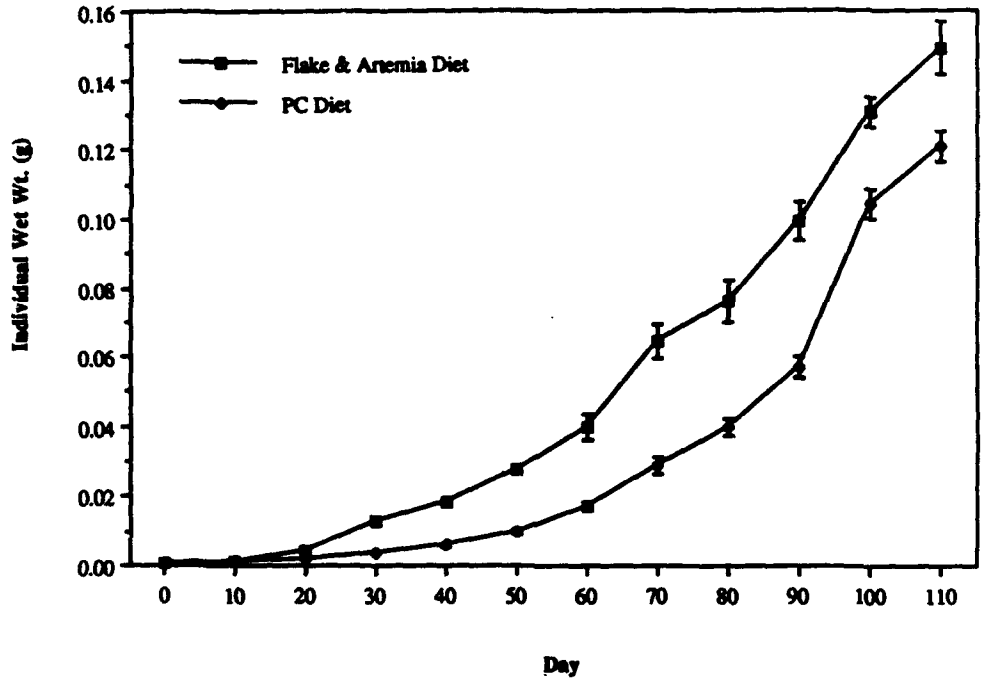

Figure 1. Effect of diet on individual wet weight.

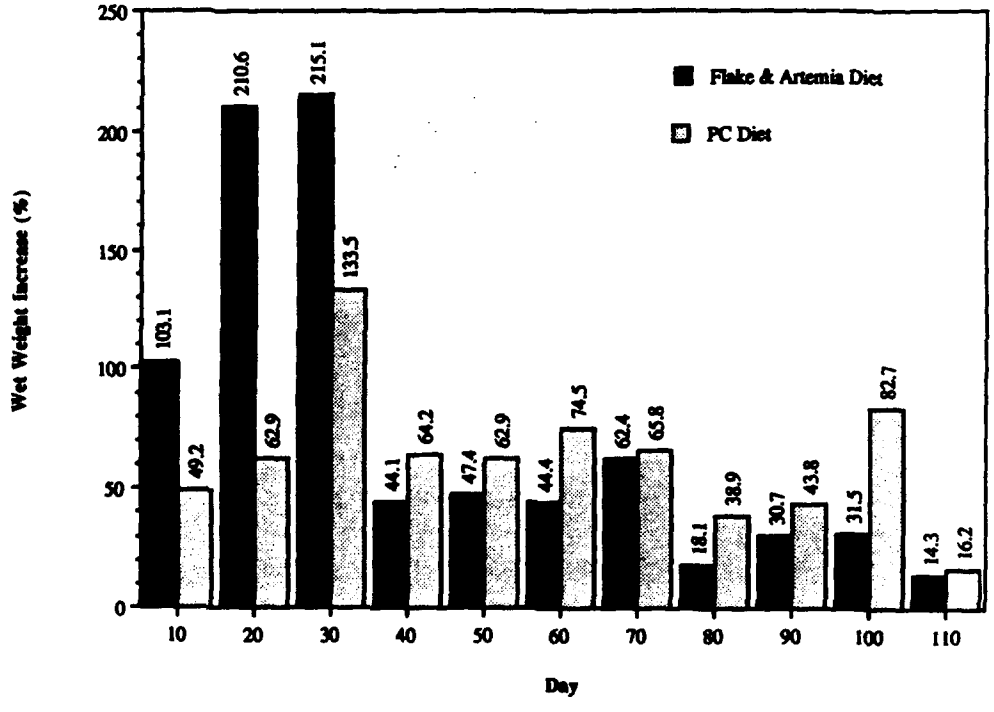

Figure 2. Effect of diet on percent body weight increase.

Morphometry

Morphometric analyses showed similar trends for total length, maximum depth, and maximum width. Between 0 and 30 days, fish fed the flake/*Artemia* diet had significantly greater IGRs for all morphometric parameters (Figure 3). However, between 30 and 60 days the fish fed PC diet showed significantly greater IGRs for total length and maximum width. Maximum depth and maximum width IGRs were also significantly greater in the PC treatment group from 60 to 90 days. There was no significant difference in IGRs between diet treatments after 60 days (total length) or 90 days (maximum width and depth).

Mortality

Mortality was low for all of the aquaria (1.6-6.8%, Figure 4). Average mortality for the PC aquaria (5.0%) was slightly higher than for the flake/*Artemia*-fed aquaria (3.3%), but statistically they are not significantly different. There were four accidental deaths, which occurred during cleaning of the aquaria. A small number of fish (1.1%) were not detected as mortalities, but were missing when all remaining fish were counted at the end of the experiment. These fish were probably lost during the initial stages of the study because of their small size.

Deformities

A significant number of skeletal deformities (67 fish, Figure 5) were found in all of the flake/*Artemia*-fed groups (average from all aquaria = 5.4%). These gross lesions ranged from slight axial deformation to multiple lateral and dorsoventral curvatures as well as incidences of cranial abnormalities. Only one fish with a very slight axial deformation was found in the PC-fed group during the final count (0.08%).

Histology

In addition to the skeletal microscopic analysis (above), livers from both groups were examined histologically at days 0, 20, 40, 60, and 80. Specimens from days 100 to 110 have been prepared, but not analyzed to date. Liver changes were noted as early as day 20 in some flake/*Artemia*-fed fish. Out of 31 flake/*Artemia*-fed fish examined, 7 had lesions, six of which varied from fatty change to hepatocellular vacuolation and necrosis. Of 29 PC-fed fish examined, none had these lesions. Two fish, one from each group, had livers with hepatocellular eosinophilic, intranuclear inclusions of unknown etiology.

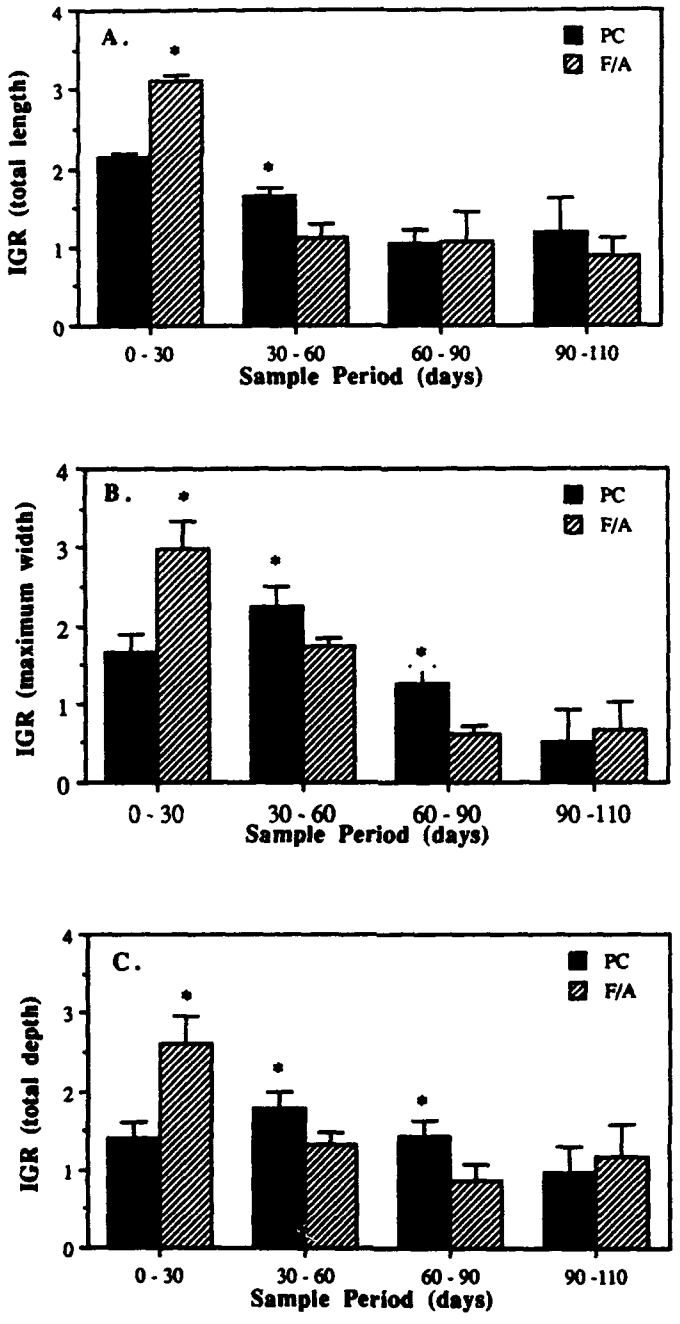

Figure 3. Average instantaneous growth rates (IGRs; ± SE) for total length [A], maximum width [B], and total depth [C] of medaka reared on purified casein (PC) and flake/*Artemia* (F/A) diets. *Denotes IGRs within same sample period that are significantly different ($p \leq 0.05$).

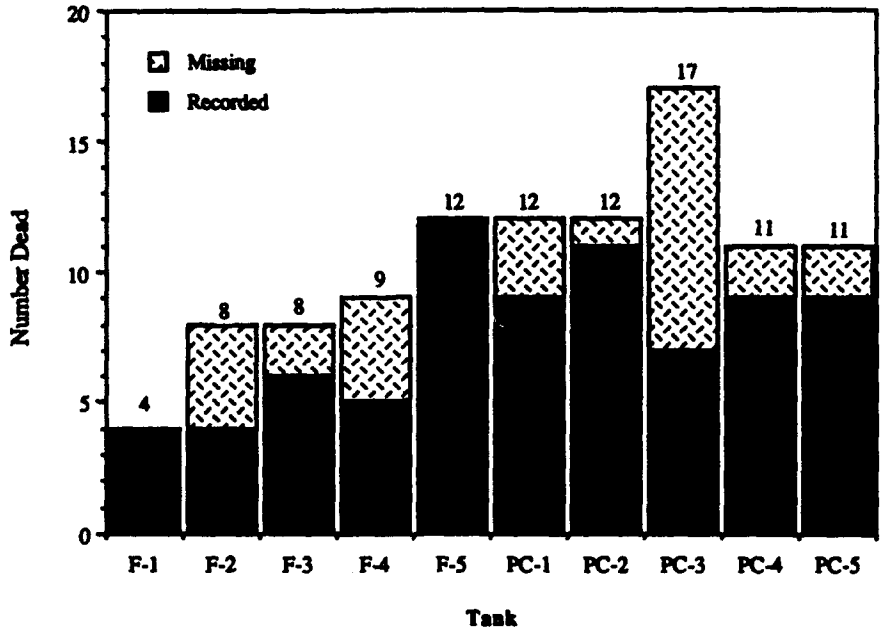

Figure 4. Mortality in individual aquaria.

Liver Enzymes

Based on a single P-450 enzyme, ECOD, it would appear that activity levels in PC-fed fish are comparable or better than those fed flake/*Artemia* regimen (Table 5). The investigators will continue those comparisons with glutathione and ethoxyresorufin *O*-deethylase. The latter will be of interest as a possible monitor of xenobiotics in *Artemia* and/or diet formulations.

Sex Differentiation

On the last day of sampling (day 110), all fish were counted and sexed including the fish sampled for morphometry/weight data. Most notable was the significant difference in the percentages of externally undifferentiated individuals (15.5% flake/*Artemia* vs. 34.1% PC, Figure 6). This difference could be due to the faster growth rate and larger size of the flake/*Artemia*-fed fish at day 110. There were also significant differences between percentages of (1) females PC and flake/*Artemia*, (2) males flake/*Artemia* and immature flake/*Artemia*, and (3) females and immature flake/*Artemia*.

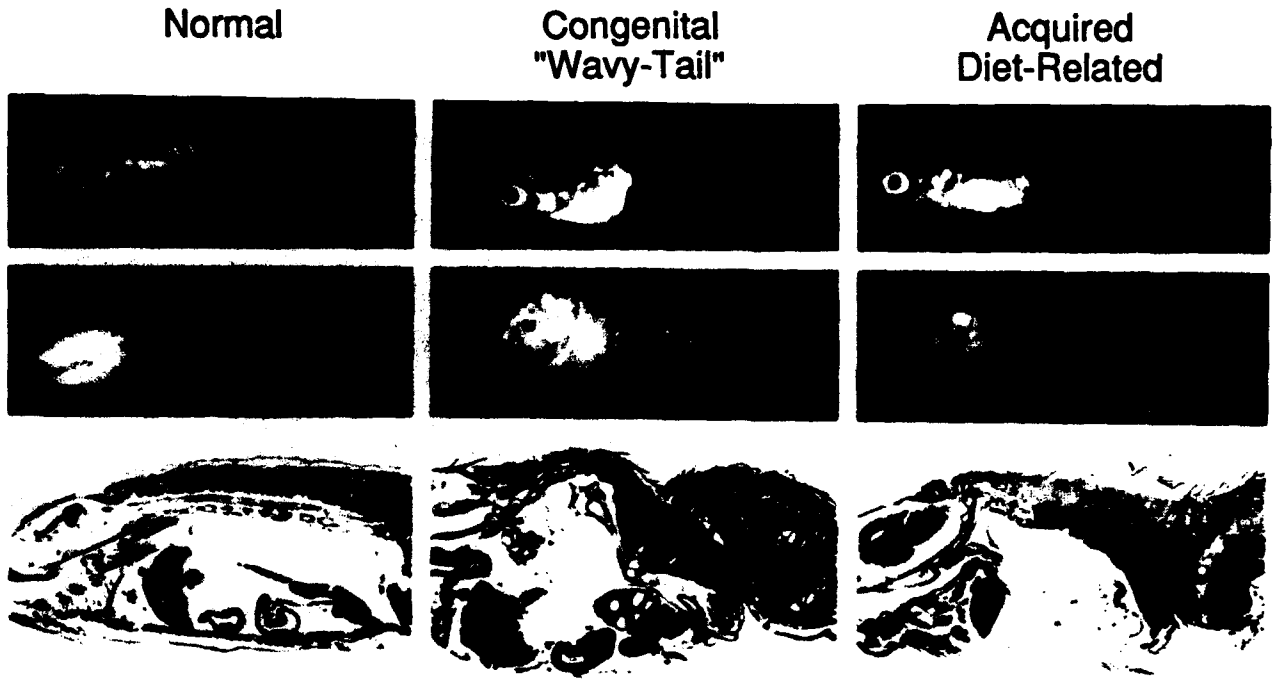

Figure 5. Gross micrographs and radiographs of medaka with normal vertebral column and those showing congenital "wavy-tail" or diet-acquired spinal defects (from flake/*Artemia* diet). Eggs from a single broodstock pool were used in this study. Therefore, we consider spinal defects (5.4% incidence in flake/*Artemia*-fed fish) of this study as diet-related. Only one fish with very slight axial deformation was found (not shown) in the PC-fed group (0.08% incidence).

Table 5. ECOD Activities* in Pooled Liver Samples

Day	Flake Diet	PC Diet
35	0.68	0.89
70	0.30	0.75
110	0.76	1.06

*Activities in nanomoles/minute/milligram protein in the S9 pooled liver fraction from 20 fish/sample.

Egg Production

There were a greater number of fertile eggs and a lower number of infertile eggs produced by PC-fed fish than were produced by the flake/*Artemia*-fish (Figure 7). Egg production was detected first and was more evenly distributed among all PC-fed aquaria. In the flake/*Artemia* treatment, two aquaria produced 70% of the eggs. There was a statistically significant difference between fertilized and unfertilized eggs in the PC-fed fish, but this difference was not significant in the flake/*Artemia* treatment.

DISCUSSION

The PC formulation studied herein appears adequate for the normal nutrition and growth of newly hatched, young, and adult medaka. In general, growth was superior in the flake/*Artemia* regimen, but on the final day of sampling, the average weight of individual fish was not significantly different in the two groups. Survivability, health, and reproduction were comparable or better in PC-fed (days 30-110) versus flake/*Artemia*-fed fish. One of the primary concerns voiced by the Consensus Committee (1984) was the need for the development of a defined diet. The possible contamination of live food (such as brine shrimp) was feared to confound carcinogen bioassay where xenobiotics might influence metabolism, carcinogen initiation, promotion, and progression.

A partial goal has been reached, but further refinements are needed. It is possible that the oil mixture (medicinal grade cod liver oil) contains contaminants. Work is needed to determine the essential fatty acid requirements of medaka, thereby permitting development of a defined diet. This and other aspects of the early (larval) nutrition are discussed below.

It is not known at this time what combination of factors might be involved in the differing growth rates during the first month of the experiment. An examination of the proximate analysis (see Table 1) of the diets used does indicate some differences. However, the significance of these differences is unknown at this time. Protein represents about half of all the feed sources. The level of 58% crude protein in *Artemia* nauplii should be viewed with some caution. Crude protein (%CP) reflects analysis based on nitrogen levels, which is then multiplied by a factor representing the average nitrogen content of protein. For crustaceans such as *Artemia*, this value can be artificially high in that chitin contains appreciable levels of nitrogen. Where possible, data from more specific protein assays are included (%P).

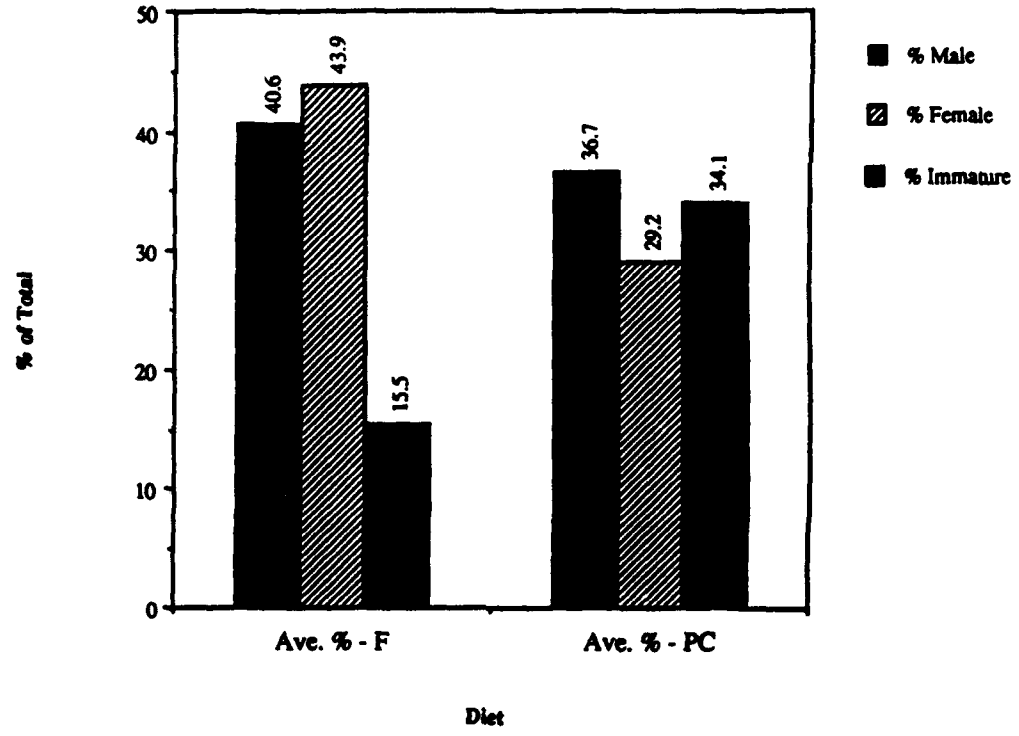

Figure 6. Effects of diet on sexual differentiation.

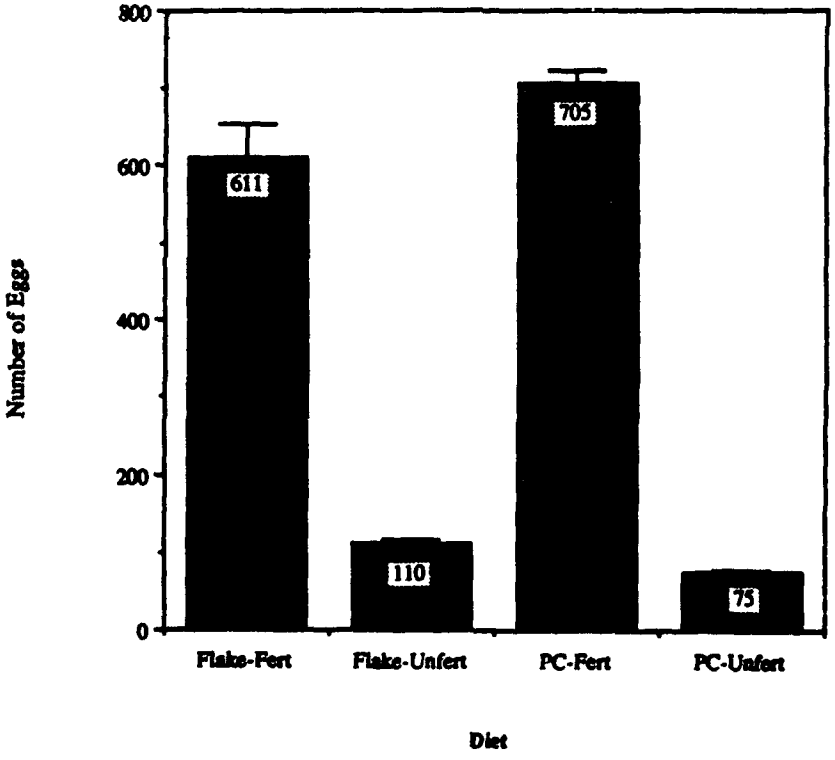

Figure 7. Effect of diet on egg production.

There are notable differences in the energy components of the diets. Carbohydrate levels are lower in *Artemia* nauplii in comparison to either of the formulated diets. While carbohydrates in the diet of fish are generally not utilized as well as lipids, some caution again must be used in examining this component. The carbohydrate fraction (%CR) is not particularly well defined and in some tabulations is produced by subtraction of everything else from initial dry weight. The carbohydrate fraction may in some cases contain undigestible fiber.

Lipids, which are quite important in the diet of aquatic animals, are highest in the case of *Artemia* nauplii (see Watanabe et al., 1983; Simpson et al., 1983; Leger et al., 1986; Dendrinos and Thorpe, 1987). It should be noted, however, that fatty acid profiles of *Artemia* vary a great deal depending upon the source of the cysts as well as on diet of the nauplii. Such variation has been shown to affect the growth and survival of some species of fish. In fact, the fatty acid composition of *Artemia* is often enhanced with regard to long-chained polyunsaturated fatty acids by feeding a diet high in these lipids. One of the most widely practiced ways of doing this is to feed a special yeast preparation, produced by adding cuttlefish liver oil (15%) to the culture medium for baker's yeast (*Saccharomyces cerevisiae*), to *Artemia* nauplii. These animals contain as a result an enhanced fatty acid profile in which specific polyunsaturated fatty acid' `0:5 n-3 and 22:6 n-3) represent about 10% of the total fatty acids or 1-2% of total dry weight. As these specific fatty acids have been shown to be important for many fish species, their role in the nutrition of medaka needs to be examined in detail.

The total ash level (%A) is quite different in the PC diet, being only a third or less of that in *Artemia* nauplii and the commercial diet formulation. While this might play a role in the lower early growth, it is doubtful. In that aquatic animals can take up minerals from the water across the gill membranes, dietary minerals have been shown to be of limited significance for cultured fish.

Essential nutrients such as water-soluble vitamins could also impact the early growth. Water-soluble vitamins rapidly leach from the small particles and consequently ingested amounts could be marginal. The deformities noted in fish fed the flake plus *Artemia* diet could indicate a problem with ascorbic acid. Work by Dabrowski et al. (1988) suggests the ascorbate level in *Artemia* nauplii rapidly decreased after hatching. In that the level of this nutrient also declines markedly during embryonic development in fish (Sato et al., 1987), tissue levels need to be rapidly restored during early feeding (Soliman et al., 1986). While the PC diet contains vitamin C, oxidation during processing and storage as well as leaching (Goldblatt et al., 1979; Soliman et al., 1987) of this vitamin could reduce levels reaching the early feeding period. Because vitamin C is particularly important to fish growth and reproduction, as well as various aspects of detoxification

(see reviews by Hilton, 1984; Lovell, 1984; Sandnes, 1984), establishment of adequate tissue levels should be undertaken.

Other factors that may be important in the feeding of larval and early juvenile fish are movement of live food, color, palatability, and digestibility. Factors involved with live prey, which have been shown to be important to larval fish such as proteolytic autolysis (Dabrowski, 1979) and free amino acid content (Holm and Walther, 1988), have not been investigated and would be difficult to replicate in formulated diets.

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DEVELOPMENT OF CARCINOGENESIS BIOASSAY MODELS: RECENT STUDIES ON THE RESPONSE OF SMALL FISH SPECIES TO VARIOUS CLASSES OF CARCINOGENS

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BACKGROUND

Several small fish species, especially the Japanese medaka (*Oryzias latipes*) and the guppy (*Poecilia reticulata*) are beginning to be widely used in experimental carcinogenesis studies (see reviews by Ishikawa et al., 1984; Zimmerer, 1984; Hawkins et al., 1988*a*; and Metcalfe, 1989). To reach one ultimate objective, the development of rapid, sensitive, and economical carcinogenicity bioassays, the field must develop along two principal fronts. On one front, the biology of tumorigenesis in the models must be understood in order to evaluate responses following exposure to test compounds and to compare and extrapolate these responses to other models. These studies are based mainly on the confirmed high sensitivity of medaka and several other small fish species to the hepatocarcinogenic effects of carcinogens such as methylazoxymethanol acetate (MAM) (Aoki and Matsudaira, 1984), benzo[*a*]pyrene (Hawkins et al., 1988*b*) and diethylnitrosamine (Kyono-Hamaguchi, 1984; Nakazawa et al., 1985; Hinton et al., 1988). Carcinogenic effects in tissues and organs other than the liver also have been examined, such as the retinal neoplasms that develop in medaka following exposure to MAM (Hawkins et al., 1986), exocrine pancreatic neoplasms in MAM-exposed guppies (Fournie et al., 1987), and melanomas and other types of tumors in medaka following exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Hyodo-Taguchi and Matsudaira, 1984; Hyodo-Taguchi and Matsudaira, 1987).

Along the other front are studies that have to do with the applicability of small fish to carcinogenesis bioassays. These studies address the histogenesis of carcinogen-induced lesions in various tissues, the classes of chemical carcinogens that induce tumorigenic responses in the test species, spontaneous tumor rates, and identifying sources of variability in carcinogenesis bioassays.

Studies described in this report mainly concern bioassays designed to establish the tumorigenic sensitivity of the medaka and guppy to compounds representing a broad spectrum of carcinogenic mechanisms. Correspondingly, the studies address tumor histogenesis, spontaneous

tumor rates, and mechanisms of carcinogenesis, with respect to hepatic metabolism of carcinogens and toxic events in the initiation of tumorigenesis.

STUDIES

Studies on the Metabolism and Carcinogenicity of the Aromatic Amine 2-Acetylaminofluorene in the Japanese Medaka (*Oryzias latipes*) and King Cobra Guppy (*Poecilia reticulata*) and Its Metabolism in the Medaka

The aromatic amines are a class of chemicals that include the carcinogens benzidine and aniline as well as 2-acetylaminofluorene (2-acetamidofluorene; *N*-2-fluorenylacetamide; 2-AAF). Although the carcinogenicity of 2-AAF in rodents is well known and 2-AAF is widely used as a model carcinogen in initiation-promotion tests, its carcinogenicity, or the carcinogenicity of any other aromatic amine, has not been extensively examined in small fish models. For 2-AAF to be carcinogenic, it must be N-hydroxylated by a cytochrome P-450-dependent, microsomal-bound enzyme. Ring hydroxylation, on the other hand, by another P-450 enzyme appears to be a detoxification step. In this series of studies, we are investigating parameters of hepatic metabolism of 2-AAF in the medaka and guppy, especially the ability of the fish to activate or to detoxify the carcinogenic compound. In addition, we examined the tumorigenic responses of 2-AAF in the medaka and guppy following waterborne exposures.

To investigate the hepatic bioactivation of 2-AAF, medaka were given a preliminary 48-hour exposure to 8.6 ppm 2-AAF, and the activities of a series of mixed-function oxidase enzymes (MFOs) were assayed. The activity of the MFO ethoxycoumarin *O*-deethylase was suppressed by 2-AAF exposure. A new protein band at approximately 49 kD was observed in the electrophoretic separation of the microsomal fraction from AAF-exposed fish but not in controls. No difference was observed in the bands of the cytosolic fractions from fish from the treated and control groups. For the definitive study, approximately 100 fish were exposed to 2.1 ppm 2-AAF for 48 hours, with 100 untreated fish serving as controls. Exposed fish were transferred to a dilute solution of 2-AAF for 24 hours and held in that exposure medium until they were sacrificed. Incubation of 2-AAF with the microsomal fractions from treated and control medaka resulted in a decrease by a factor of 4 in the total pool of 2-AAF metabolites in the 2-AAF-pretreated microsomes relative to that of the controls (Table 1). The major AAF metabolite formed *in vitro* was 7-OH-AAF, followed by 5-OH-AAF, both of which indicate ring hydroxylation. The carcinogenic intermediate, *N*-OH-AAF, was also produced demonstrating, at least qualitatively, the capability of medaka hepatic P-450 enzyme systems of activating aromatic amines. In summary, 2-AAF depresses hepatic microsomal oxidative enzyme activities in the

Table 1. Biotransformation Pathways in Control and AAF-treated Medaka and Guppy

Parameter Measured	Medaka		Guppy	
	Control	Treated	Control	Treated
Protein Yield (mg/g liver)				
Microsomes	9.6	15.4	6.5	6.8
Cytosol	52.0	44.4	28.6	29.1
Oxygenation of AAF (pmole/min/mg protein)				
Total	256	68.5	510	130
7-OH	210	48.4	n.c.[a]	n.c.
5-OH	20	8.8	n.c.	n.c.
3-OH	4	1.9	n.c.	n.c.
1-OH	2	1.3	n.c.	n.c.
N-OH	5	0.7	n.c.	n.c.
Glucuronyltransferase (pmole/min/mg protein)				
4-Methylumbelliferone	556	680	430	576
3-Hydroxy-AAF	181	244	133	102
7-Hydroxy-AAF	n.d.[b]	n.d.	74	46
Epoxide Hydrolase (nmole/min/mg protein)	1.5	1.1	6.6	4.6
GSH-*S*-Transferase (nmole/min/mg protein)	1,370	1,844	4,079	4,350
Sulfotransferase (pmole/min/mg protein)				
4-Methylumbelliferone	129.6	40.0	86.2	118.5
3-Hydroxy-AAF	25.2	30.0	7.6	83.5
7-Hydroxy-AAF	n.d.	n.d.	120.8	43.8
N-Hydroxy-AAF	184[c]	75[c]	11	16.6

[a]n.c. - Assay not completed.
[b]n.d. - Assay not done.
[c]Results not reliable because of problems with the assay.

medaka, whereas it increases glutathione *S*-transferase activity. Exposure to 2-AAF does not seem to affect the activities of epoxide hydrolase or of glucuronyltransferase. Medaka unexposed to 2-AAF appear to be able to hydroxylate AAF *in vitro* mainly to ring metabolites and to a lesser extent to the N-metabolite, *N*-hydroxy-AAF, the proximate carcinogen.

The approach used to investigate the hepatic metabolism of AAF in the guppy was similar to that used for the medaka, and the interim results are presented in Table 1. Thin-layer

chromatographic analyses of AAF metabolites in guppies are not complete, but it appears that the AAF-treated guppies have more *N*-hydroxy-AAF than do controls; but in both treated fish and controls, 7-hydroxy-AAF is the major metabolite. Overall, AAF monooxygenation is significantly lower in treated than in control guppies possibly because of the presence of residues of AAF or its metabolites that were isolated along with the microsomes. Epoxide hydrolase activity was also significantly lower in treated than in control livers, which is counter to the usual effects of AAF in rats and mice, in which AAF-induced liver nodules have high epoxide hydrolase activity. The effect of epoxide hydrolase could also possibly be due to direct inhibitory effects of residues of AAF metabolites. Other effects were a significant decrease in 7-hydroxy-AAF-glucuronyltransferase activity and a decrease in 3-hydroxy-AAF-glucuronyltransferase in treated fish. Decreased conjugation with glucuronide, which was not observed in medaka, would tend to increase toxicity as glucuronidation is a major detoxification pathway. There was also a striking increase in sulfotransferase activity in the guppy with 3-hydroxy-AAF as a substrate. Since sulfation is a major toxification pathway, an increase in sulfotransferase activity would explain the increased toxicity of AAF to the guppy. A striking increase in 3-OH-AAF sulfotransferase did not occur in medaka, and the 7-OH-AAF assay was not done in that species.

For the tumorigenesis studies, medaka and guppy were exposed to AAF by two mechanisms: (1) single or multiple static pulse exposures and (2) continuous prolonged static-renewal exposure. All exposures were performed in the dark or subdued light. Pulse exposure groups were as follows: (a) 1 × 6 hours; (b) 1 × 12 hours; (c) 2 × 12 hours, 1-week intervals; (d) 3 × 12 hours, 1-week intervals; and (e) 4 × 12 hours, 1-week intervals. For the continuous static renewal exposure, one group was exposed for 168 hours (7 days) with renewal of AAF and control solutions every 24 hours. Appropriate controls were included for each treatment group. Pulse exposures were conducted in one control aquarium and one AAF treatment aquarium. Medaka and guppies were exposed simultaneously in the same treatment aquarium with specimens for each treatment contained in separate mesh chambers. For each weekly pulse exposure, AAF was added to well water to produce a 10 mg/l nominal concentration, the mixture was stirred in the dark at room temperature for 4 days and filtered through a 0.2-micron Nuclepore membrane filter, and the resulting suspension was diluted 1:1 with well water and transferred to the exposure aquarium. Concentrations for both types of exposures were about 1.0 mg/l. Following exposure, the specimens were placed in grow-out aquaria for sampling at 6, 9, and 12 months after initial exposure. Histopathological evaluations followed protocols described above. AAF appears to cause neoplastic lesions in livers of the guppy earlier and in higher incidences that in the medaka. The incidences of those lesions are shown in Tables 2 and 3 for the guppy and medaka,

Table 2. Incidence of Combined Hepatocellular Neoplastic Lesions (Altered Focus, Adenoma, and Carcinoma) in the Japanese Medaka (*Oryzias latipes*) Exposed to AAF

Exposure Groups	24 wk	36 wk	52 wk
Control 12 hr × 4	0/62	0/75	0/1
Constant Control (no pulse)	0/65	1/76	1/104
AAF 6 hr × 1	0/76	0/75	0/0
AAF 12 hr × 1	0/73	1/76	0/0
AAF 12 hr/wk × 2	3/74	0/71	0/1
AAF 12 hr/wk × 3	0/1	1/31	0/0
AAF 12 hr/wk × 4	0/65	0/74	3/87
AAF Constant (24 hr × 7)	3/77	0/75	0/83

Table 3. Incidence of Combined Hepatocellular Neoplastic Lesions (Altered Focus, Adenoma, and Carcinoma) in the Guppy (*Poecilia reticulata*) Exposed to AAF

Exposure Groups	24 wk	36 wk	52 wk
Control 12 hr × 4	0/89	1/70	0/1
Constant Control (no pulse)	0/84	0/69	1/81
AAF 6 hr × 3	0/95	1/74	5/84
AAF 12 hr × 1	0/94	0/72	0/0
AAF 12 hr/wk × 2	2/89	1/74	2/33
AAF 12 hr/wk × 3	4/91	3/72	8/68
AAF 12 hr/wk × 4	3/97	7/72	3/66
AAF Constant (24 hr × 7)	3/86	7/75*	9/98*

*Statistically significant at $p \leq 0.05$ when compared by Fisher's exact test with the incidence in the constant control group.

respectively. The response in the two highest exposure levels in the guppy tests was statistically significant by Fisher's exact test. The hepatic neoplastic lesions diagnosed in the guppy, however, were not considered malignant. Most of the lesions were either foci of cellular alteration (altered

foci) or hepatocellular adenomas. None were diagnosed as hepatocellular carcinomas.

To our knowledge, these studies on the hepatic metabolism of AAF in medaka and guppy represent the first time that the relative capabilities of these fish species of metabolizing the carcinogen AAF have been investigated and compared with tumorigenic effects. The comparative inability of the medaka to adequately metabolize 2-AAF to its proximate carcinogen by N-hydroxylation relative to its ability to detoxify the compound by ring hydroxylation is possibly related to the lack of a tumorigenic effect. Studies on the metabolism of AAF by the guppy are still incomplete but the decreased conjugation with glucuronide that was observed might have served to increase the toxicity of AAF to the guppy. AAF caused a statistically significant increase in combined hepatic neoplastic lesions in the guppy. The low carcinogenic potency of this compound, however, was evidenced by the fact that many of the induced lesions, in spite of the fact that they persisted for 6 and 9 months postexposure, did not appear to be robust or actively progressing lesions. This is the first time in our studies that we have observed the apparent regression of carcinogen-induced lesions, even ones such as those we designate as altered foci most of which regress in rodents following cessation of the stimulus that induced them. This poses some interesting questions regarding the nature of the neoplastic initiation in small fish models. Perhaps the persistence of neoplastic lesions is related to the dose × time or to the strength of the test carcinogen. Nevertheless, a metabolic explanation for tumorigenic sensitivity of the guppy compared with the insensitivity of the medaka was not seen.

Carcinogenesis Bioassay with the Halogenated Hydrocarbon Vinylidene Chloride (1,1-Dichloroethylene) on the Japanese Medaka (*Oryzias latipes*) and the King Cobra Guppy (*Poecilia reticulata*)

Vinylidene chloride (1,1-dichloroethylene), an air and drinking water contaminant (U.S. EPA, 1982; U.S. EPA 1986), is a monomer used to produce polyvinylchloride resins and plastic food wraps (Quast et al., 1986). Its potential for contamination in the workplace and for consumer exposure and its structural similarity to other compounds of environmental interest such as vinyl chloride, trichloroethylene, 1,2-dichloroethane, 1,2-dibromoethane, and tetrachloroethylene make vinylidene chloride (VC) a likely candidate for carcinogen bioassay. Recently, the International Agency for Research on Cancer (IARC) has reevaluated VC and concluded that it, along with trichloroethylene, belongs to the group 3 animal carcinogens, meaning that there is limited evidence for the carcinogenicity of those compounds to animals (Van Duuren, 1989). The term "limited evidence" is applied because, whereas 2-year bioassays of Fischer 344 rats and B6C3F$_1$ mice did not result in significant increases in tumors of treated as compared with control animals

(see Chu and Milman, 1981), a study on Swiss mice resulted in high incidences of kidney tumors in the male specimens (Maltoni, 1977; Maltoni et al., 1977).

We conducted carcinogenesis bioassays with VC in the medaka and the guppy in flow-through exposures. Histopathological examination of specimens exposed to VC for 3 months then grown-out in clean water for additional periods of 3, 6, or 9 months indicate that this compound is hepatocarcinogenic to both species. Although the incidences are in the range of 15% to 20%, which we consider a moderate carcinogenic response in these models, the lesions were histologically well advanced and would certainly affect the health of the organisms.

Medaka and guppy were exposed to VC under flow-through conditions for 3 months (Aldrich Chemical Company, Milwaukee, WI; 99% purity). Following a series of range-finding tests, a targeted exposure concentration of 40 ppm was chosen. Three hundred 6- to 7-day posthatch medaka and 300 guppies 24 to 48 hours old were impartially distributed to control and treatment aquaria.

The tests incorporated the following treatment groups: (1) aquarium control group (situated outside the exposure system); (2) flow-through control group (situated inside the exposure system and thus subject to low levels of volatile test compounds); (3) low concentration exposure group in which VC was delivered once for 24 hours every 6 days; (4) intermediate concentration exposure group in which VC was delivered twice for 24 hours every 6 days (a 24-hour exposure followed by 48 hours in toxicant-free water); (5) high concentration exposure group in which the nominal 40 ppm concentration was delivered continuously for a 12-day period and terminated because of excessive mortality; and (6) high concentration exposure in which 30 ppm VC was delivered continuously for 90 days. VC values measured by gas chromatography are depicted in Figures 1 and 2 for the medaka and guppies, respectively.

Several studies aimed at determining the acute and chronic toxicological effects of VC on medaka and guppy were conducted. At day 28, 15 randomly sampled fish from each retention chamber were fixed for histopathological analysis and electron microscope analysis. Another 25 fish from each retention chamber were examined for compound-related growth reduction. Each fish was measured to the nearest millimeter standard body length, and individually wet and dry weighed. A one-way analysis of variance, followed by a Newman-Keuls multiple comparison test, was applied to the data for each species. Results presented in Table 4 indicate that intermittent exposure to 40 ppm VC did not affect the growth of young medaka.

However, as results in Table 5 suggest, intermittent exposure to 40 ppm VC significantly retarded the growth (length, wet weight, and dry weight) of guppies.

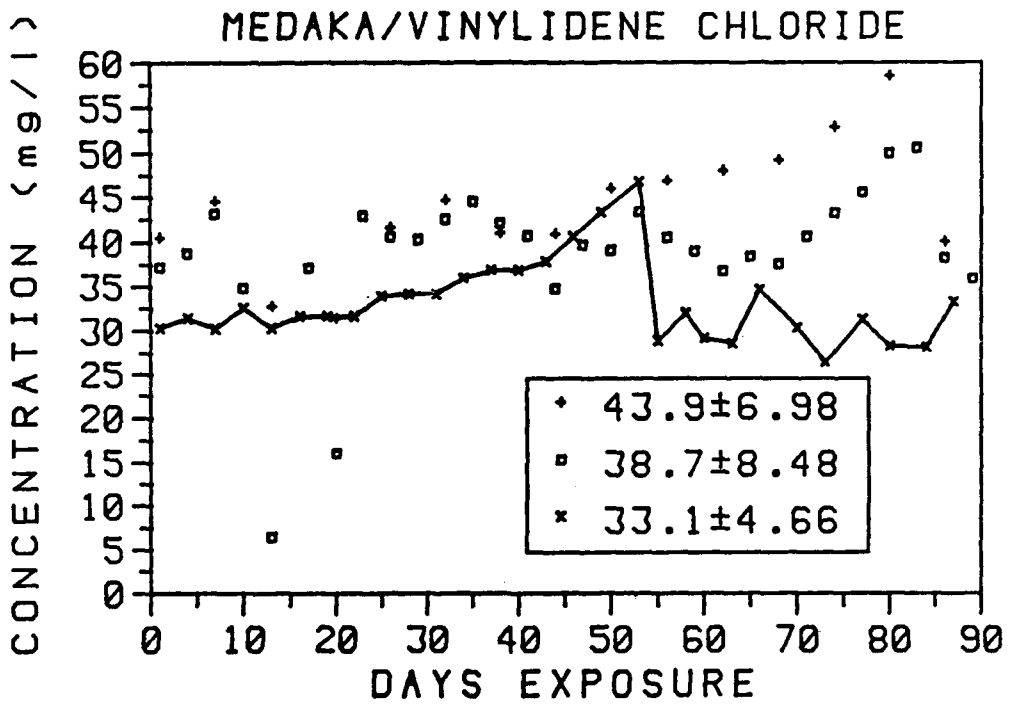

Figure 1. Measured concentrations of vinylidene chloride during 90-day exposure in medaka (*Oryzias latipes*).

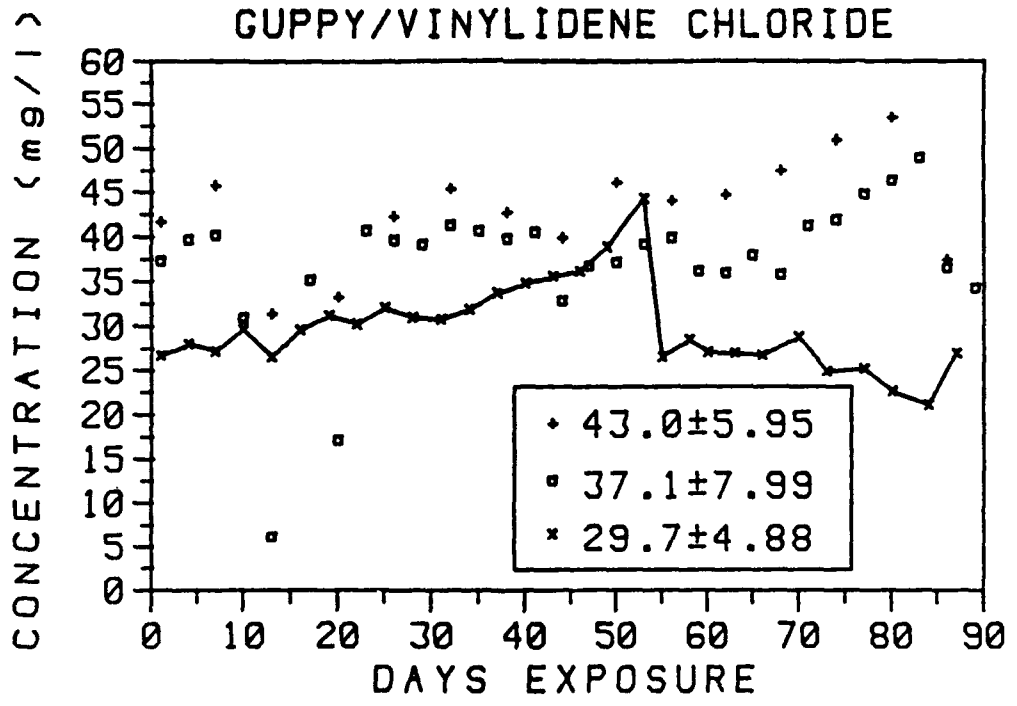

Figure 2. Measured concentrations of vinylidene chloride during 90-day exposure in the guppy (*Poecilia reticulata*).

Table 4. Mean Body Measurements of Medaka from Retention Chambers at Day 28 of Exposure to Vinylidene Chloride (VC)

Exposure Group	Length (mm)	Wet Weight (mg)	Dry Weight (mg)
Control (no exposure)	12.3[1**]	22.9[1]	5.9[1]
Control (flow-through)	11.4[1]	17.4[1]	4.5[1]
Low VC	11.9[1]	24.5[1]	5.3[1]
Intermediate VC	12.0[1]	23.2[1]	5.5[1]

**Within each category, treatment means with different numbers were determined to be statistically different at the 0.01 level.

Table 5. Mean Body Measurements of Guppy from Retention Chambers at Day 28 of Exposure to Vinylidene Chloride (VC)

Exposure Group	Length (mm)	Wet Weight (mg)	Dry Weight (mg)
Control (no exposure)	12.0[2**]	30.5[2]	6.7[2,3]
Control (flow-through)	12.2[2]	31.2[2]	8.0[3]
Low VC	11.2[1]	25.0[1]	5.5[2]
Intermediate VC	10.8[1]	22.2[1]	3.8[1]

**Within each category, treatment means with different numbers were determined to be statistically different at the 0.01 level.

At the termination of the exposures, fish were removed to grow-out aquaria to be sampled at 24, 36, and 52 weeks from the time they entered the exposure system. Results of ongoing histopathological analyses are summarized in Tables 6 and 7.

Table 6. Incidence of Hepatocellular Neoplastic Lesions (Altered Focus, Adenoma, and Carcinoma) in the Japanese Medaka (*Oryzias latipes*) Exposed to Vinylidene Chloride (VC)

Exposure Group	24 wk (%)
Control (no exposure)	0/19
Control (flow-through)	0/0
Low VC	0/0
Intermediate VC	0/0
40 ppm VC[a]	1/24 (4)
30 ppm VC	8/76 (11)

[a]Exposure was interrupted after 2 weeks because of excessive mortality, and specimens were removed to grow out; a new exposure at 30 ppm VC was initiated and continued for 90 days.

Table 7. Incidence of Hepatocellular Neoplastic Lesions (Altered Focus, Adenoma and Carcinoma) in the Guppy (*Poecilia reticulata*) Exposed to Vinylidene Chloride (VC)

Exposure	24 wk (%)
Control (no exposure)	0/71
Control (flow-through)	0/0
Low VC	0/0
Intermediate VC	0/0
40 ppm VC[a]	10/61 (16)[a]
30 ppm VC	12/70 (17)[a]

[a]Exposure was interrupted after 2 weeks because of excessive mortality, and specimens were removed to grow out; a new exposure at 30 ppm VC was initiated and continued for 90 days.
[a]Statistically significant at $p \leq 0.05$ when compared by Fisher's exact test with the incidence in the flow-through control group.

Analysis of interim data indicates that vinylidene chloride (VC) is carcinogenic to both medaka and guppy when administered in flow-through exposures for 90 days, with specimens examined at 24 weeks after initial exposure. In the guppy, incidences of hepatocellular neoplastic

lesions (total of altered foci, adenomas, and carcinomas) in the high concentration (30 ppm continuous 90-day exposures) were statistically significant. In similarly conducted tests, we have examined the carcinogenicity of three other compounds, 1,1,2,2-tetrachloroethane (TeCE), 1,2-dibromoethane (EDB), and trichloroethylene (TCE), that are structurally similar to VC. Neither TeCE nor TCE was carcinogenic to either the guppy or medaka; whereas, EDB was highly carcinogenic to the medaka and not yet tested in the guppy. The metabolic activation of EDB involves conjugation with glutathione, a pathway normally associated with detoxification. Although the metabolism of VC in fish has not been examined, in rodents VC is metabolized by MFOs to an unstable epoxide that can form chloroacetyl chloride, a highly reactive compound, which can be detoxified by glutathione, bind to macromolecules, or be further metabolized to a dihydrodiol (see D'Souza and Andersen, 1988). The studies suggest that these small fish species are useful for examining the carcinogenicity of halogenated hydrocarbons that have great environmental importance. Further studies on the metabolic activation of these compounds correlated with their carcinogenicity to these models will yield important information regarding the fate and effects of these compounds.

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PATHOLOGY ASSESSMENT ISSUES

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INTRODUCTION

Experimental Pathology Laboratories, Inc. (EPL), is working with the U.S. Army on several projects in addition to providing pathology support services for the bioassays conducted in the field with medaka (*Oryzias latipes*) as the test animal. These projects include method development for optimal histologic sectioning of medaka, compilation of two atlases (normal histology of the medaka and histopathology of the medaka), and an evaluation of the U.S. Army's fish culture facilities and procedures for the conduct of bioassays.

METHOD DEVELOPMENT FOR HISTOLOGIC SECTIONING OF MEDAKA

The sectioning method used currently for preparing medaka for histologic evaluation is to remove the tail at the caudal peduncle, embed the medaka left side down, and cut five step sections in a sagittal plane to include two left paramedian sections, one midsagittal section, and two right paramedian sections. Thirty tissues are examined routinely in each medaka. There are nine tissues for which there is less than optimal accountability when the above sectioning method is used. These tissues are pituitary gland, pineal organ, corpuscle of Stannius, spleen, thymus, urinary bladder, chromaffin tissue, interrenal tissue, and gallbladder.

Possible optional sectioning methods that might improve tissue accountability include cutting transverse sections or cutting frontal (horizontal) sections.

Five medaka were gross trimmed for transverse sectioning. The whole fish body was transected cranial to the eye, caudal to the eye, at the caudal edge of the operculum, at the cranial edge of the pectoral fin, at the attachment of the pelvic fin, halfway between the pelvic fin and the anus, at the anus, and about 2-mm caudal to the anal opening. These eight body sections were placed caudal side down, four in each of two cassettes. The microtomist cut five step sections through the different areas of the body. This resulted in 40 transverse sections on two glass slides. These sections from the five medaka were examined for tissue accountability. All but three of the 30 required tissues were observed in all five medaka. The corpuscle of Stannius was seen in only one of the medaka. Gallbladder and pseudobranch were present in three of the five fish. Swim

bladder was present in all five fish, but the epithelial cells near the cranial rete of the swim bladder were not always present in the section.

A comparison was made between the longitudinal step section method and the transverse step section method with regard to the various stages in the preparation of glass slides.

Gross Trimming

Longitudinal Sections

Gross trimming is accomplished rather quickly and easily as the tail only is trimmed from the fish, and the fish is placed whole in the cassette.

Transverse Sections

Gross trimming requires more time and more materials for transverse sections than for longitudinal sections as the fish is transected into eight pieces and is placed in two cassettes. There is an opportunity for loss of tissues as the body sections are transferred to the cassettes. There is considerable reliance on the technician to cut the sections consistently from fish to fish and to place them in the proper order in the cassettes.

Embedding

Longitudinal Sections

Embedding is accomplished easily as there is only one whole fish to handle. Care must be taken, however, that the fish are embedded consistently with the left side down and such that the midsagittal plane of the fish is parallel to the bottom of the embedding pan.

Transverse Sections

Embedding is more time consuming when transverse sections are prepared than it is when whole fish are handled. There is opportunity for misplacement of sections relative to one another and reversal of the cranial and caudal faces of the tissue mass.

Microtomy

Longitudinal Sections

Whole fish are difficult to section as tissues of varying density and texture must be cut through simultaneously. The consistency with which the five levels are chosen for placement on the slides is highly dependent on the skill of the technician. The pigmented eye serves as a useful

marker for the proper specimen depth. The proper order of placement of the various step section levels on the slide is not a trivial task.

Transverse Sections

Microtomy is easy relative to sectioning the whole fish. There is no good anatomic marker to recognize the proper levels of the sections to be taken, but this can be overcome somewhat by establishing an approximate distance between levels and discarding approximately the same number of sections between levels for each fish.

The transverse section method of cutting medaka has several advantages over the longitudinal section method. In the five medaka cut transversely, there was marked improvement in tissue accountability over medaka sectioned longitudinally. Both organs of a pair (for example, pseudobranch, thymus, eye, nares) were usually present in the transverse sections. Often in longitudinal sections only one of a paired organ (especially thymus and nares) is present for examination. In transverse sections of the head, skeletal structures were easy to assess as they were present on left and right sides symmetrically. On the negative side, transverse sections are not ideal for small organs like the pituitary gland that have a varying structure (specific cell types for secretion of a variety of hormones) that differs from cranial to caudal aspects of the organ. Transverse sections also do not illustrate the cranial to caudal relationships of one organ to another in a single section as, for example, in the pharynx, esophagus, intestine sequence and in the kidney, mesonephric duct, urinary bladder sequence.

The ultimate comparison of the longitudinal section method versus the transverse section method might be made if a study were conducted in which fish are exposed to a chemical, such as methylazoxymethanol acetate, that induces a variety of lesions. One-half of the fish would be sectioned longitudinally and the other half sectioned transversely. The results would be compared as to percentage of fish with various lesions and as to tissue accountability.

The method of sectioning medaka in a frontal, or horizontal, plane that is perpendicular to the sagittal plane and parallel to the dorsal and ventral surfaces of the fish has not yet been fully explored as to its advantages or disadvantages compared to the longitudinal section method.

MEDAKA ATLASES

The U.S. Army and EPL are cooperating to compile two atlases. One will illustrate the normal anatomy of the medaka, and the other will illustrate spontaneous and induced lesions of the medaka. These atlases are intended to be used by the U.S. Army and its contractors in the pathologic assessment of medaka from bioassays. The emphasis will be on light microscopy and

hematoxylin-and-eosin-stained tissues. Special stains will be illustrated to corroborate a diagnosis or depict a tissue type.

Atlas of Normal Histology of the Medaka

This atlas will be compiled by Marilyn Wolfe, Suzanne Neuenschwander, and Henry Gardner. It will illustrate medaka of three ages: 14 days, approximately 3 months, and approximately 6 months. These ages correspond to the age at which medaka are placed on test (14 days), the approximate age of medaka at the first, or interim, sacrifice during a bioassay (3 months), and the approximate age of medaka at the final sacrifice (6 months) during a bioassay. Medaka will be sectioned in sagittal, transverse, and frontal planes to illustrate the relationships of the organ systems. Chapters in the atlas will be organized by organ systems, and there will be a brief introduction to each system. The atlas will consist mainly of color photomicrographs with explanatory captions. Line drawings will be included as necessary to illustrate concepts or anatomy.

Atlas of Histopathology of the Medaka

This atlas will have a systems arrangement with introductory material for each system, as is also planned for the atlas of normal histology. The individuals who will compile the atlas are Henry Gardner, Jerry Hendricks, Tracie Bunton, William Hawkins, John Harshbarger, and Marilyn Wolfe. The atlas will be reviewed by various specialists in the different systems. The photomicrographs will be in color, and the captions will contain as much information about the fish with the lesion as it is possible to obtain. This information will include such facts as age, sex, diet, water temperature, and chemical exposure, if applicable, to include concentration of the chemical and duration of the exposure. Interesting lesions will be included in the atlas even if all of the above information is not available for the medaka.

One object of this process of assembling an atlas of histopathology will be to make an attempt at standardization of nomenclature for similar lesions that occur under a variety of conditions. In addition, information on the incidence of common and unusual lesions will be gathered.

EVALUATION OF FISH CULTURE FACILITIES AND BIOASSAY PROCEDURES

Experimental Pathology Laboratories, Inc., will evaluate the U.S. Army Biomedical Research and Development Laboratory for facilities and procedures related to fish culture and use of fish, particularly medaka, in field bioassays. The standards for evaluation will be the Good Laboratory

Practices (GLP) legislation for nonclinical laboratory studies. The U.S. Army is not required to operate its laboratory facilities under the rules of the GLP Act. Compliance with GLP regulations, however, could only strengthen the credibility of the data obtained in the field bioassays with the medaka as the test animal.

One of the major elements of EPL's evaluation will be an examination of the Standard Operating Procedures (SOPs) that pertain to fish culture and use. SOPs detail how specific laboratory procedures are to be performed by laboratory personnel. These procedures include, among others, animal room care, diet preparation, storage and handling of laboratory chemicals, specimen collection and identification, necropsy procedures, data handling, and equipment maintenance and calibration.

Another element of the evaluation will be an audit of data from a fish bioassay that has been completed. The study protocol is the key to such a data audit as the protocol outlines the purpose of the study and states precisely how the study is to be conducted. By an examination of the raw data generated during the study, one should be able to determine if, in fact, the study was conducted according to the protocol.

This evaluation of facilities and procedures will include recommendations for improvements in the existing system if improvements are necessary.

ACUTE EXPOSURE OF MEDAKA TO CARCINOGENS: AN ULTRASTRUCTURAL, CYTOCHEMICAL, AND MORPHOMETRIC ANALYSIS OF LIVER AND KIDNEY*

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INTRODUCTION

Several species of fish are recognized as acceptable and effective nonmammalian models for toxicity and carcinogenicity studies and as useful as complementary extensions of current mammalian testing systems (Couch and Courtney, 1987; Hinton et al., 1988). Fish have exhibited relative degrees of susceptibility to a variety of unrelated carcinogens. The Japanese medaka, *Oryzias latipes*, has served as a popular model for the investigation of chemically induced hepatic and renal tumors (Kyono, 1978; Norton and Gardner, 1987; Hinton et al., 1988). Many such studies have included the use of diethylnitrosamine, a site-specific carcinogen for hepatic neoplasms, and methylazoxymethanol acetate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, two compounds that are less distinct in their target sites (Ishikawa et al., 1975; Aoki and Matsudaira, 1977; Kimura et al., 1981).

Critical information concerning the characterization and classification of chemically induced tumors in fish has been acquired (Aoki and Matsudaira, 1984; Hawkins et al., 1986). Nevertheless, because the employment of fish as a bioassay system is a relatively new approach to the investigation of cellular transformation, there are considerable voids in the basic knowledge of the biochemical, cytochemical, and ultrastructural manifestations of exposure to selected carcinogens. Specifically, there is a paucity of information regarding cellular alterations that result from fish being exposed to different carcinogens in a sequential fashion to determine initiation/promotion relationships.

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*The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

MATERIALS AND METHODS

The primary objective of this study is to determine the ultrastructural and selected cytochemical effects of an initial acute exposure of medaka to diethylnitrosamine (48 hours at 200 mg/l) followed by a chronic exposure of the fish to trichl[illegible]hylene (10 mg/l). Randomly selected fish (exposed and control) will be sacrificed at 6, 16, 32, and 52 weeks subsequent to the initial exposure to diethylnitrosamine. Recognizable hepatic and renal lesions will be investigated by light microscopy (hematoxylin and eosin stains) and conventional transmission and scanning electron microscopy. Cellular relationships, cytoplasmic traits unique to preneoplastic and neoplastic cells, and pertinent intercellular markers will be analyzed by transmission electron microscopy. A scanning electron microscopy evaluation of the chemically induced lesions should provide valuable complementary information on a three-dimensional level. Such an analysis may be particularly beneficial in the investigation of spongiosis hepatis, a hepatic lesion characterized by the presence of cyst-like cavities.

Trichloroethylene was chosen as a carcinogen of interest primarily because the compound is considered a significant contaminant of various toxic waste sites and also represents a serious risk to aquifers. Relatively little is known of the cytochemical and ultrastructural effects of exposure of fish model systems to trichloroethylene. The acquisition of such basic information may prove valuable to scientists studying chemically induced carcinogenesis and investigators attempting to predict the consequences of exposure to contaminants of toxic waste sites containing trichloroethylene.

DISCUSSION

Preneoplastic lesions and hepatocellular carcinomas that develop subsequent to the administration of diethylnitrosamine have demonstrated a suppressed activity of certain enzymes, including magnesium-dependent, adenosine triphosphatase (Hinton et al., 1988). Additional studies involving carcinomas have revealed pronounced differences between normal and transformed cells with regard to the activity of enzymes such as acid phosphatase, glucose-6-phosphatase, and uridine-diphosphate-glucuronyl transferase (Taper and Bannasch, 1976; Zaki, 1982; Fischer et al., 1983). By binding electron-dense markers to reaction products, selective cytochemical techniques allow, at the microscopic level of cellular organelles, the detection of specific sites of enzyme activity. The liver and kidney of medaka will be monitored ultrastructurally and cytochemically, since both organs contain the oxidative enzyme systems necessary for the bioactivation of potential carcinogens (Hinton et al., 1978; Stegeman, 1981; Hard et al., 1984).

Three specific enzyme systems, acid phosphatase, peroxidase, and magnesium-dependent adenosine triphosphatase will be investigated ultrastructurally by means of electron-dense visual markers. Investigators have demonstrated decreased activity of acid phosphatase and membrane-bound adenosine triphosphatase in acidophilic cell foci induced in livers of both rats and fish exposed to *N*-nitrosomorpholine and diethylnitrosamine, respectively (Scherer and Emmelot, 1976; Hinton et al., 1988). Suppressed adenosine triphosphatase activity has also been documented in cells of resultant tumors (Hinton et al., 1988). Normally, adenosine triphosphatase activity is localized in the basal, apical, and lateral plasma membranes of hepatocytes. One current theory regarding the decreased activity of adenosine triphosphatase among exposed hepatocytes relates to the demonstrative accumulation of intracellular calcium (Bannasch et al., 1985). Peroxidase activity will be analyzed, cytochemically, primarily to substantiate results obtained from preliminary investigations (unpublished data) which indicate that significant quantitative changes occur in peroxisomes of medaka hepatocytes and bile preductile cells as a consequence of exposure of diethylnitrosamine. To provide a quantitative analysis of the ultrastructural observations, a morphometric determination of nuclear to cytoplasmic ratios, lipid content, and selected cellular organelles, such as the endoplasmic reticulum and lysosomes, will be conducted.

The development of electron-dense probes for electron-microscopy-related studies concerning immunocytochemistry has enabled investigators to identify, with a high degree of confidence, the location of specific antigenic sites. Many of the initially described immuno-electron microscopic techniques were unnecessarily complex and often resulted in variable results with excess background labeling. However, recent improvements in various aspects of the procedures have resulted in the establishment of techniques characterized by high degrees of specificity, minimal background staining, and reproducibility (Knibbs et al., 1990).

A variety of electron-dense markers are available with ferritin (Takamiya et al., 1979), peroxidase (Raybould et al., 1981), and colloidal gold (de Harven and Soligo, 1986; de Harven and Christensen, 1988) representing the more consistently employed markers. Ferritin is particulate in nature and small in size, both favorable traits for potential immunocytochemical markers; however, the molecule has such a low density that it is unsuitable for many studies. Peroxidase markers are readily detectable, but the electron-dense material can obliterate the fine structure of cells. The various physical and chemical properties of colloidal gold make it one of the more highly desired markers.

CONCLUSIONS

The procedures require the use of specific antibodies conjugated to colloidal gold particles of a predetermined diameter (5-20 nm). Such a technique has proven valuable for determining the origin of cells from which various tumors are formed (Viale et al., 1988; Cosgrove and Fitzgibbons, 1989). Unfortunately, antibodies currently available commercially for investigations of this nature are derived from and utilized primarily in mammalian systems. The antibodies of interest may or may not function effectively in a fish model. However, the potential benefits warrant an attempt to test several of the antibodies generated against specific cellular filaments of different tissue types. The proposed protocol includes an attempt to investigate primary antibodies that have been generated against the proteins vimentin, actin, and desmin. If successful, the technique will be employed at all but the initial time points.

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TUMOR IMMUNODIAGNOSIS IN FISH

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ABSTRACT

The use of fish species in aquatic bioassays for chemical carcinogenesis has produced neoplasms difficult to diagnose based on histologic criteria alone. Immunocytochemistry techniques to identify intermediate filament proteins, which include cytokeratin, desmin, vimentin, neurofilament proteins, and glial fibrillary acidic protein, have been routinely used in mammals to identify cellular differentiation along certain cell lines in tumors. To exploit this technique for aquatic bioassays in order to aid in the diagnosis of problematic tumors in fish, a streptavidin biotin horseradish peroxidase method was used on normal medaka (*Oryzias latipes*) and striped bass (*Morone saxatilis*) tissues. Skin, gills, cornea, gastrointestinal tract, and other epithelial tissues showed good cross-reactivity with cytokeratin antibodies AE1/AE3 (Boehringer Mannheim, Indianapolis, IN) and MAK-6 (Triton Biosciences, Alameda, CA). This cross-reactivity indicates that these and other antibodies to intermediate filaments may assist in tumor immunodiagnoses in fish.

INTRODUCTION

Chemically induced or naturally occurring neoplasms in fish are sometimes difficult to diagnose because of cellular characteristics or patterns of distribution different from what is typically described in the mammalian literature (Bunton, 1990). There are also potentially preneoplastic lesions in fish of unknown significance that may be important to recognize as such because of the need to establish risk. Although ultrastructural examination may assist in identifying some of these lesions and neoplasms, there is a need for the development of additional tests. Immunocytochemistry techniques to identify intermediate filament proteins, which include cytokeratins, vimentin, neurofilament proteins (NF), desmin, and glial fibrillary acidic protein (GFAP), are used in mammalian tumor diagnosis to determine differentiation along cell lines (Battifora, 1988; Bhan, 1988). The current study examined the use of a streptavidin biotin horseradish peroxidase method (Cartun and Pedersen, 1989), three commercially available anticytokeratin antibodies, and two fixatives on normal medaka and striped bass tissues to establish the use of this technique in fish tumor diagnosis (Bunton, in press).

METHODS

All major tissues from striped bass and longitudinally sectioned medaka were fixed in Bouin's or formalin fixatives and routinely processed for paraffin embedment, and 5.0-μm sections were produced and stained with hematoxylin and eosin. Paraffin was removed from serial unstained sections in changes of xylene, followed by dehydration in alcohol. The sections were placed in a solution of hydrogen peroxidase to eliminate nonspecific tissue peroxidase reaction. A primary antibody to cytokeratin, AE1/AE3, MAK-6, or CAM 5.2 (Becton Dickinson, Mountain View, CA), was applied to slides at 4.0°C overnight. A secondary biotinylated antibody, streptavidin peroxidase, and chromogen were applied on the second day.

RESULTS

The primary antibody CAM 5.2 did not react in fish tissues. However, both AE1/AE3 and MAK-6, which consist of mouse monoclonal antibodies against human cytokeratins, showed good cross-reactivity in both species of fish. Epithelium in skin, gills, gastrointestinal tract, kidney, bile duct, cornea, and thymus were positive for cytokeratin, whereas nonepithelial tissues were uniformly negative. Bouin's fixative produced cleaner, stronger staining than formalin produced. Predigestion of tissues with either pepsin or protease also produced stronger staining intensity. Preliminary studies using *AE1/AE3 on neoplasms from* medaka exposed to methylazoxymethanol acetate have shown cytokeratin reactivity in many epithelial neoplasms including cholangiocarcinoma (Bunton, submitted). Preliminary studies with antibodies to other intermediate filament proteins including GFAP and NF have also shown good results in fish tissues.

CONCLUSION

The results show that good cross-reactivity with commercially available antibodies to cytokeratin is present in fish and that these antibodies may be applied to problematic tumors to determine their differentiation along epithelial cell lines. Antibodies to other intermediate filament proteins also show promise and will continue to be studied.

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MOLECULAR ANALYSIS OF MEDAKA TUMORS: NEW MODELS FOR CARCINOGENICITY TESTING

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ABSTRACT

These studies were initiated to examine the role of oncogene activation in carcinogen-induced tumors in the Japanese medaka (*Oryzias latipes*). Medaka fry were exposed in separate experiments to single doses of two known mammalian liver carcinogens: methylazoxymethanol acetate (MAMA) or diethylnitrosamine (DEN). Liver DNA was analyzed in transfection assays for the presence of transforming genes. DNAs isolated from a MAMA-induced cholangiocarcinoma and a mixed hepatocholangiocarcinoma were able to induce foci of altered cell morphology in NIH 3T3 cells as well as in colonies in a colony selection assay. The identity of the transforming gene(s) in the tumor DNAs has not yet been determined. DNA isolated from a DEN-induced cholangiocarcinoma was also positive in both transformation assays and induced tumors in nude mice (Van Beneden et al., 1990). Sequence analysis has been initiated. Although data are preliminary, we have not detected homology to known gene sequences deposited in GenBank or the European Molecular Biology Laboratory (EMBL), suggesting the possibility of a novel oncogene.

INTRODUCTION

This report of oncogene activation in chemically induced tumors in the Japanese medaka is part of an ongoing study to evaluate the use of nonmammalian species for carcinogenicity testing. The medaka, which has been used for carcinogenicity testing for well over a decade (Hoover, 1984), is well suited for these studies. Its small size allows treatment of large numbers of animals in a small space, which provides statistically relevant numbers. Medaka can be induced to breed prolifically year-round and are easy to culture. Induction of tumors is rapid and has been reported in nearly every organ by agents known to be carcinogenic to humans. Unlike some

strains of rodents now used for testing, the incidence of spontaneous tumor formation in the medaka is nearly zero.

Examination of changes at the genetic level, however, is just beginning. Modern molecular oncology has focused on the interactive roles of two classes of genes involved in tumor development: (1) cellular oncogenes—dominant cellular genes with key roles in the control of cell growth and differentiation; and (2) suppressor genes—recessive genes that act as negative regulators of cellular proliferation. The functions of these genes have been extensively studied in human and other mammalian tumors, in *Drosophila*, *Xenopus*, and yeast. Research at the molecular level in teleost fish, however, has lagged far behind. It was not until 1986 that the first oncogenes from fish—*ras* (Nemoto et al., 1986) and *myc* (Van Beneden et al., 1986)—were cloned and sequenced. Since that time, the field of teleost oncogene research has virtually exploded (Van Beneden, in press) with efforts concentrated on the roles of these genes in tumor formation. The use of fish models promises to provide important contributions to the field of cancer research and highlights the value of knowledge of species differences and similarities in comparative carcinogenesis.

The results of the studies reported here on the molecular basis of tumor induction in the medaka provide further clues in the ongoing investigation of the role of oncogenes in the development of chemically induced tumors. The data further indicate that the medaka is an excellent candidate for testing the potentially carcinogenic effects of waterborne toxicants.

MATERIALS AND METHODS

Tumor Induction

Fourteen-day-old medaka fry were exposed to methylazoxymethanol acetate (MAMA) at 20 mg/liter for 2 hours. Animals were then transferred to aquaria containing clean water. Fish were sacrificed at 3 and 6 months postexposure. Livers were excised, and a portion was preserved by fixation in Bouin's solution and subsequently stained with hematoxylin and eosin for histopathological analysis. The remaining tissue was immediately frozen in liquid nitrogen and stored at -70°C until DNA was extracted.

Exposure of 14-day-old fry to diethylnitrosamine (DEN, 200 mg/liter) and subsequent sacrifice of the fish was by a similar procedure, described previously in detail by Van Beneden and associates (1990).

DNA Preparation

High-molecular-weight DNA was prepared by the quick-Dounce homogenization method as described previously (Van Beneden et al., 1990; Van Beneden et al., in press). DNA concentration was estimated by absorbance at 260 nm, and integrity was examined by electrophoresis on low-percentage agarose gels.

Transfection Analysis

A co-transfection assay using mouse fibroblast (NIH 3T3) cells modified from Graham and van der Eb (1973), was used to identify oncogenes in fish tumors. Up to 100 μg of high-molecular-weight fish DNA was co-transfected with the pSV2neo plasmid in the presence of calcium phosphate. Cells were exposed to G418 (geneticin) for 2 weeks and drug-resistant colonies harvested by trypsinization. The cells were pooled and divided among three assays: (1) standard focus assay, (2) nude mouse assay, and (3) colony selection assay.

In the standard focus assay, cells were replated and grown to confluency. Selected foci were picked and expanded, and DNA was isolated for further analysis. Cells from the same pool were injected into athymic mice (Blair et al., 1982). Mice were examined for tumor formation at the site of injection (usually in 6-8 weeks). In the colony selection assay, cells were replated in a defined serum-free medium (QBSF, Quality Biologicals) both in the presence and in the absence of a low amount (0.1%) of fetal calf serum. Transformed cells formed colonies in the absence of serum, usually within 2 weeks.

To confirm that cells picked as foci in the standard focus assay or as colonies in QBSF selection were true transformants, the researchers expanded these cells and grew them in soft agar (McPhearson and Montegnier, 1964). Although NIH 3T3 cells, which normally require a hard surface to attach, are unable to grow in this medium, some transformed cells will grow to form small colonies.

Sequence Analysis

A genomic DNA library was previously prepared in a lambda-based vector (EMBL4) using DNA from NIH 3T3 cells co-transfected with DEN-induced medaka tumor DNA and pSV2neo DNA (Van Beneden et al., 1990). Several positive clones were isolated, and C-7 was selected for further analysis.

C-7 phage DNA was isolated (Sambrook et al., 1989) and subjected to restriction analysis. Restriction enzymes were selected based on their frequency of sites (either low or absent) in the EMBL4 vector and the co-transfectant, pSV2neo DNA. A ±9-kb fragment, which does not

hybridize to either EMBL4 or pSV2neo, was isolated by agarose gel electrophoresis and purified by using glass beads (Gene Clean, Bio 101; Vogelstein and Gillespie, 1979). This fragment was then ligated into the Sac I site of pBluescript KS. The C-7 Sac I 9-kb fragment was digested with the restriction enzymes, Bam HI and Xba I, and the resulting fragments ligated into Bam HI-digested and Xba I-digested pBluescript SK, respectively. Three subclones were isolated for further investigation. Recombinant plasmid DNA was purified by the alkaline-SDS method (Birnboim and Doly, 1979) and partially sequenced using the dideoxy-chain-termination method (Sanger et al., 1977). DNA sequence analysis was performed using both the McVector and the UWGCG sequence analysis programs.

RESULTS

MAMA Studies

Transfection assays were initiated using DNA samples from 14 individual medaka that had been exposed to MAMA and sacrificed 3 months postexposure. Table 1 lists the histopathological findings on the tissues used in this study. During the transfection analysis, the samples were coded to conceal their identity and thus ensure objectivity in the scoring of the results. Data from the primary transfection (TR23) are summarized in Table 2. DNA from three MAMA-exposed individuals induced significant numbers of foci in NIH 3T3 cells in the standard focus assay. These same cells were also positive in the colony selection assay when grown in QBSF medium with the addition of 0.1% supplemented calf serum (Gibco). No growth was observed in QBSF medium alone.

Tumorigenicity testing was initiated by injection of G418-resistant cells into nude mice. These assays are currently in progress. A second primary transfection was initiated using DNA isolated from fish sacrificed at 6 months postexposure. To confirm these results, transformed cells identified in the primary transfection described above are being expanded and will be used for isolation of DNA for a secondary transfection cycle.

DEN Studies

Results of the primary and secondary transfection assays using DNA isolated from DEN-induced tumors have been described in detail (Van Beneden et al., 1990; Van Beneden et al., in press). DNA isolated from a cholangiocarcinoma was highly transforming in all assays. Transformed cells rapidly induced tumors in nude mice. Southern blot analysis of DNA from transformed cells revealed bands that hybridized to a medaka DNA probe. No homology, however, was detected to any of the oncogene sequences used as probes. Secondary and tertiary

transfections were repeated without the addition of the plasmid pSV2neo. Southern blot analysis of DNA digests from these cells revealed the presence of pSV2neo-homologous bands that were present only in DNA from transformed cells. A library was prepared from DNA from a tertiary transfectant and screened with pSV2neo, which appears to be tightly linked to the medaka transforming gene.

Table 1. Identification of Tumors in Livers of MAMA-Exposed Fish Used in Transfection Analysis

Fish	Histopathology
Medaka Controls	
AA-91-351-1-1	ND
AA-91-351-1-6	Hepatocellular vacuolation
AA-91-351-1-11	ND
AA-91-351-1-18	ND
MAMA-Exposed Medaka	
AA-91-351-5-18	Spindle cell proliferation Cyst degeneration Hepatocellular vacuolation, mild One locus of vacuolated hepatocytes
AA-91-351-4-18	hepatocellular vacuolation Moderately severe bile duct Hyperplasia
AA-91-351-5-19	Cholangiocarcinoma
AA-91-351-4-4	Mixed hepatocholangiocarcinoma
AA-91-351-4-17	Cholangiocarcinoma
AA-91-351-4-21	Hepatocellular vacuolation, moderate Bile duct hyperplasia
AA-91-351-5-1	ND

Histopathological findings reported here are part of a draft report prepared by Experimental Pathology Laboratories, Inc. (Herndon, VA). ND indicates that the samples were not examined.

Table 2. Transfection Analysis of Liver DNA from MAMA-Exposed Medaka

DNA Source	Standard Focus Assay (number of foci/μg DNA)	Colony Selection Assay[a] (QBSF + 0.1% serum)
Calf thymus	0	+
AA-91-351-1-1	0	-
AA-91-351-1-6	0.07	-
AA-91-351-5-18	0.14	++
AA-91-351-1-11	0	++
AA-91-351-4-18	0	0
AA-91-351-5-19	26.9	++++
AA-91-351-1-18	0	0
AA-91-351-4-4	2.0	+++
AA-91-351-4-17	0	0
AA-91-351-4-21	0	-
AA-91-351-5-1	0	-

[a]Growth relative to *mos*-transformed NIH 3T3 cells.

Sequence Analysis of a Transforming Gene from a DEN-Induced Tumor

In order to select additional clones, the screening of the genomic library was repeated under low stringency conditions (35% formamide at 37°C) using Pvu II-digested pSV2neo DNA labeled with [^{32}P]dATP and [^{32}P]dCTP by the random primer method (Feinberg and Volgelstein, 1983). Putative positive plaques were isolated from approximately 1 x 10^5 total recombinants. A second round of screening of these recombinants resulted in the selection of 67 recombinants that hybridize to the pSV2neo DNA. When these clones were rescreened under high stringency conditions (50% formamide at 42°C), no positive clones were detected. Examination of the pSV2neo DNA probe indicated possible degradation of the DNA; therefore, reisolation of the pSV2neo DNA from the plasmid is currently in progress.

The clone designated as C-7 that was isolated in a previous screen was examined for the presence of DNA fragments that did not hybridize to either the pSV2neo DNA or to the EMBL4 vector DNA. It was assumed that these fragments would contain a portion of the DNA sequence responsible for the transformation of the NIH 3T3 cells. Restriction digests of C-7 were analyzed by agarose gel electrophoresis (1% agarose in TBE buffer) and transferred to a nitrocellulose filter. The filter was then hybridized sequentially to the ^{32}P-labeled pSV2neo and EMBL4 DNA and exposed to film. An approximately 9-kb Sac I fragment was identified that had no homology

either to lambda arms or to pSV2neo. The C-7 Sac I fragment was subcloned into pBluescript KS and recombinants selected by plating transformed XL1-Blue cells on X-gal/IPTG, LB_{amp} plates.

In addition, three subclones were generated from the Sac I 9-kb fragment by restriction digestion and subcloning into pBluescript SK: a Bam HI fragment, a large Xba I fragment (Xba-7), and a small Xba fragment (Xba-3). The orientation of the 533 nucleotide Xba-3 fragment within the Sac I fragment is unknown. The Xba-7 fragment that was partially sequenced overlaps the 5′ end of the C-7 Sac I 9-kb sequence and is approximately 6.0-6.5 kb. The Bam HI fragment is approximately 4-5 kb with an unknown orientation within the C-7 Sac I 9-kb fragment (Figure 1).

At this time, the correct orientation and reading frame for the putative transforming gene is unknown. All sequence information is discussed relative to the orientation of the sequencing vector, pBluescript KS. Open reading frame (ORF) analysis in the forward direction shows several possible short reading frames. Analysis of the reverse and the reverse complement also indicates small ORFs. However, more information is required before the analysis can be completed. Sequence analysis of restriction sites that may be useful in subcloning is shown in Figure 2. Preliminary analysis revealed no significant homology to sequences deposited in GenBank or EMBL. A highly enriched GT(CA) region in the Xba-3 fragment showed some homology to various genes (e.g., catfish immunoglobulin gene, rat c-*myc* gene, and a rat embryonic skeletal muscle gene). However, no definitive conclusions may be drawn.

DISCUSSION

MAMA is the stable aqueous form of methyazoxymethanol (MAM), the active carcinogenic component of the naturally occurring glucoside carcinogen cycasin. MAMA appears to be metabolically activated in tissues by esterases and NAD-dependent dehydrogenases (Grab et al., 1977). The carcinogenicity of MAMA in higher animals is well documented (Zedeck and Sternberg et al., 1977; Sieber et al., 1980). MAMA has also been reported in previous studies to induce tumors in fish (Aoki and Matsudaira, 1981; Hawkins et al., 1986; Fournie et al., 1987; Van Beneden et al., 1990).

The identification of the transforming gene detected in the MAMA-induced tumors is also still unknown. To confirm that the transformation of NIH 3T3 cells is due to fish sequences, restriction digests of DNA isolated from transfected cells will be analyzed on Southern blots for the presence of fish-specific sequences. Duplicate Southern blots will also be hybridized to radiolabeled probes of known oncogenes in order to identify activated oncogenes. These studies are in progress.

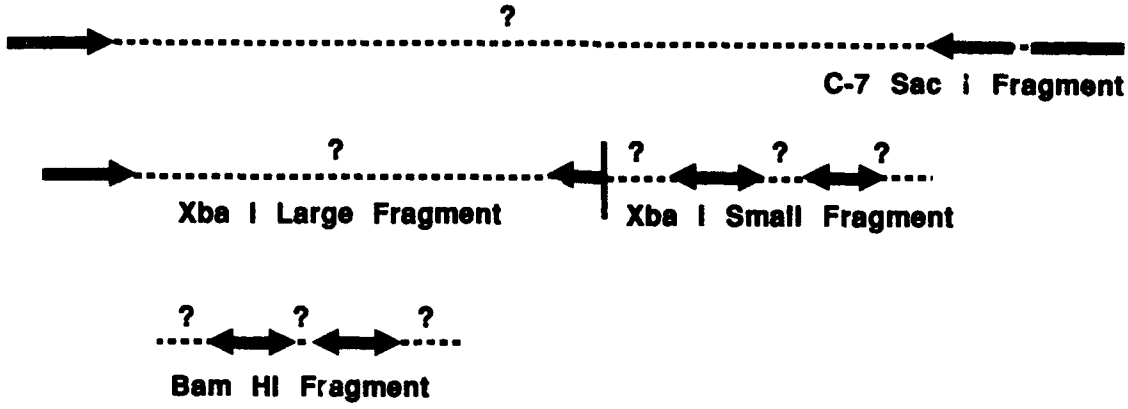

Figure 1. Subclones of transforming DNA. A graphic representation of the subclones generated by restriction enzyme digestion and ligation into pBluescript KS and pBluescript SK. This diagram shows the relative positioning of the Xba I and Bam HI fragments as they occur in the C-7 Sac I 9-kb fragment.

DEN is one of the most potent and extensively studied mammalian carcinogens. Metabolic activation of this compound via α-hydroxylation to the N-nitroso compound results in an electrophilic metabolite that is able to alkylate a variety of sites in DNA. Exposure to DEN has been shown to lead to the development of liver cancers in rats and mice. Lung cancer, however, is the most prevalent lesion seen in hamsters (Williams and Weisburger, 1991). This suggests the involvement of species-specific, and possibly organ-specific, factors. In a recent study by Stowers et al. (1988), DNAs isolated from DEN-induced tumors in $B6C3F_1$ mice and Fischer 344 rats were examined for the presence of activated cellular oncogenes using a transfection technique similar to the one described here. Somewhat unexpectedly, the incidence of activated *ras* oncogenes detected (14/33) in $B6C3F_1$ mouse liver tumors was significantly lower that reported for other chemically induced mouse liver tumors. The authors suggested the probability that multiple pathways exist for the formation of liver tumors in this strain of mouse. Activation of the H-*ras* oncogene may be one event in some but not all of these pathways. In contrast, DNA isolated from only one of the Fischer 344 rats was able to produce foci in NIH 3T3 cells. These results were supported by data from previous studies that reported that *ras* activation was not consistently observed in tumors in Fischer rats induced by a variety of chemicals.

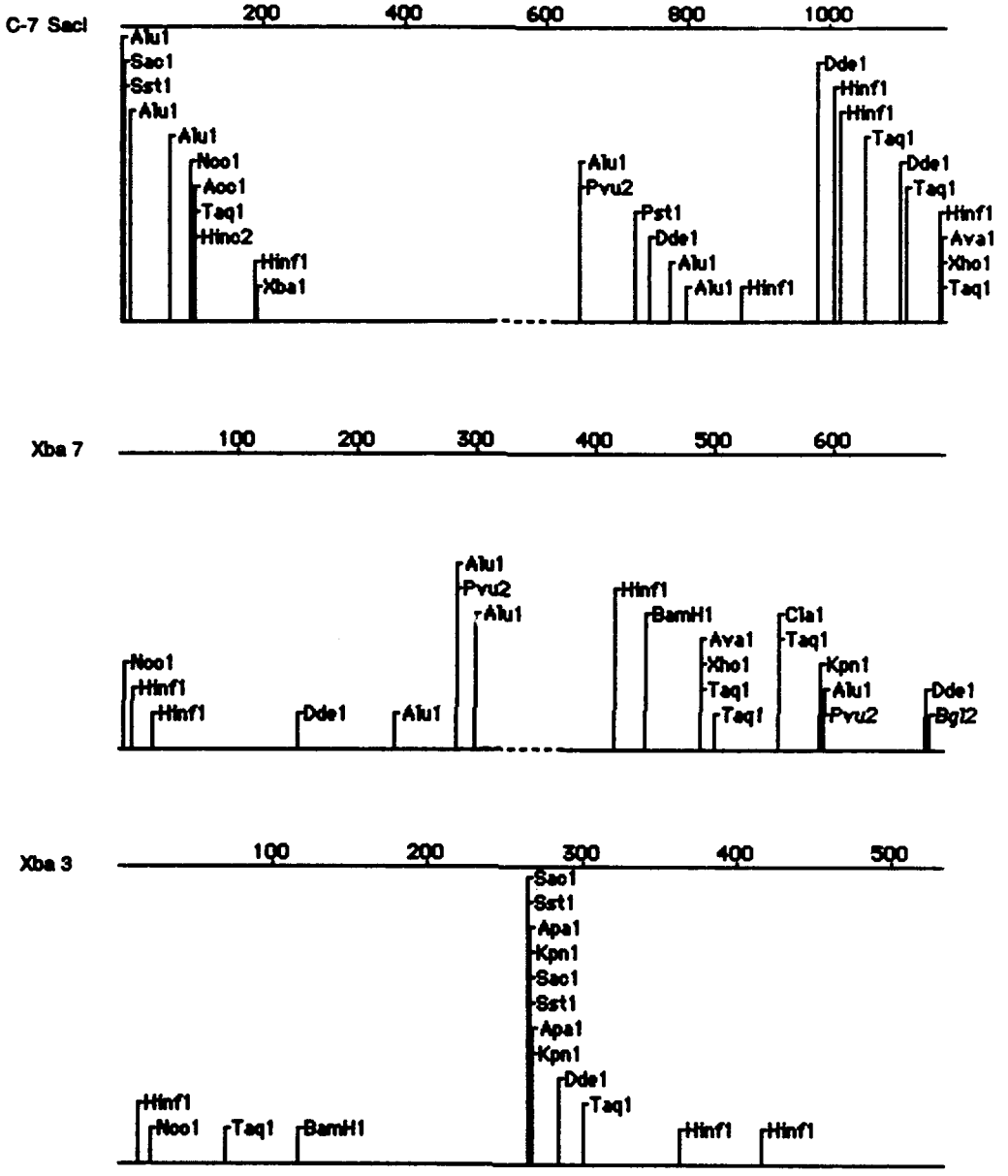

Figure 2. Restriction map of C-7 Sac I 9-kb subclones. This figure shows selected restriction sites present in each of the subclones sequenced thus far. The Bam HI subclone is not included at this time. All sequence analysis was performed using the McVector DNA Analysis Program.

DEN has been used to induce a variety of tumors, also primarily of hepatic origin, in several species of fishes (Park and Kim, 1984; Schultz and Schultz, 1988; Grizzle and Thiyagarajah, 1988; Lee et al., 1989; McCarthy et al., 1991). Activated *ras* oncogenes have been detected by transfection analysis of DNA from several fish tumors (Wirgin et al., 1989; McMahon et al., 1990; Chang et al., 1991). No other studies of molecular analysis of DEN-induced tumors in fish have been reported. The gene detected in the DEN-induced cholangiocarcinoma does not appear to be homologous by Southern blot analysis to any of the known oncogenes that were used as probes. Sequence data to date support this conclusion. This strongly suggests that it may be a novel oncogene. This supports the conclusions of Stowers et al. (1988) of the existence of multiple pathways that do not involve the activation of *ras* genes.

Results of the transfection analysis of tumor DNA from both MAMA and DEN-exposed fish indicate that, like mammals, fish tumors have activated transforming genes that are able to transform NIH 3T3 mouse fibroblasts *in vitro*.

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DNA BIOMARKERS AS EARLY INDICATORS OF GENETIC INJURY AND CANCER RISK

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ABSTRACT

Four modifications of the nucleotide base structure of DNA have been shown to be associated with cancer formation: 8-hydroxyguanine (8-OH-Gua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G), 8-hydroxyadenine (8-OH-Ade) and 4,6-diamino-5-formamidopyrimidine (Fapy-A). These modifications result from the attack of the highly reactive hydroxyl radical on the DNA base structure. The base modifications have a high potential for serving as sensitive biomarkers for the early detection of genotoxic changes in animals and humans exposed to environmental chemicals, as well as for the prediction of cancer risk.

INTRODUCTION

Formation of the Hydroxyl Radical

Oxygen radicals (e.g., the hydroxyl radical [·OH]), formed in the body metabolically (Slater, 1978; Ames 1983; Troll and Wiesner, 1985), produce oxidative injury to somatic cells (Slater, 1978; Ames, 1983; Troll and Wiesner, 1985; Adelman et al., 1988; Schneider et al., 1988; Blount et al., 1989; Klausner et al., 1989; Lee et al., 1989) that is reflected in changes in the base structure of DNA. The biological significance of these changes has been eloquently stated in a recent publication of the National Academy of Sciences: DNA base modifications, if unrepaired, will result in mutations that "will usually be passed on to all descendants of the mutated cell, and even if the mistake is confined to a single DNA base, massive adverse consequences may result if important genetic information has been altered in a way that affects its function" (Ahmed, 1991). Thus, if the DNA base damage can be defined, quantified, and linked to cellular change, it has great potential for the early assessment of genotoxic injury and cancer risk.

Several ·OH-induced DNA base lesions have been identified, mostly in *in vitro* systems. These include thymine glycol, 5,6-dihydroxythymine (Aruoma et al., 1989) and 8-hydroxydeoxyguanosine (8-OH-dG) (Dizdaroglu and Bergtold, 1986; Kasai et al., 1987; Kuchino et al., 1987). Moreover, a significant advance in understanding the association between

·OH-modified base structures and cancer was obtained when it was found that 8-OH-dG was misread in a DNA synthesis system with *Escherichia coli* (Kuchino et al., 1987). In fact, the presence of the 8-OH-dG in the DNA was considered "an important cause of mutation and carcinogenesis" (Kuchino et al., 1987). Thus, a firm basis exists (Adelman et al., 1988; Schneider et al., 1988; Blount et al., 1989; Klausner et al., 1989; Lee et al., 1989) for implicating ·OH-induced DNA injury in carcinogenesis.

The reduction of molecular oxygen in all aerobic eukaryotic cells results in intermediates that are highly toxic. These include the superoxide ion (O_2^-), H_2O_2, and ·OH (Imlay et al., 1988). Although O_2^- and H_2O_2 individually may not be particularly damaging, their combined action leads to reactive and damaging ·OH:

$$O_2^- + H_2O_2 \text{ ------> } \cdot OH + OH^- + O_2$$

The above reaction can be relatively slow; however, when catalyzed by metal ions (e.g., Fe[II]), it is accelerated and becomes relevant in the initiation of biological damage (Imlay et al., 1988). Specifically, the conversion of H_2O_2 to ·OH is accelerated through the iron Fe(II)-catalyzed Fenton reaction:

$$Fe(II) - EDTA + H_2O_2 \text{ ------> } Fe(III) - OH^- + \cdot OH$$

The proliferation of ·OH may then result in an attack on most molecules in living systems with deleterious consequences (Ames, 1983; Troll and Wiesner, 1985; Kuchino et al., 1987; Imlay et al., 1988). A major defense against ·OH-induced damage is provided by enzymes that catalytically scavenge the intermediates of oxygen metabolism. For example, O_2^- is eliminated by superoxide dismutase (SOD), and this oxygen species and H_2O_2 are eliminated by catalase and glutathione peroxidase (Schneider et al., 1988). The H_2O_2 can result from redox cycling of endogenous chemicals (e.g., estrogen) via the cytochrome P-450 system, as well via xenobiotics (e.g., polychlorinated biphenyls [PCBs]) to which an organism is exposed (Guengerich, 1991).

MATERIALS AND METHODS

Analysis of Nucleotide Bases

Trimethylsilyl (TMS) derivatives of the nucleotide bases in tissue DNA were analyzed by gas chromatography-mass spectrometry with selected ion monitoring (GC-MS/SIM), essentially as previously described (Malins et al., 1990; Malins and Haimanot, 1990). Briefly, the nucleotide

bases were allowed to react with acetonitrile-bis(trimethylsilyl)trifluoracetamide (BSTFA) (2:1 v/v) for 45 minutes at 80°C. Quantification of DNA base derivatives was undertaken on the basis of the mass-to-charge ratio (m/z): 354, 352, 442, and 440 for Fapy-A, 8-OH-Ade, Fapy-G, and 8-OH-Gua, respectively. Analyses were performed with a 5890A gas chromatograph equipped with an autosampler interfaced to a Hewlett Packard mass-selective detector model 5970B. A fused silica capillary column coated with 5% phenylmethylsilicone gum phase (15 m; 0.2 mm i.d., and 0.3-μm film thickness) was used for the separation of the DNA base derivatives. The column temperature was maintained at 120°C for 1.5 minutes, increased to 176°C at 3°/min, and then to 250°C at 6°/min. The injection port and ion source were kept at 250°C throughout the analysis. Helium was the carrier gas, and mass spectra were obtained with 70 eV ionizing energy. The GC-MS/SIM profiles of the TMS nucleotide bases are given in Fig. 1.

The Fapy-A and 8-bromoadenine were purchased from Sigma Chemical Company, and 8-hydroxyguanine was obtained from the Chemical Dynamics Corporation. The 8-OH-Ade and the Fapy-G were synthesized in our laboratories from 8-bromoadenine and 4,5,6-triaminopyrimidine sulfate, respectively, and purified by recrystallization (Cavalieri and Bendich, 1950).

CONCLUSIONS

Studies in Fish

English Sole

Despite persuasive evidence implicating the ·OH in DNA damage, such damage had not been found in living systems (e.g., tissue) until recently when GC-MS/SIM was applied for the first time to the problem (Malins et al., 1990; Malins and Haimanot, 1990; Malins and Haimanot, 1991*a*). This work, sponsored by the U.S. Army Medical Research and Development Command and conducted in our laboratories, initially revealed high concentrations of the DNA base lesion Fapy-G in liver tumors of English sole environmentally exposed to carcinogens (Malins et al., 1990). No evidence was found under the microscope for this DNA lesion in the normal tissues surrounding the tumors. Subsequent studies revealed three other DNA base lesions in liver tumors of environmentally exposed fish from a highly contaminated site and the normal tissues (as seen under the microscope) of minimally exposed (compared to nonexposed) fish from a population with a small incidence of liver cancer. The DNA lesions were the purine derivatives 8-OH-Gua, Fapy-G, 8-OH-Ade, and Fapy-A (Fig. 1).

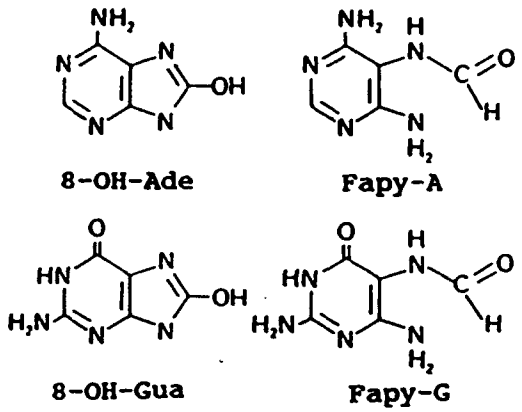

Figure 1. Modifications arising from the attack of the ·OH on C-8 of the purine ring of adenine and guanine.

The DNA lesion concentrations in the tumor tissue were substantially higher than those of the exposed fish with livers that appeared to be normal when viewed through a microscope, and the latter lesion concentrations were statistically higher than those of normal control fish from essentially uncontaminated areas (Fig. 2). Thus, there was a clear indication for the first time that the DNA lesions could occur in microscopically normal tissue of exposed fish and thus may be intimately involved in tumor formation. The findings also indicated that the DNA lesions may serve as sensitive biomarkers for the early prediction of cancer risk (Malins and Haimanot, 1991*a*).

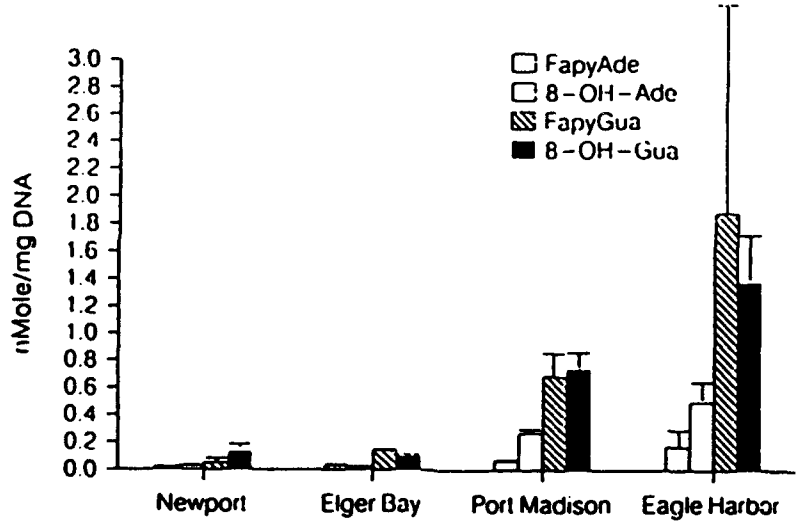

Figure 2. DNA lesions in normal livers of English sole from reference areas (Newport and Elger Bay) and the chemically contaminated areas of Port Madison (normal tissue as seen under the microscope) and Eagle Harbor (hepatic tumor tissue) (Malins and Haimanot, 1991*a*).

Medaka

A *preliminary* experiment was conducted at the U.S. Army Biomedical Research and Development Laboratory (BRDL) with Medaka exposed to trichloroethelene (TCE), diethylnitrosomine (DEN), and groundwater. Analyses of DNA base modifications in the liver revealed that the base concentrations were often higher after exposure to groundwater, DEN, and TCE, (Table 1). Thus, the DNA bases are potentially useful biomarkers for chemically induced genotoxic changes in medaka, although further experiments are required to firmly establish a cause-and-effect relationship between exposure and increases in the DNA base concentrations.

Table 1. Modified Base Products Formed in the Liver DNA of Medaka Exposed to TCE, DEN, and Groundwater

Sample No.		Concentrations (nmol/mg DNA)		
		8-OH-Ade	Fapy-G	8-OH-Gua
1A	Control	0.037	ND	0.097
2A	Control + 10 mg DEN/liter	0.083	ND	0.173
3A	Control	0.053	0.197	0.330
4A	Control + 10 mg DEN/liter	0.057	0.220	0.177
5A	50% Groundwater	0.053	0.357	0.337
6A	50% Groundwater + 10 mg DEN/liter	ND	0.467	0.197
7A	10% Groundwater	0.200	0.553	0.280
8A	10% Groundwater + 10 mg DEN/liter	ND	ND	0.350
9A	100% Groundwater	0.263	0.490	1.053
10A	100% Groundwater + 10 mg DEN/liter	0.037	0.240	0.087
11A	5 ppm TCE	0.210	0.547	0.257
12A	5 ppm TCE + 10 mg DEN/liter	0.337	0.517	0.263

ND, Not detected.

Studies of Breast Cancer

The encouraging results with the fish studies led to the interesting question of whether similar results would be obtained with human tissues. In this regard, female breast carcinomas of the invasive ductal type, microscopically "normal" tissue from the cancerous breast, normal blood lymphocytes, and a normal DNA control (calf thymus DNA) were analyzed for the same DNA lesions found in the fish. The findings were most encouraging, indicating that the breast carcinomas contained high concentrations of Fapy-G, 8-OH-Gua, and 8-OH-Ade, whereas the adjacent normal tissue concentrations were not statistically different from those of the control (calf thymus DNA) (Fig. 3).

The normal tissue did not show any evidence for either preneoplastic or neoplastic lesions when examined by microscope. Overall, it was concluded that the DNA base modifications "likely play a major role in initiation and probably contribute to the further transformation of neoplastic cells in cancer of the female breast" (Malins and Haimanot, 1991*b*). In this respect, given that cancer cells generally have a constitutive propensity to generate their own H_2O_2 (Szatrowski and Nathan, 1991), it seemed likely that the breast cancer cells also generated the ·OH that progressively damaged the DNA base structure. Thus, it is likely that a mechanism is provided to

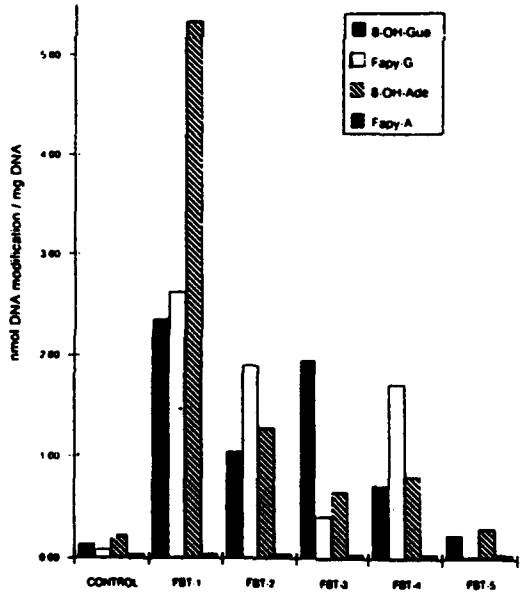

Figure 3. DNA base modifications (nanomoles per milligram of DNA) in five female breast tumors (FBT) of the invasive ductal carcinoma type are compared to those of a (calf thymus) control. There were significant differences among the concentrations of 8-OH-Gua ($p \leq 0.01$), Fapy-G ($p \leq 0.02$), and 8-OH-Ade ($P \leq 0.05$) with respect to the carcinoma tissue and the control (five replicate analyses) (Malins and Haimanot, 1991b).

generate heterogenicity in tumor cells resulting in pleomorphism and the acquisition of metastatic potential. In this regard, we have found elevated concentrations of the base lesions in the *microscopically normal* epithelium from the cancerous colon (Fig.4).

In addition, it is likely that the observed oxidative damage to the DNA bases leads to mutational events by induced mispairing of the modified bases during DNA replication. One likely consequence is mutations in the tumor suppressor p53 gene product that inactivate its tumor suppressor function. Studies of p53 in lung cancer, for example, have indicated a high mutational frequency (Miller et al., 1992). Investigations of the direct effect of oxidative damage have indicated that 8-OH-Gua leads to G-->T transversions with a mutational frequency of 0.7% (Cheng et al., 1992). These properties of Fapy-G or 8-OH-Ade, for example, are presently unknown. Given the apparent importance of the base modifications in carcinogenesis in the breast and other tissues, analyses of modified base replication, coupled with the mutational status (or incidence of modified bases) present in p53 is of prime significance and a high priority for future research.

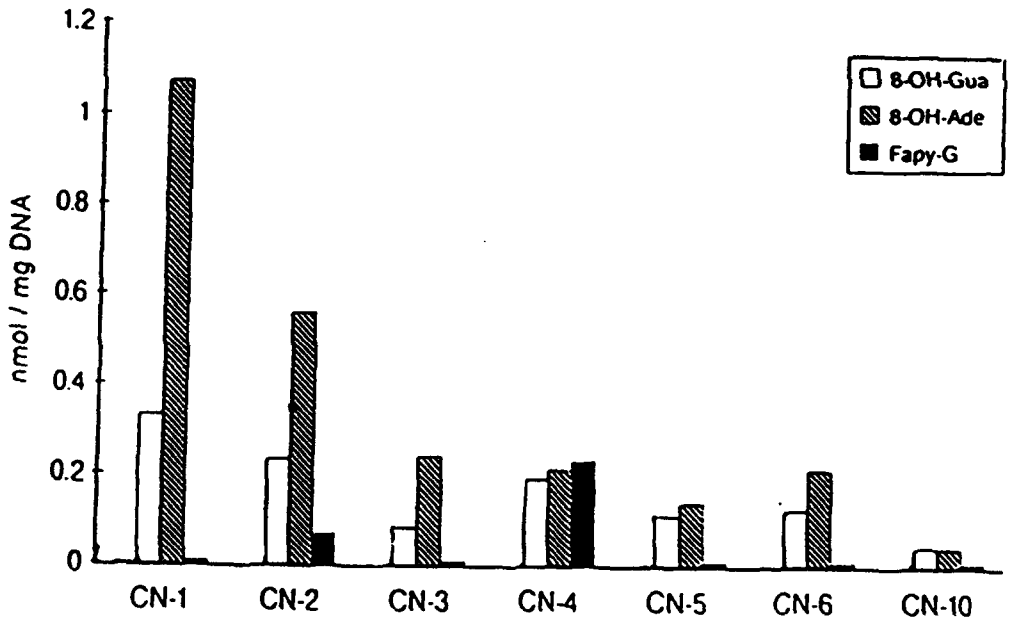

Figure 4. DNA base modifications in microscopically normal colon epithelium in human cancerous colons.

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ANALYSIS OF CHROMATIN STRUCTURE IN BLOOD CELL NUCLEI OF MEDAKA, BLUEGILL SUNFISH, AND MOSQUITOFISH

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ABSTRACT

The Japanese medaka (*Oryzias latipes*) has been found to be resistant to alkaline denaturation of DNA. However, single-strand breaks are induced to a greater degree following exposure to genotoxic compounds. Protein, tightly complexed with extracted DNA, is associated with this resistance to denaturation, which is overcome by proteinase K treatment. Japanese medaka were exposed to benzo[*a*]pyrene (BaP, 4 μg/liter) for 52 days. Dissociation of proteins from blood cell chromatin by full-strength buffer showed that medaka not exposed to BaP have a greater variety of nonhistone proteins than bluegill sunfish. These proteins were depleted after medaka were exposed to BaP for 52 days. Exposed medaka also have a higher histone 5/histone 1 ratio, indicating a possible shift in red cell maturational state. Dissociation of chromatin proteins by half-strength buffer showed that bluegill core histones were more easily extracted than those of medaka, which may be indicative of a stronger nucleosomal-DNA association. Treatment of fish red cell nuclei with micrococcal nuclease showed that medaka chromatin is more resistant to endonuclease digestion than chromatin from bluegill sunfish or mosquitofish. This observation supports a proposed model in which DNA and protein in the chromatin of blood cells from Japanese medaka are packaged in a nuclease-resistant structure.

INTRODUCTION

To understand the effects of genotoxic compounds in eukaryotic organisms, damage to and modifications of target cell DNA must be detected and quantified. Single-strand breaks in cellular DNA that are induced by exposure to genotoxic agents have been quantified by a number of techniques such as alkaline elution (Kohn *et al.*, 1976), DNA precipitation (Olive, 1988), alkaline unwinding (Kanter and Schwartz, 1979; Shugart, 1988), and gel electrophoresis (Singh *et al.*, 1988; Olive *et al.*, 1992; Theodorakis *et al.*, 1992). The alkaline unwinding assay (AUA), a relative measure of DNA single-strand breakage, has been used successfully to measure DNA damage in response to genotoxic agents for a number of species such as fish (Shugart, 1988), rodents (Morris and Shertzer, 1985), turtles (Meyers-Schone *et al.*, 1992), and marine mussels

(Nacci and Jackim, 1989). The Japanese medaka (*Oryzias latipes*), a small killifish species endogenous to Southeast Asia, is currently being studied as one of several alternate animal model systems for toxicological and carcinogenesis research. Of particular interest, in studies using medaka, is the measurement of DNA damage induced by genotoxic agents such as benzo[*a*]pyrene (BaP) and diethylnitrosamine.

Attempts to quantitate relative DNA strand breaks in medaka, by means of AUA, have proven difficult using the procedure of Shugart (1988). Medaka DNA shows resistance to heat and alkali denaturation during the standard AUA. Although bluegill sunfish and mosquitofish DNA was significantly denatured at 37°C, medaka DNA remains in the duplex configuration (Shugart *et al.*, 1991). It may be inferred from this observation in the medaka that an usual molecular biological phenomenon underlies resistance to denaturation in this species. Preliminary observations indicated that the medaka whole-body, liver, and blood cell DNA is resistant to denaturation.

The studies reported here tested the hypothesis that the unwinding inhibitor is proteinaceous and is a component of medaka cell nuclei. The experimental approach focused on (1) removing the agent(s) responsible for inhibiting alkaline unwinding so as to permit measurement of DNA strand breaks and (2) beginning an investigation to identify the biochemical/structural basis of resistance to denaturation in medaka. The second approach concentrated on a comparative study of blood cell chromatin structure and protein characteristics in medaka, bluegill sunfish, and mosquitofish. The effects of chronic exposure to BaP on medaka alkaline unwinding and chromosomal proteins was also examined.

MATERIALS AND METHODS

Animals

Japanese medaka (*Oryzias latipes*) and bluegill sunfish (*Lepomis macrochirus*) were obtained from laboratory stocks maintained at the Environmental Sciences Division (ESD) aquarium facilities. Mosquitofish (*Gambusia affinis*) were captured in a dipnet from a pond located near the ESD research facilities. Control, chemically exposed, and nonexperimental fish were sacrificed by cervical scission prior to cellular and biochemical analysis.

Exposure Conditions

Adult Japanese medaka were exposed to BaP dissolved in water for 7, 10, and 52 days in three separate experiments. In the first exposure experiment, medaka were divided into four groups with six fish per tank and two replicates in each group (three males and three females).

The four treatment groups were as follows. Group A was untreated controls; group B was exposed to BaP. BaP was added to 3.5 liters of water in 4-liter aquaria at a concentration of 4 μg/liter by passage through a column of 1-mm diameter glass beads coated with BaP on the first day. The BaP was not renewed; this treatment was the same for all groups exposed to BaP. Group C was exposed to verapamil (1 μg/liter) added daily, plus BaP (4 μg/liter). Verapamil is a beta-blocking heart drug, reported to block the vertebrate cellular xenobiotic pump (Kurelec and Pivcevic, 1989). Group D was exposed to verapamil (1 g/liter), added daily. The second exposure experiment was the same as the first but lasted for 10 days, and a constant BaP concentration of 4 μg/liter was maintained in the water. In a third exposure experiment, lasting 52 days, the procedure was similar to the first, but lacking the BaP + verapamil and verapamil exposure groups. The fish were sacrificed by cervical scission at the end of each exposure experiment.

DNA Purification and Alkaline Unwinding Analysis

DNA was extracted from medaka carcass and blood, mosquitofish carcass and blood, and bluegill liver and blood tissues for AUA. The intact tissue was homogenized at 40°C in 1 N NH_4OH and 0.2% Triton X-100, followed by phenol/chloroform/isoamyl alcohol extraction. Further purification prior to the AUA was accomplished by passing the DNA extract through a Sephadex G-50 column.

To obtain protein-free DNA, a procedure modified from Maniatis *et al.* (1982) was used. About 300 mg of intact tissue was homogenized (blood cells were washed twice in homogenization buffer) with a Teflon pestle in 50 mM Tris-HCl, pH 7.4, 100 mM EDTA, and 250 mM NaCl. To the homogenate was added 250 μl of 10% Sarkosyl and 50 μl of RNase (2 mg/ml stock) followed by incubation at 50°C for 30 minutes. Proteinase K was added to a concentration of 50 μg/ml, and the incubation was continued for another 60 minutes. The cellular lysates were then extracted with an equal volume of phenol/chloroform/isoamyl alcohol, 25:24:1, followed by extraction with an equal volume of chloroform. DNA in the aqueous supernatant was precipitated by addition of two volumes of cold 100% ethanol. The DNA was pelleted by centrifugation, and the pellets were resuspended in alkaline unwinding buffer. Alkaline unwinding was performed according to the method of Shugart (1988).

Isolation of Blood Cell Nuclei

Bluegill sunfish were bled into heparinized Vacutainer tubes that were kept on ice; medaka and mosquitofish were sacrificed by cervical scission. Individual fish or pooled fish (15 medaka and 30 mosquitofish) were cleanly cut into three pieces with a new razor blade. Individual fish

were sectioned in a petri dish containing 3 ml of physiological saline; pooled sectioned fish were placed in a beaker with 12 ml of saline. Blood was allowed to perfuse from the tissues for 5 minutes and collected into a Pasteur pipette through a spherical glass wool filter. Fifty microliters of fresh bluegill blood was diluted in 3 ml of fish physiological saline. The collected cells were almost exclusively nucleated erythrocytes. The blood cells were pelleted by centrifugation (700 rpm, Dynac table-top centrifuge). The pelleted blood cells occupied a volume of 20-50 μl.

Nuclei were isolated by a modification of the procedure of Hewish and Burgoyne (1973). The pelleted blood cells were washed in 3 ml of buffer 1 (15 mM Tris-HCl, 15 mM NaCl, 15 mM β-mercaptoethanol, 60 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 0.34 M sucrose, 2 mM EDTA, and 0.5 mM EGTA, pH 7.4). The cells were then pelleted by centrifugation at 500 × g for 10 minutes (Dynac table-top centrifuge) and resuspended and twice washed in 3 ml of buffer 2 (same as buffer 1 plus 0.5% NP-40). Nuclei were pelleted each time by centrifugation at 1,000 rpm in a tabletop centrifuge. The nuclei were then twice washed in buffer 3 (buffer 1 minus EDTA and EGTA) and collected by centrifugation at 1,000 rpm. All three buffers also contained 1 mM phenylmethylsulfonyl fluoride (PMSF), added just before use. Nuclei isolated for protein analysis were either processed fresh or frozen at -20°C.

Chromatin Protein Analysis

Chromatin proteins were extracted from blood cell nuclei by means of a procedure adapted from Miller (1988). Nuclei were suspended in 500 μl ddH_2O with 20 μl 2.0% sodium dodecyl sulfate (SDS), to facilitate lysis. Lysates were cleared by centrifugation in a microcentrifuge at 6,500 rpm for 1 minute. Two volumes of 100% ethanol was added to the supernatant to precipitate chromatin, which was then pelleted by centrifugation in a microcentrifuge. The precipitates were dissolved for 2 hours in 50 μl of full strength chromatin dissociation buffer modified from Laemmli (1970) [10 mM Tris-HCl, pH 7.4, 20% glycerol, 2.0% SDS, 2.0% β-mercaptoethanol, bromophenol blue (0.01 mg/ml)] or partial dissociation conditions (precipitated chromatin was suspended in 50 μl of half-strength chromatin dissociation buffer (10mM Tris-HCl, pH 7.4, 1 mM EDTA, 20% glycerol, 1% SDS, and 1% β-mercaptoethanol). Samples were boiled for 5 minutes in a water bath, and the proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Gels were stained with Coomassie brilliant blue and scanned with a laser densitometer.

Chromosomal Structural Analysis

Freshly isolated nuclei from unexposed medaka, bluegill sunfish, and mosquitofish were suspended in 0.5 ml of buffer 3. Nuclei were counted in a hemocytometer and the concentrations per milliliter were adjusted by addition of more buffer 3. SDS was added to aliquots (50 μl of nuclei suspension) containing an equal number of nuclei until 0.5% SDS was reached in order to lyse nuclear membranes. Total DNA content was determined by fluorimetry using Hoechst dye 22358. Because of different genomic DNA content in the three fish species studied, nuclei preparations were diluted with buffer 3 to equalize the DNA concentrations for each reaction. To 0.5 ml of nuclei preparation (kept on ice) was added 55 μl of 10× reaction buffer (100 mM Tris-HCl and 10 mM $CaCl_2$, pH 7.4), and 10 μl of micrococcal nuclease diluted 1/5 in 1× reaction buffer (30,000 U/ml stock). The reaction mixtures were incubated at 37°C in a water bath. Sixty-microliter aliquots were removed at 0, 30 sec, and minutes 1, 2, 4, 8, 16, and 32 (Lewin, 1980), and a sample from the reaction mix prior to micrococcal nuclease addition was set aside. The aliquots were placed into 1.5-ml microcentrifuge tubes containing 10 μl of 2% (w/v) SDS and 2% (v/v) β-mercaptoethanol solution and were placed on ice. The samples were then extracted with an equal volume of chloroform/isoamyl alcohol/phenol (CIP) and centrifuged for 2 minutes at 6,500 rpm in a microcentrifuge, and the aqueous top layer was transferred to another 1.5-ml microfuge tube. Ten microliters of extract was analyzed on a 1% agarose gel, stained with ethidium bromide, and photographed under ultraviolet transillumination.

RESULTS

DNA extracted in 1 N NH_4OH/0.2% Triton X-100, according to the method of Shugart (1988), from unexposed whole medaka, resisted denaturation until AUA incubation temperatures exceeded 55°C (Figure 1). The percentage of DNA molecules remaining in the double-stranded form remained high compared to DNA extracted by the same procedure from bluegill sunfish liver and whole mosquitofish. Similar results were observed in DNA extracted from blood of these three species (data not shown). The AUA has been routinely performed at 37°C for most species that have been previously studied. The resistance of medaka DNA to alkaline denaturation at 60°C increased when fish were exposed for 7 days to BaP, verapamil, and BaP + verapamil (Figure 2). This showed that the phenomenon of resistance to alkaline unwinding may be induced by xenobiotic exposure. Extraction of medaka DNA under conditions which extensively deproteinate blood cell chromatin, using proteinase K, showed that the resistance to alkaline unwinding had been overcome (Figure 3).

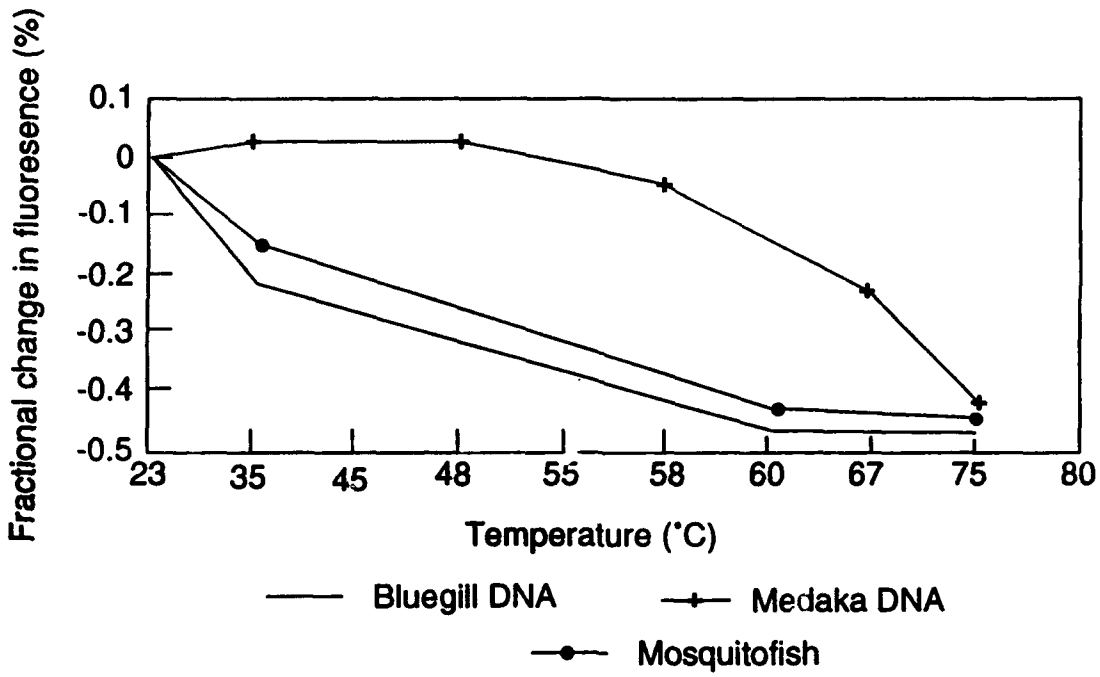

Figure 1. Thermal denaturation of medaka, bluegill, and mosquitofish DNA under alkaline conditions. The fractional change in fluorescence was determined by binding of Hoechst dye 33258 to DNA with increasing temperature.

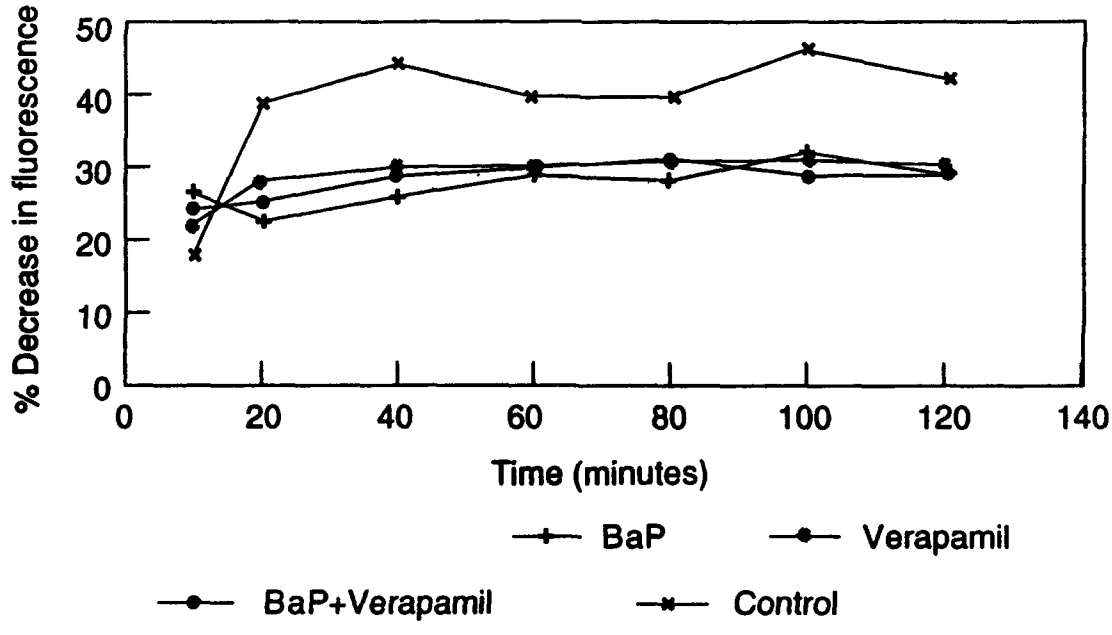

Figure 2. Comparison of alkaline unwinding of medaka DNA from control (untreated), BaP (4 μg/L) and verapamil (1 μg/L) exposed fish. Fish were exposed for 7 days. The degree of alkaline unwinding was determined as the percent decrease in fluorescence (DNA + Hoechst dye 33258) over time.

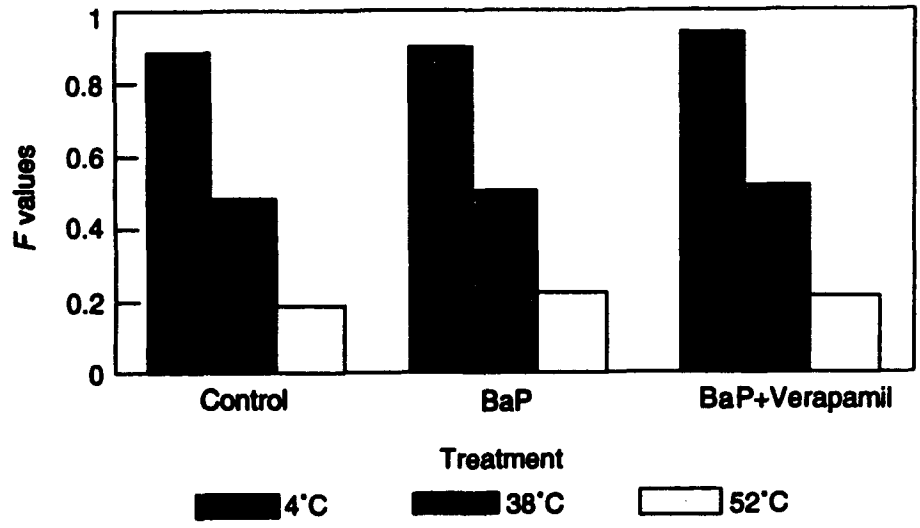

Figure 3. Comparison of alkaline unwinding *F* values from medaka exposed to a constant BaP concentration of 4 μg/L for 42 hours. DNA was purified by a modified procedure which extensively deproteinated the DNA during purification.

A comparison of chromosomal proteins extracted from precipitated chromatin from erythrocyte nuclei, according to a procedure adapted from Miller (1988), for bluegill, medaka, and medaka exposed to BaP for 52 days is shown in Figure 4. Both the core and H1-H5 histone profiles are similar for control bluegill (Figure 4A), and medaka (Figure 4B), with medaka H1-H5 histones being more negatively charged than bluegill H1-H5 proteins. The H5 histones are present in higher concentrations relative to H1 histone in mature erythrocytes of fish and birds (Van Holde, 1989). There is a greater variety of nonhistone proteins in medaka chromatin relative to bluegill. In medaka exposed to BaP for 52 days (Figure 4C), most nonhistone proteins are depleted, most H5 histone is decreased and H1 histone is increased, and most positively charged core histone protein(s) are decreased. A small 14 kD protein(s) is seen migrating negative to the core histone group.

A comparison of nuclear chromosomal proteins extracted from bluegill, medaka, and BaP-exposed medaka blood cells under partial dissociation conditions is shown in Figure 5. The H1-H5 histone group does not dissociate as readily from the DNA as the core histones. Bluegill core histones (Figure 5A) are extracted from DNA more readily than from both control and BaP-exposed medaka DNA (Figure 5B). The protein or proteins with an apparent mass of 14 kD are detected in greater amounts in BaP-exposed medaka red cell nuclei.

When isolated blood cell nuclei from medaka, bluegill, and mosquitofish are subjected to digestion with micrococcal nuclease (Figure 6), bluegill and mosquitofish chromatin are digested

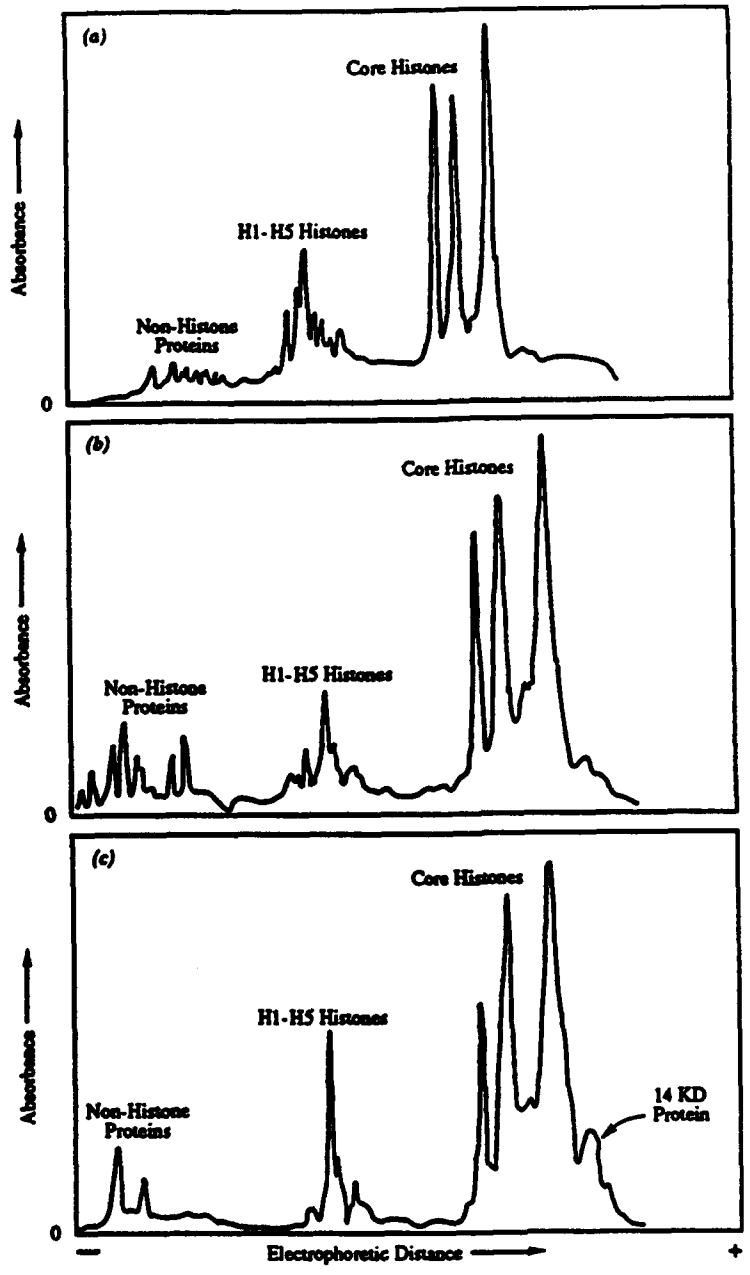

Figure 4. Scanning densitometric profiles of completely dissociated bluegill and medaka chromosomal proteins. Blood cell nuclei were purified and lysed, and the chromatin was precipitated in ethanol. Protein was dissociated from DNA by boiling in full-strength SDS-ß-mercaptoethanol sample solution. Proteins were analyzed on 13% SDS-PAGE gels and stained with Coomassie blue. (*a*) Bluegill control. (*b*) Medaka control. (*c*) Medaka-exposed to BaP for 51 days.

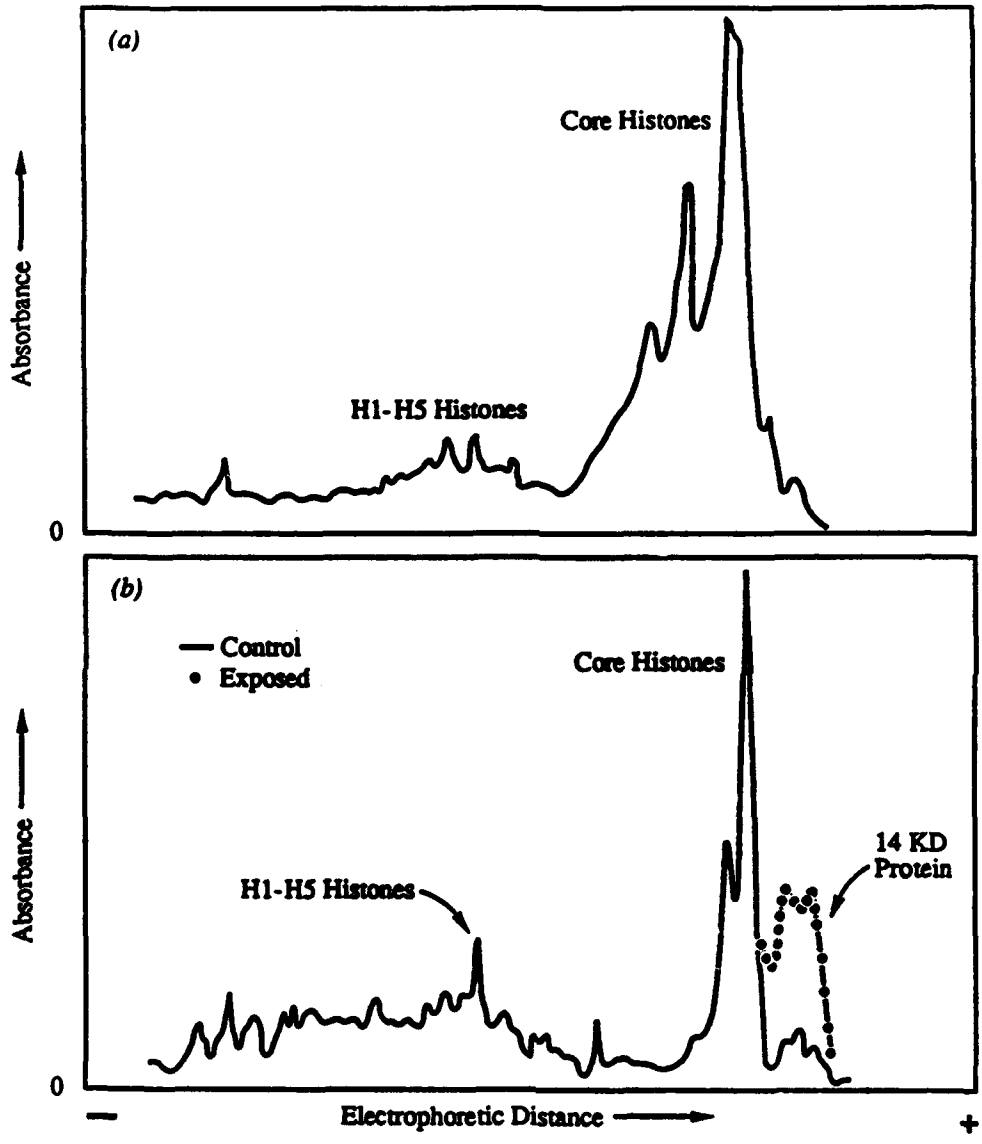

Figure 5. Scanning densitometric profiles of partially dissociated bluegill and medaka nuclear proteins. Blood cell nuclei were purified and lysed. The lysate was 10 mM Tris buffer, pH 7.4, plus an equal amount of SDS-ß-mercaptoethanol dissociation solution, and boiled. Proteins were analyzed on 13% SDS-PAGE gels and silver stained. (*a*) Bluegill. (*b*) Medaka.

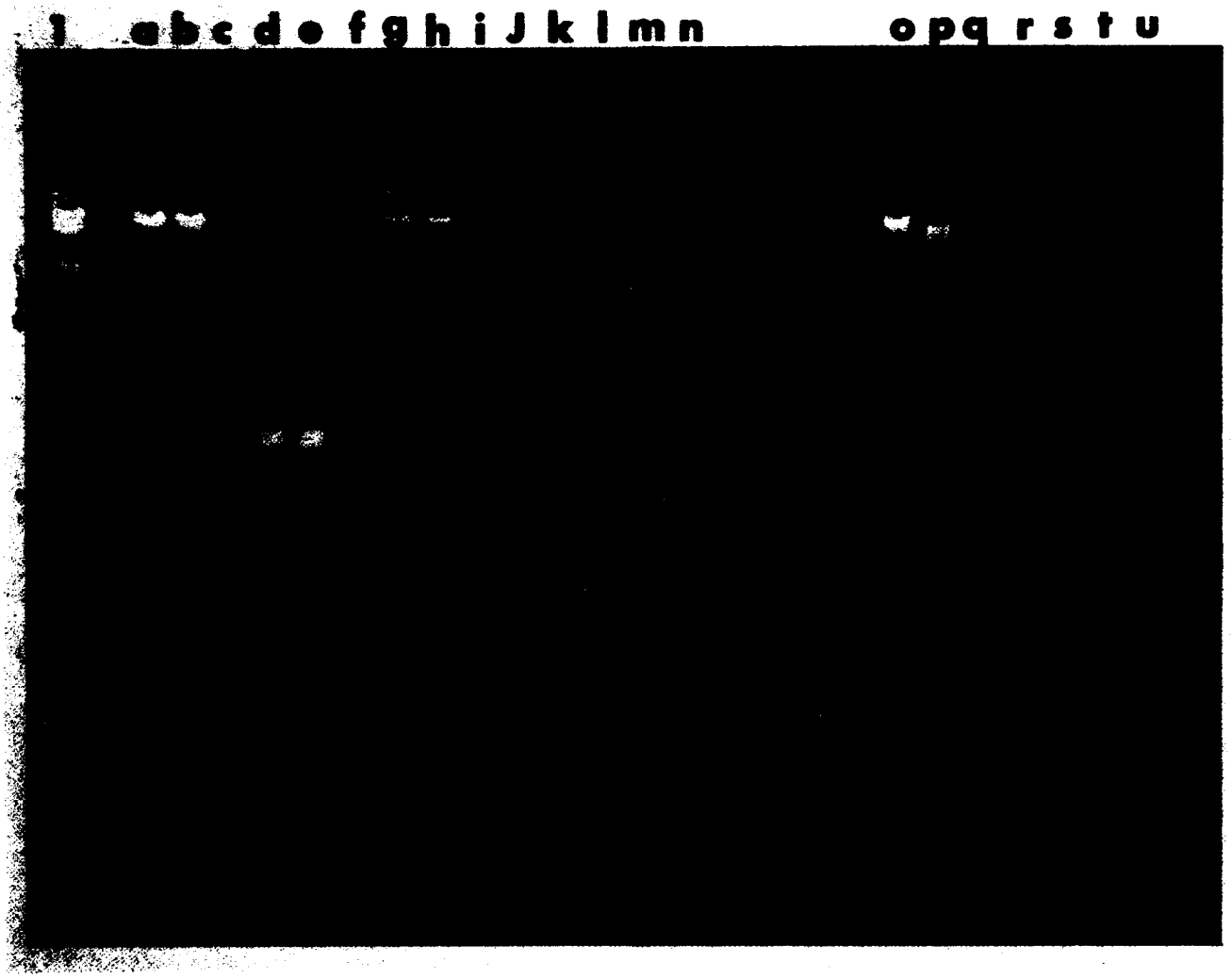

Figure 6. Agarose gel electrophoresis of DNA molecular length fragments of bluegill, medaka, and mosquitofish blood cell nuclei treated with micrococcal nuclease. Nuclei were treated with enzyme, and aliquots were removed at specified time intervals. The reactions were terminated, and DNA was extracted from protein and analyzed on 1% agarose gels. Lane 1, DNA HinD III molecular weight markers. Lanes a-f, bluegill DNA treated with enzyme for (a) no enzyme, (b-f) 0, 0.5, 1, 2, and 4 minutes, respectively. Lanes g-n, medaka DNA treated with enzyme for g) no enzyme, (h-n) 0, 0.5, 1, 2, 4, 8, and 16 minutes, respectively. Lanes o-u, mosquitofish DNA treated with enzyme (o-u) 0, 0.5, 1, 2, 4, 8, and 16 minutes, respectively.

to nucleosomal multimers quite readily (lanes c and p, respectively). The presence of DNA monomers is easily seen 30 seconds into micrococcal nuclease digestion. However, digestion of medaka chromatin is kinetically slower: DNA monomers are not seen till after 1 minute of digestion (lane j). Clearly, medaka chromatin is resistant to micrococcal nuclease digestion.

DISCUSSION

Medaka DNA extracted using ammonium hydroxide and Triton X-100 is resistant to alkaline unwinding. This resistance is induced at higher temperatures in fish exposed to xenobiotics such as verapamil and BaP. Extensive salt extraction and proteinase K treatments overcome the resistance to unwinding in medaka DNA, indicating that proteinaceous materials are responsible for this phenomenon. Removal of this protein facilitates normal unwinding (denaturation) of medaka DNA with heat and alkali.

Several hypotheses to account for this observed resistance in medaka, previously not seen with alkaline unwinding in other fish species (bluegill, mosquitofish) can be proposed: (1) medaka have an efficient DNA repair mechanism (Shugart *et al.*, 1991) and (2) proteins tightly associated with medaka DNA may function in a protective/repair capacity to protect the genome from DNA damaging agents. High mobility group proteins of eukaryotes (Van Holde, 1989) and Rec A-like proteins, which function in bacterial DNA repair, might be studied in light of this phenomenon. The coating of damaged DNA with proteins (Rec A) prior to repair steps is well known in *Escherichia coli* and may have analogous processes in eukaryotes.

Under conditions of strong dissociation, medaka blood cell chromatin contains relatively more nonhistones than bluegill. Nonhistone proteins comprise about 40% of the chromosomal protein in most cells (Van Holde, 1989), except in nucleated erythrocytes where histone comprises 80% of total chromosomal protein. Nonhistone proteins comprise more than 100 different protein species each in low abundance relative to the five or six histone proteins which are abundant in eukaryotic cells. The loss of nonhistone proteins in medaka exposed to BaP for 52 days may reflect an overall decrease in DNA regulatory or gene expression activity in chronically exposed fish. Similar observations have been seen in bluegill exposed for 8 weeks to contaminated sediment (Theodorakis *et al.*, 1992). The different electrophoretic mobilities of the H1-H5 histones for medaka and bluegill indicate that different functional variants may exist in the two species.

Chromatin dissociation under conditions of partial extraction shows that medaka core histones are more resistant to extraction than bluegill core histones. The H2A, H2B, H3, and H4 histones, which comprise the eukaryotic nucleosome structures, are regularly spaced along

chromosomal DNA (Van Holde, 1989). Histones function to effect higher order of chromosomal structure and packaging of DNA into compact units. Medaka, bluegill, and mosquitofish chromatin was digested with micrococcal nuclease. Digestion of chromatin with this enzyme cleaves exposed DNA between nucleosomes, which protect associated DNA from digestion. A limited digestion reveals a distinct ladder of DNA fragments when analyzed by agarose gel electrophoresis after protein extraction (Lewin, 1980). Medaka chromatin was resistant to digestion by this enzyme, which digested bluegill and mosquitofish chromatin twice as fast. On the basis of the resistance of medaka histones to partial extraction, resistance to micrococcal nuclease digestion, and the induction of protein(s) that confer resistance to alkaline unwinding, medaka chromosomal DNA may be uniquely packaged in a novel DNA-protein complex not seen with other eukaryotic organisms to date. Further study of this unique phenomenon is warranted to understand the biological significance of chromatin containing tightly associated DNA-protein complexes in this species.

ACKNOWLEDGMENTS

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ORNITHINE DECARBOXYLASE ACTIVITY IN THE LIVER OF INDIVIDUAL MEDAKA OF BOTH SEXES

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ABSTRACT

Ornithine decarboxylase (ODC) activity has been determined in the liver of individual medaka (*Oryzias latipes*) ($N = 48$). The results indicate a mean ODC activity of 1.69 ± 1.77 nmoles $^{14}CO_2$/hr/mg protein. The fish displayed a large interindividual variation of normal ODC activity with a range of 140-fold. However, no differences attributable to sex were observed. The findings also indicate that the mean ODC activity in this fish model is approximately 100-fold greater than observations in the published literature as well as in our laboratory for commonly employed rodent models.

INTRODUCTION

Polyamines play an important role in the regulation of cellular development and differentiation (Tabor and Tabor, 1984). Ornithine decarboxylase (ODC) catalyzes the rate-limiting step in the polyamine biosynthesis pathway (Russell and Snyder, 1969; Snyder and Russell, 1970; Raina et al., 1976) and shows a rapid and dramatic increase in response to many different growth stimuli (Russell et al., 1976). Increased ODC activity was observed in regenerating liver (Russell and Snyder, 1969; Janne and Raina, 1968; Holtta and Janne, 1972; Walker et al., 1978) and is associated with hepatic tumor promotion in rats (Olson and Russell, 1979). In addition, an increase in hepatic ODC was observed in rats following single or multiple exposures to the peroxisome proliferators clofibrate (Russell, 1971; Eliassen and Osmudsen, 1984; Fukami et al., 1986) and trichloroacetic acid (TCA) (Parnell et al., 1988).

To date, ODC activity in fish has been characterized in only two species, the European sea bass (*Dicentrarchus labrax* L.) (Corti et al., 1987, 1988) and the goldfish (*Carassius auratus*) (Devalli et al., 1989, 1990). The purpose of this research was to determine hepatic ODC levels in medaka (*Oryzias latipes*), a small aquarium fish, as part of an overall project to assess factors affecting liver carcinogenesis in this model. The results of this preliminary investigation were

striking in that the ODC activity was determined to be approximately 100-fold greater than seen in rodent liver.

MATERIALS AND METHODS

Two lots of medaka, 8 months in age, were obtained from Carolina Biological Supply Company (Burlington, NC). Medaka were maintained at 22°C on a 12-hour light/dark schedule and were fed twice daily alternately with Tetra Fin Flake Food and Tetra Brine Shrimp Treat (Tetra Sales, Inc., Morris Plains, NJ). Medaka were sacrificed by exposure to 3-aminobenzoic acid ethyl ester (Sigma Chemical Co., St. Louis, MO). Livers were removed and homogenized in a 0.1 M phosphate buffer, pH 7.2, containing 0.1 mM pyridoxal-5-phosphate, 2.0 mM EDTA, 5 mM dithiothreitol (DTT) and 100 μM phenylmethylsulfonyl fluoride (PMSF). Homogenates were centrifuged at 15,000 x g for 30 minutes. Supernatants were assayed for ODC by the method of Davis and Paulus (1983), which measures the amount of $^{14}CO_2$ liberated from L-[1-^{14}C]ornithine. The final concentration of reagents in the enzyme assay mix was 100 mM potassium phosphate buffer, pH 7.2, 100 μM PMSF, 5 mM DDT, 1 mM EDTA, 5 mM pyridoxal-5-phosphate, 2 mM L-ornithine, and 5 μCi of L-[1-^{14}C]ornithine (59 mCi/mmol). Also, DL-α-(difluoromethyl)-ornithine hydrochloride monohydrate (DFMO), a gift of Marion Merrill Dow Research Institute (Cincinnati, OH), was added to some samples at a final concentration of 5 mM. The $^{14}CO_2$ was collected on 1 x 2 cm pieces of Whatman #1 filter paper spotted with 10 μl of monoethanolamine-methylcellusolve (1:2, v/v). Next, 200 μl of the enzyme assay mixture was added to 100 μl of enzyme and incubated at 37°C for 60 minutes. The reaction was stopped by the addition of 200 μl of 10% TCA. Tubes were incubated for an additional 90 minutes at 37°C to collect the $^{14}CO_2$. Filter papers were counted in Ecoscint A fluor (National Diagnostics, Manville, NJ) on a 1217 Rackbeta liquid scintillation spectrophotometer (LKB Instruments, Inc., Gaithersburg, MD). Protein content of the samples was determined by the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL).

RESULTS

The mean hepatic ODC activity for all the fish was 1.69 $\pm$ 1.77 nmoles $^{14}CO_2$/hr/mg protein (Table 1). Considerable interindividual variation was seen in the fish, ranging from a low of 0.06 to a high of 8.43 nmoles $^{14}CO_2$/hr/mg protein (i.e., a range of 140-fold). Some variation was also seen between the two lots of fish. The mean ODC activity of lot 1 was 2.21 $\pm$ 2.04 nmole $^{14}CO_2$/hr/mg protein, and the mean ODC activity of lot 2 was 1.69 $\pm$ 1.77 $^{14}CO_2$/hr/mg protein. The mean ODC activity of the males was 1.60 $\pm$ 1.84 nmoles $^{14}CO_2$/hr/mg protein, and the mean

ODC activity in the females was 1.84 ± 1.67 nmoles $^{14}CO_2$/hr/mg protein. DFMO effectively inhibited 100% of ODC activity (Table 2). ODC activity was linear over time (Figure 1).

Table 1. Mean ODC Activity (nmoles $^{14}CO_2$/hr/mg protein) in Individual Medaka

	Sample Size	Mean	Standard Deviation
ODC in medaka: mixed sexes; lots 1 & 2	48	1.69	1.77
ODC in female medaka: lots 1 & 2	17	1.84	1.67
ODC in male medaka: lots 1 & 2	31	1.60	1.84
ODC in medaka: mixed sexes; lot 1	24	2.21	2.04
ODC in female medaka: lot 1	9	2.36	1.82
ODC in male medaka: lot 1	15	2.11	2.22
ODC in medaka: mixed sexes; lot 2	24	1.17	1.29
ODC in female medaka: lot 2	8	1.26	1.37
ODC in male medaka: lot 2	16	1.12	1.29

Table 2. Effect of DFMO on ODC Activity (nmoles $^{14}CO_2$/hr/mg protein) in Pooled Medaka Livers

	ODC Activity	
Sample Number	Without DFMO	With DFMO
1[a]	1.48	0.06
2[a]	2.32	0.00
3[a]	3.74	0.00
4[b]	0.93	0.00
5[b]	0.07	0.00
6[b]	1.40	0.00
7[b]	0.58	0.00
8[b]	0.65	0.00
9[b]	0.10	0.00

[a]each sample represents a pool of four medaka livers.
[b]each sample represents a pool of two medaka livers.

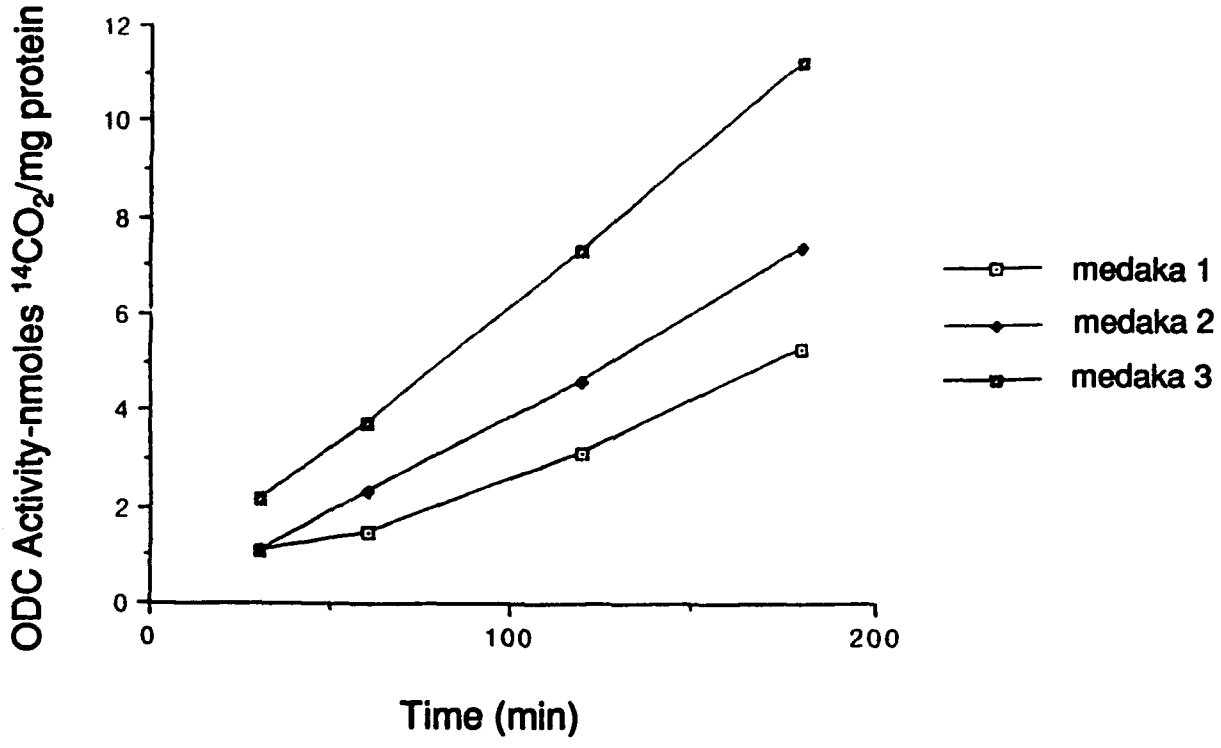

Figure 1. Ornithine decarboxylase (ODC) activity (nanomoles $^{14}CO_2$/milligram of protein) in pooled medaka liver samples (4 livers/sample) over time.

DISCUSSION

The present study represents the first documentation of hepatic ODC activity in medaka. The large standard deviation observed appears to be due to interindividual variation and not to the sex of the animal (Table 1). It should be emphasized that these findings were reported for individual medaka, which represents a significant improvement over our earlier attempts utilizing pooled samples of up to four fish. Having the capacity to obtain reliable data on individual fish will provide both significantly improved opportunities to understand chronic disease processes in this model species and assistance in the statistical design and planning of future investigations.

Compared with rats, medaka have higher hepatic ODC activity by a factor of 100 (Bisschop et al., 1981; Fukami et al., 1986; Olson and Russell, 1979; Pereira et al., 1982; Savage et al., 1982). This elevated level of ODC activity was not a procedural artifact, as demonstrated by its linear response over time (Figure 1) and its inhibition by DFMO (Table 2).

Other investigators have demonstrated higher activity of hepatic ODC in fish compared with rats (Corti et al., 1987; Devalli et al., 1989, 1990), although not to the extent observed in medaka. Sea bass, in a comparison study with rats, were shown to have higher levels of hepatic ODC by a factor of approximately 3 (Corti et al., 1987). Goldfish exhibit maximum hepatic ODC activity, subject to seasonal variation, higher than rats by a factor of approximately 10 (Devalli et al., 1989, 1990).

The ability to inhibit ODC with DFMO will provide an opportunity to assess experimentally the role of ODC in tumor promotion and progression. This assay has been exploited with great success in both rodent and human carcinogenesis. Similar use in fish could yield significant insights concerning features controlling the process of hepatocarcinogenesis.

The significance of elevated activity of normal hepatic ODC in medaka is at present unknown, although it has been suggested that ODC and polyamines are involved in adaptive metabolic responses to environmental changes or stress stimuli (Devalli et al., 1990). Clarification of the relationship of elevated levels of hepatic ODC activity both to normal metabolic processes and to the susceptibility of medaka to chemically induced hepatocarcinogenesis is strongly recommended.

CONCLUSION

Normal hepatic ODC activity has been determined for the first time in individual medaka. The activity was approximately 100-fold greater than that observed in normal rat liver.

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THE EFFECT OF PEROXISOME PROLIFERATORS ON S-PHASE SYNTHESIS IN PRIMARY CULTURES OF FISH HEPATOCYTES

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ABSTRACT

The ability of seven structurally diverse peroxisome proliferators to induce S-phase synthesis was studied in primary cultures of rainbow trout and medaka hepatocytes. Cells were maintained in serum-free conditions designed to facilitate attachment, viability, and function. Lead nitrate, a well-known and potent rodent liver mitogen, was used *in vitro* in trout and medaka and *in vivo* in trout to evaluate the capacity of the species in our system to respond to mitogenic stimuli. S-phase synthesis induced by peroxisome proliferators did not result in any statistically significant increase over that in control cultures. These *in vitro* results are supported both by *in vivo* data previously obtained and by the present work with lead nitrate.

INTRODUCTION

Cell proliferation has been implicated as a mechanism important to tumor promotion and can result from direct mitogenic stimulation (Butterworth et al., 1987). A number of agents that have the capacity to cause peroxisome proliferation have also been shown to have mitogenic properties in rat hepatocytes (Bieri et al., 1986, 1987*a*; Marsman et al., 1988). It has been demonstrated that peroxisome proliferation and replicative DNA synthesis can be stimulated independently and that the mitogenic potency of these substances cannot be predicted from their effects on the peroxisomal compartment (Bieri et al., 1987*b*).

The *in vivo* and *in vitro* inducibility of peroxisome proliferation in rainbow trout is considerably less pronounced than that observed in rodents (Henderson and Sargent, 1983; Yang, 1990; Yang et al., 1990; Scarano, 1991; Donohue et al., 1992). Since the carcinogenic potency of peroxisome proliferators has been more closely associated with their mitogenic potency than with their capacity for peroxisome proliferation (Bieri et al., 1986, 1987*a*,*b*; Marsman et al., 1988), it is important to clarify this response in aquatic species to better understand the carcinogenic process in such organisms. The effect of peroxisome proliferators on DNA synthesis under defined

conditions of primary culture is one approach that can be used in the characterization of this type of nongenotoxic carcinogen.

Kocal et al. (1988*a*) suggested that trout hepatocytes *in vitro* rapidly catabolize thymidine to a product that is not incorporated into DNA, thereby invalidating the use of [^{3}H]thymidine as a marker of DNA synthesis under certain conditions. The present study with rainbow trout and medaka was designed to overcome this limitation by conducting 2 hour-pulse labeling during periods of expected DNA synthesis to maintain a concentration of [^{3}H]thymidine sufficient to mark cells in S-phase over time. Lead nitrate, a known liver mitogen in rats (Columbano et al., 1983, 1985; Ledda-Columbano, 1983), was selected as a positive control since the *in vivo* verification of positive results *in vitro* was possible. Seven structurally diverse peroxisome proliferators were tested for their mitogenic potency in primary hepatocyte cultures with rainbow trout and medaka. In addition, an *in vivo* validation of the *in vitro* results with lead nitrate was attempted with rainbow trout.

MATERIALS AND METHODS

In Vitro Study

Animals

Rainbow trout (*Salmo gairdneri*) weighing 300-350 grams were obtained from the Massachusetts Division of Fisheries and Wildlife fish hatchery in Sunderland, Massachusetts. Medaka (*Oryzias latipes*), 8 months in age, were obtained from Carolina Biological Supply Company, Burlington, North Carolina.

Test Chemicals

Ciprofibrate was obtained from Sterling Research, Rennselaer, New York. Lead nitrate, lead acetate, gemfibrozil, clofibric acid, and trichloroacetic acid (TCA) were purchased from Sigma Chemical Company, St. Louis, Missouri. Nafenopin was obtained from CIBA-GEIGY Corp., Summit, New Jersey. Mono-2-ethylhexyl phthalate acid ester (MEHP) was obtained from American Tokyo Kasei, Inc., Portland, Oregon, and 2,4-dichlorophenoxyacetic acid dimethyl amine (2,4-D) was purchased from Dow Chemical, Co., Midland, Michigan.

Hepatocyte Isolation

Rainbow trout. Hepatocytes were isolated by collagenase perfusion based on the procedure for rodents (Seglen, 1973) and modified for fish (Moon et al., 1985; Lipsky et al., 1986; Maitre et al., 1986; Kocal et al., 1988*b*). In order to facilitate the perfusion technique, the perfusion was not

done *in situ*. Trout were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co.), placed on a surgical board, and incised from the urogenital pore to the gills. The bile was removed from the gall bladder with a 1-cc tuberculin syringe. The liver was then separated from the gall bladder and transferred to a sterile petri dish. The sinus venosus was cannulated using a blunt 18 gauge needle and ligated. Initially the liver was perfused with 10 mM HEPES buffer, pH 7.4, containing 136.9 mM NaCl, 3.4 mM KCl, 0.4 mM KH_2PO_4, 4.1 mM $NaHCO_3$, 5.5 mM glucose, and 0.1 mM EGTA. A perfusion with the same buffer followed, but with the EGTA omitted and containing 1 mg/ml collagenase (Type II, Worthington Biochemical Corp., Freehold, NJ) and 5.1 mM $CaCl_2$. During perfusion with the dissociating solutions, the liver was gently massaged to increase both blood clearance and hepatocyte yield (Moon et al., 1985). At the end of the perfusion, the liver was washed with HEPES buffer, and the hepatocyes were dissociated, by using a sterile pipet and forceps. The hepatocytes were passed sequentially through sterile 500, 250, 150, and 75 gauge Nitex screening (Tetko, Inc., Elmsford, NY). The cell suspension was then centrifuged for 2 minutes at 35 × g. The pellet was resuspended in culture medium and centrifuged for 2 minutes at 35 × g. The final pellet was resuspended in culture medium. Viability was determined by trypan blue exclusion test; only preparations with 95% viability were utilized.

Medaka. Medaka were anesthetized with MS-222, transferred to a dissecting tray, and incised from the urogenital pore to the gills. The liver was removed, separated from the gall bladder, and transferred to a sterile beaker containing 2 ml of 0.5% trypsin, 5.3 mM EDTA (Gibco Laboratories, Grand Island, NY), reconstituted in HEPES buffer, pH 7.4, containing 136.9 mM NaCl, 3.4 mM KCl, 0.4 mM KH_2PO_4, 4.1 mM $NaHPO_3$, and 5.5 mM glucose, and stirred for 10 minutes at room temperature. The cell suspension was transferred to microcentrifuge tubes and centrifuged for 2 minutes at 35 × g. The pellets were resuspended in culture medium and centrifuged for 2 minutes at 35 × g. The final pellet was resuspended in culture medium. Viability was determined by the trypan blue exclusion test; only preparations with 95% viability were utilized.

Culture Conditions

Chemicals were obtained from Gibco Laboratories unless otherwise stated. The culture medium, based on previous trout studies (Lipsky et al., 1986; Maitre et al., 1986; Kocal et al., 1988*b*) consisted of Leibowitz L-15, 10 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml, Sigma), and insulin (1 μg/ml, pH 7.6). Trout hepatocytes, plated in 0.2 ml medium at a density of 7×10^4 cells/chamber in an 8-chamber Permanoz slide

(Baxter Scientific Products, McGraw Park, IL), were incubated at 15°C in 100% air. Medaka hepatocytes, plated in 0.2 ml of medium at densities dependent on liver size of 1.6 to 18.0 × 10^4 cells/chamber, were incubated at 22°C in 100% air. Plating density was less than confluent to reduce cell-to-cell contact and optimize S-phase response (Nakamura and Ichihara, 1985). Stock solutions of agents were prepared in dimethyl sulfoxide (DMSO) and added to the medium to give a final DMSO concentration of 0.4%. Concentrations were determined by preliminary dose-range-finding experiments. The highest concentrations that did not show toxicity by trypan blue exclusion following 48 hours of exposure were chosen. Control cultures were exposed to medium containing 0.4% DMSO without the agent. Treatment was initiated 4 hours after plating.

Replicative DNA Synthesis

Replicative DNA synthesis was assessed based on the procedures of Klaunig (1984) and Kelly and Maddock (1985). Pulse labeling was conducted 18 to 30 hours following exposure to test chemicals, during which time medium containing [^{3}H]thymidine (10 μCi/ml; 50 Ci/mmol; New England Nuclear, Boston, MA) was changed every 2 hours. Preliminary studies with lead nitrate demonstrated a 1.9-fold increase in cumulative percentage of cells in S-phase over control in the 18- to 30-hour time period. Less dramatic treatment-related increases were observed in the 8- to 18- and 34- to 44-hour time periods that gradually decreased over time to 68 hours. Two hours prior to treatment with [^{3}H]thymidine (Bieri et al., 1988), 10 mM hydroxyurea, a specific inhibitor of replicative DNA synthesis (Lammers and Follman, 1983), was added to the medium of selected cultures. At the end of this time, hepatocytes were washed in Hanks' Balanced Salt Solution (HBSS) containing 1 mM thymidine, followed by two washes with HBSS. Cells were fixed with methanol at -20°C for 10 minutes (Baserga, 1989). Slides were air-dried, coated with NTB-3 emulsion (Eastman Kodak Co., Rochester, NY), diluted 1:1 with distilled water, and kept in a light-tight box at -20°C for 12 days. S-phase synthesis was determined by dense nuclear labeling, and the cumulative percentage of cells in S-phase was quantified by counting 1,000 consecutive morphologically unaltered hepatocytes in each of three replicative chambers for each treatment. To verify that the DNA synthesis was replicative and not repair, these results were compared with the number of cells in S-phase synthesis observed in cultures pretreated with hydroxyurea.

Statistical Analysis

Data were analyzed by one-way analysis of variance for trout and by a paired *t* test for medaka, because of the difference in the study designs.

In Vivo Study

Animals

Rainbow trout (*Salmo gairdneri*), average body weight of 250 grams, were obtained as previously described and transferred to holding tanks for study at the Division's Sunderland fish hatchery.

Test Chemicals

Lead nitrate and lead acetate were purchased from Sigma Chemical Co.

Treatment

Trout were anesthetized in water containing 100 ppm MS-222. Lead nitrate and lead acetate were dissolved in distilled water and filter-sterilized. A final volume of 0.2 ml was injected into the peritoneal cavity through the center region of the ventral side of the trout. Controls received 0.2 ml of distilled water only. Pilot studies were conducted to determine concentrations of lead compounds that did not cause hepatotoxicity. Lead nitrate was administered at concentrations of 250 or 375 mg/kg body weight. To ensure that the response observed was not due to the chemical form of the salt used, lead acetate was administered at a concentration of 375 mg/kg body weight. Five fish were used in each treatment group. Fish were sacrificed by exposure to water containing 300 ppm MS-222 64 hours after injection. Whole fish were weighed and the livers removed and weighed.

Liver-to-Body Weight Ratio

Body weight was measured at the end of the study period. Livers were removed at termination and weighed. Data were expressed as grams of liver per 100 grams of body weight.

DNA Content

Hepatic DNA content was measured by the diphenylamine method of Schneider (1957). Data were expressed as milligrams of DNA per 100 grams of body weight.

Statistical Analysis

Data were analyzed by a one-way analysis of variance.

RESULTS

In Vitro Study

Replicative DNA Synthesis

Rainbow trout. No statistically significant increase in cumulative percentage of cells in S-phase over control was observed in any treatment group (i.e., peroxisome proliferators) (Table 1). The lead nitrate treatment that was to serve as a positive control displayed a response 1.7-fold greater than the control. Treatment with clofibric acid elicited a 1.4-fold increase over control.

Medaka. Statistically significant increases over control in cumulative percentage of cells in S-phase were observed only in the groups treated with lead acetate ($p < 0.05$) and lead nitrate ($p < 0.10$) (Table 2). Treatment with ciprofibrate approached statistical significance ($0.20 < p < 0.10$) with a 1.7-fold increase over control.

In Vivo Study

Liver-to-Body Weight Ratio

No statistically significant increase in liver-to-body weight ratios over control group was observed in any treatment group (Table 3).

DNA Content

No statistically significant increase in hepatic DNA content over control group was observed in any treatment group (Table 3).

DISCUSSION

The present study demonstrates that S-phase synthesis induced by peroxisome proliferators in primary cultures of rainbow trout and medaka hepatocytes does not result in any statistically significant increase over control cultures. However, it is possible that clofibric acid in the trout and ciprofibrate in the medaka stimulate S-phase synthesis, but the sample size precluded a more definitive appraisal. The standard serum-free culture conditions used in this study were designed to promote attachment, viability, and function. Hepatic function was demonstrated by the ability of these cells to metabolize acetaminophen (via cytochrome P-450) and to respond to peroxisome proliferators by induction of acyl-CoA oxidase and the peroxisomal bifunctional enzyme (Donohue et al., 1992). Evidence that the [^{3}H]thymidine incorporation observed was mainly replicative was demonstrated by the 80-90% inhibition of incorporation by 10 mM hydroxyurea.

These *in vitro* results are supported by *in vivo* data on liver-to-body weight ratios in rainbow trout exposed to peroxisome proliferators. No statistically significant increases in liver-to-body

Table 1. Cumulative Percentage of Rainbow Trout Hepatocytes in S-Phase Synthesis During the 18- to 30-Hour Time Period Following Exposure to Test Chemical

Test Chemical	Concentration (mM)	Cumulative % Cells in S-Phase*	*x*-Fold Control
Control		0.07 ± 0.05	
Lead Acetate	0.001	0.12 ± 0.07	1.7
Lead Nitrate	0.001	0.12 ± 0.07	1.7
Clofibric Acid	2.750	0.10 ± 0.09	1.4
Ciprofibrate	1.000	0.08 ± 0.07	1.1
Nafenopin	0.160	0.08 ± 0.07	1.1
MEHP	0.500	0.07 ± 0.08	1.0
Gemfibrozil	1.250	0.03 ± 0.05	0.4
2,4-D	0.500	0.03 ± 0.05	0.4

*The value represents the mean ± SD of six cultures; three cultures from each of two fish on two different days; 1,000 hepatocytes were counted in each culture.

weight ratios were observed in rainbow trout treated via intraperitoneal injection for 2 to 4 weeks with clofibrate, lactofen, di-(2-ethylhexyl)phthalate (DEHP), 2,4-D, or trichloroethylene (TCE) (Yang, 1990; Scarano, 1991). In the same studies, statistically significant increases were observed only in the highest dose gemfibrozil group (Scarano, 1991), and only marginally significant increases were observed in the highest dose ciprofibrate group (Yang, 1990). A 7-week feeding study with trout on a high-fat diet plus DEHP also revealed no statistically significant increase in liver-to-body weight ratio (Henderson and Sargent, 1983).

Further evidence that rainbow trout do not respond to mitogen stimulation in a manner similar to rodents was shown in the *in vitro* and *in vivo* experiments with lead compounds. Columbano et al. (1983, 1985) demonstrated a significant increase in liver-to-body weight ratios and hepatic DNA content within 48 hours in rats administered a single injection of lead nitrate compared with control rats. No lead-induced increases in liver-to-body weight ratio or hepatic DNA content were observed in trout within 64 hours following a single injection of lead nitrate or lead acetate. Despite the fact that lead nitrate and lead acetate *in vitro* showed the highest S-phase response over control compared with the seven peroxisome proliferators, the overall cumulative percentage of cells in S-phase was low.

Table ?. Cumulative Percentage of Medaka Hepatocytes in S-Phase Synthesis During the 18- to 30-Hour Time Period Following Exposure to Test Chemicals

Test Chemical	Conc. mM	Cumulative % Cells in S-Phase[a] Control	Treated	d[b]	$\bar{d}$[c]	x-Fold Control
		0.07	0.13	0.06		
Lead Acetate	0.001	0.03	0.07	0.04	0.053[d]	1.9
		0.07	0.13	0.06		
		0.07	0.13	0.06		
Lead Nitrate	0.001	0.07	0.13	0.06	0.050[e]	1.7
		0.07	0.10	0.03		
		0.07	0.10	0.03		
Ciprofibrate	1.000	0.10	0.13	0.03	0.043	1.5
		0.10	0.17	0.07		
		0.07	0.07	0.00		
Nafenopin	0.160	0.10	0.17	0.07	0.033	1.4
		0.07	0.10	0.03		
		0.17	0.27	0.10		
Clofibric Acid	2.750	0.13	0.10	-0.03	0.037	1.3
		0.13	0.17	0.04		
		0.07	0.10	0.03		
TCA	5.000	0.07	0.07	0.00	0.010	1.1
		0.07	0.07	0.00		
		0.07	0.03	-0.04		
MEHP	0.500	0.07	0.10	0.03	0.010	1.2
		0.03	0.07	0.04		
		0.03	0.03	0.00		
2,4-D	0.500	0.03	0.07	0.04	0.000	1.0
		0.07	0.03	-0.04		
		0.13	0.07	-0.06		
Gemfibrozil	1.250	0.13	0.13	0.00	-0.020	0.8
		0.10	0.10	0.00		

[a]Each value represents the average cumulative percentage of cells in S-phase of three replicate cultures; 1,000 hepatocytes were counted in each culture.

[b]d = difference in cumulative percentage of cells in S-phase between control and treated cultures from the same fish.

[c]$\bar{d}$ = average of the differences (d) from the three fish in each treatment group.

[d]Statistically significant from control ($p < 0.05$) as determined by paired t test.

[e]Statistically significant form control ($p < 0.10$) as determined by paired t test.

Table 3. Effect of Single Intraperitoneal Injection of Lead Nitrate or Lead Acetate on Liver-to-Body Weight Ratio and Hepatic DNA Content in Rainbow Trout

Treatment	Dose (mg/kg)	Liver-to-Body Weight Ratio[a]	Hepatic DNA Content[b]
Control	0	1.50 ± 0.13	3.32 ± 0.22
Lead Nitrate	250	1.39 ± 0.09	3.23 ± 0.18
Lead Nitrate	375	1.41 ± 0.23	3.30 ± 0.28
Lead Acetate	375	1.40 ± 0.11	3.48 ± 0.24

[a]Units = mean ± SE grams of liver/100 grams body weight, $N = 5$.
[b]Units = mean ± SE mg DNA/100 grams body weight, $N = 5$.

The present study was designed to address two principal questions: whether trout hepatocytes respond to rodent mitogenic stimuli *in vitro* and *in vivo*, and whether selected rodent peroxisome proliferators induce S-phase synthesis in primary cultures of trout and medaka hepatocytes. The data indicate no convincing evidence of hepatic mitogenic stimulation *in vivo* with lead nitrate and lead acetate, with possible weak responses *in vitro* in both trout and medaka. While either weak or lack of response in the *in vitro* system of the trout and medaka suggests that further efforts should be directed toward optimizing the conditions of the culture system to facilitate the S-phase response, the lack of an apparent mitogenic response *in vivo* in trout to lead compounds suggests that the trout and medaka may not be sensitive to these mitogenic stimuli.

CONCLUSION

Peroxisome proliferators do not significantly induce S-phase synthesis in primary cultures of rainbow trout or medaka hepatocytes under the conditions described.

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COMPARATIVE RESPONSE OF RAINBOW TROUT AND RAT TO THE LIVER MITOGEN, LEAD

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ABSTRACT

Lead nitrate and lead acetate are potent mitogens in the liver of the Wistar rat. Prior exposure to these agents has been found to alter the rat's susceptibility to hepatotoxins. The present study assessed the capacity of both lead compounds to cause mitogenicity in the liver of adult male and female rainbow trout. Groups treated with a single intravenous or intraperitoneal injection of lead nitrate or lead acetate had no significant alterations in liver-to-body weight ratio nor hepatic DNA content. Results provide evidence of significant interspecies variation between rainbow trout and Wistar rats in their mitogenic response to lead in the liver.

INTRODUCTION

Cell proliferation from a variety of mitogenic stimuli has become a major interest in the field of chemical carcinogenesis. For example, Ames and Gold (1990) argued that chemically induced liver cell death that results in a mitogenic cell-replacement response strongly promotes the process of carcinogenesis. Cohen and Ellwein (1988) have established a strong association between saccharin-induced hyperplasia in the urinary bladder and the subsequent occurrence of bladder cancer. Similarly, cell proliferation resulting from direct mitogenic stimulation has been considered more closely related to hepatic tumor promotion than peroxisome proliferation in the epigenetic carcinogens and hypolipidemic drugs such as clofibrate and ciprofibrate (Bieri et al., 1986).

Lead nitrate has been employed as a chemical to produce a potent mitogenic response in the rodent liver in order to assess the role of mitogenicity in chemically induced hepatocarcinogenicity (Ledda-Columbano et al., 1983; Columbano et al., 1984). The present study was designed to assess if the rainbow trout, a model extensively used in fish carcinogenesis studies (Lee et al., 1968; Sinnhuber et al., 1968, 1974; Hendricks et al., 1980; Shelton et al., 1984; Bailey et al., 1988), would also be sensitive to lead as a mitogen.

MATERIALS AND METHODS

Rainbow trout (*Salmo gairdneri*) were obtained from the Massachusetts Division of Fisheries and Wildlife fish hatchery in Sunderland, Massachusetts. The trout were maintained at this facility during experiments in 1,100-liter fiberglass tanks with a fresh water flow-through exchange rate of 110 liters per minute. Trout ranged in age from 18 to 24 months and in weight from approximately 80 to 400 grams. Fish were separated into individual tanks by treatment group and fed a standard diet of trout feed (Zeigler Brothers, Gardners, PA) prior to, but not during, experiments. Water temperatures ranged from 8°C to 11°C during experiments.

Lead nitrate and lead acetate were obtained from Sigma Chemical Company (St. Louis, MO). Lead acetate was also administered to ensure that any response observed was not due to the chemical form of salt used. Both intravenous (IV) and intraperitoneal (IP) injections of lead nitrate were administered to account for potential variation in results due to route of exposure. Trout were anesthetized in water containing 100 ppm 3-aminobenzoic acid ethyl ester (MS-222) for dosing. Lead nitrate and lead acetate were dissolved in distilled water and filter-sterilized. A final volume of 0.2 ml was injected via a 26 gauge needle into the peritoneal cavity through the center region of the ventral side of the trout. Intravenous injections consisted of a 0.1-ml volume of lead nitrate solution administered through a 26 gauge needle into the jugular vein at the base of the first gill arch. Controls received equal volumes of distilled water only. Equal numbers of control fish were sacrificed each time fish from treatment groups were sacrificed. Hepatic DNA content was measured by the diphenylamine method and expressed as milligrams of DNA per 100 grams of body weight (Schneider, 1957).

RESULTS AND DISCUSSION

A significant change in mean liver-to-body weight ratio was observed in 3 of 20 groups treated with a single intraperitoneal injection of lead nitrate (Table 1). However, two of the three statistical observations actually represent a reduction in liver size relative to body weight and the third, only a modest (14%) increase. The combination of small sample size ($N = 3$ or 4) and variation in size of individual fish (142-283 grams) may account for these observations. Further evidence of no treatment effect was also observed after administration of higher doses of lead nitrate and lead acetate (Table 2). A single intraperitoneal injection of lead nitrate does not result in any statistically significant changes in liver-to-body weight ratio between control and treatment groups after 64 hours ($p < 0.05$). The effect of a single intravenous injection of lead nitrate yields similar results (Table 3).

Table 1. Effect of a Single Intraperitoneal (IP) Dose of Lead Nitrate on Liver-to-Body Weight Ratio in Rainbow Trout

Time after dosage (hours)	Dose (mg/kg body weight)				
	25	50	75	100	125
43	1.15 ± 0.06	1.24 ± 0.05	1.14 ± 0.09	1.24 ± 0.04	1.27 ± 0.07
67	1.21 ± 0.02	1.26 ± 0.04	1.22 ± 0.07	1.31 ± 0.04	1.39 ± 0.07*
91	1.21 ± 0.04	1.30 ± 0.15	1.15 ± 0.12	1.12 ± 0.05	1.19 ± 0.04
115	1.12 ± 0.02*	1.20 ± 0.04	1.10 ± 0.02*	1.29 ± 0.03	1.27 ± 0.05

Values = mean ± SE; units = grams of liver/100 grams body weight; N = 3 or 4 animals; combined control (0 mg lead nitrate/kg body weight, N = 16) value = 1.22±0.05.
*Significantly different from control ($p < 0.05$).

Table 2. Effect of Lead Nitrate and Lead Acetate on Liver-to-Body Weight Ratio and DNA Content from a Single IP Injection After 64 Hours in Rainbow Trout

Treatment	Dose mg/kg	Liver-to-Body Weight Ratio[a]	Hepatic DNA Content[b]
Control	0	1.50 ± 0.13	3.32 ± 0.22
Lead nitrate	250	1.39 ± 0.09	3.23 ± 0.18
Lead nitrate	375	1.41 ± 0.23	3.30 ± 0.28
Lead acetate	375	1.40 ± 0.11	3.48 ± 0.24

[a]Values = mean ± SE; units = grams of liver/100 grams body weight; N = 5.
[b]Values = mean ± SE; units = mg DNA/100 grams body weight; N = 5.

Table 3. Effect of a Single Intravenous Injection of Lead Nitrate on Liver-to-Body Weight Ratio in Rainbow Trout After 72 and 115 Hours

Treatment	Dose (mg/kg)	Liver-to-Body Weight Ratio[a]	
		72 hour	115 hour
Control	0	1.20 ± 0.09	1.30 ± 0.07
Lead nitrate	0.5	1.27 ± 0.15	
Lead nitrate	1.0	1.23 ± 0.04	
Lead nitrate	5.0	1.48 ± 0.14[b]	1.35 ± 0.08

[a]Values = mean ± SE; units = grams of liver/100 grams body weight; $N = 6$.
[b]Significantly different from control ($p < 0.05$).

The weak capacity of lead to induce cell proliferation or hyperplasia in rainbow trout was in contrast to the Wistar rat model of Columbano (1984, 1985). A single intravenous injection (100 μmole/kg body weight) resulted in a substantial increase in the liver-to-body weight ratio (i.e., increased by 70% within 48-72 hours). The increased liver-to-body weight ratio is sustained for several days until the process of apoptosis begins, resulting over the next several days in a dramatic decrease in liver cell number such that by days 7-10 the liver-to-body weight ratio returns to normal.

This study provides evidence of significant interspecies variation between rainbow trout and Wistar rats in their response to mitogenic stimuli such as lead. To what extent this differential response is related to susceptibility to chemically induced carcinogenicity remains to be explored.

CONCLUSION

The inability of lead to induce cell proliferation in the rainbow trout is in marked contrast to its effect on the Wistar rat.

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conducting this research, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, revised 1985).

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EFFECT OF HYPOLIPIDEMIC DRUGS, GEMFIBROZIL, CIPROFIBRATE, AND CLOFIBRIC ACID, ON PEROXISOMAL β-OXIDATION IN PRIMARY CULTURES OF RAINBOW TROUT HEPATOCYTES

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ABSTRACT

Primary cultures of hepatocytes were established from sexually mature male and female rainbow trout (*Salmo gairdneri*) and treated with the hypolipidemic drugs gemfibrozil (0.25-1.25 mM), clofibric acid (2.25-3.00 mM), or ciprofibrate (0.33-1.00 mM). Significant dose-related increases in peroxisomal fatty acyl-CoA oxidase (FACO) were seen after exposure for 48 hours to clofibric acid ($p < 0.01$) and ciprofibrate ($p < 0.05$) but not gemfibrozil ($p = 0.08$). Positive correlations were obtained between increased acyl-CoA oxidase activity and relative amount of peroxisomal bifunctional enzyme (PBE), further supporting evidence of a proliferative effect. These studies demonstrate that peroxisomal β-oxidation can be induced *in vitro* in a primary rainbow trout hepatocyte culture.

INTRODUCTION

The phenomenon of hepatic peroxisome proliferation has received much attention over the last 20 years. A wide variety of agents have been shown to induce this condition in rodent models both *in vivo* and *in vitro* (Reddy et al., 1973; Reddy and Qureshi, 1979; Foxworthy and Eacho, 1986; Elcombe and Mitchell, 1986; Eacho et al., 1989). Some agents that have been shown to induce peroxisome proliferation have also produced hepatocellular carcinoma after long-term administration to rodents *in vivo* (Reddy and Rao, 1977; Reddy and Qureshi, 1979; Svoboda and Azarnoff, 1979). These agents have been shown to be nongenotoxic and, therefore, may represent a distinct class of chemical carcinogens.

It has been proposed that significant peroxisome proliferation may be a phenomenon observed only in rodents (de la Iglesia et al., 1981). For example, proliferation was not seen in marmosets (Holloway et al., 1982) after administration of various hypolipidemic drugs *in vivo*. Chickens, pigeons, cats, and cynomolgus monkeys exhibited little response to ciprofibrate at doses that caused high responses in rodents (Reddy et al., 1984). In addition, researchers studying primary hepatocyte cultures of the dog and rhesus monkey for induction of peroxisome

proliferation found less than a twofold increase in peroxisomal β-oxidation, whereas a tenfold increase was observed with rat hepatocytes, indicating possible hyperresponsiveness in the rodent model (Foxworthy et al., 1990).

Although much information on rodent responses to peroxisome proliferators is available, there is generally little known about the teleost response to these epigenetic carcinogens. Recent *in vivo* findings by Yang (1989) and Yang et al. (1990) demonstrated that fatty acyl-CoA oxidase (FACO) was induced in trout exposed to hypolipidemic drugs. In addition to this, Baldwin et al. (1990) were able to isolate and confirm the presence of peroxisomal bifunctional enzyme (PBE) in rainbow trout liver. The present study was designed to extend the work of Yang et al. (1990) by assessing the peroxisome proliferation potential of primary cultures of rainbow trout hepatocytes exposed to gemfibrozil, ciprofibrate, or clofibric acid.

MATERIALS AND METHODS

Animals

Sexually mature rainbow trout (*Salmo gairdneri*), weighing 300 to 500 grams, were obtained from the Massachusetts Division of Fisheries and Wildlife fish hatchery in Sunderland, Massachusetts.

Chemicals

Leibowitz L-15 medium, insulin, and antibiotics were obtained from Gibco Laboratories (Grand Island, NY) and 60-mm Primaria tissue culture dishes from Becton-Dickinson Co. (Oxnard, CA). Ciprofibrate was purchased from Sterling Research (Rennselaer, NY) and 2,7-dichlorofluorescein diacetate (DCF) from Eastman Kodak Co. (Rochester, NY). Collagenase, Type II, 176 U/mg, was obtained from Worthington Biochemical Corp. (Freehold, NJ). Gemfibrozil, clofibric acid, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Hepatocyte Isolation

Hepatocytes were isolated by collagenase perfusion based on the procedure for rodents (Seglen, 1973) and modified for fish (Moon et al., 1985; Lipsky et al., 1986; Maitre et al., 1986; Kocal et al., 1988). To facilitate the perfusion technique, the perfusion was not done *in situ*. Trout were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co.), placed on a surgical board, and incised from the urogenital pore to the gills. The bile was removed from the gall bladder with a 1-cc tuberculin syringe. The liver was then separated from

the gall bladder and transferred to a sterile petri dish. The sinus venosus was cannulated by using a blunt 18 gauge needle and ligated. Initially the liver was perfused with 10 mM HEPES buffer, pH 7.4, containing 136.9 mM NaCl, 3.4 mM KCl, 0.4 mM KH_2PO_4, 4.1 mM $NaHCO_3$, 5.5 mM glucose, and 0.1 mM EGTA. Perfusion with the same buffer followed, but without EGTA, and containing 1 mg/ml collagenase (Type II, Worthington Biochemical Corp.) and 5.1 mM $CaCl_2$. During perfusion with the dissociating solutions, the liver was gently massaged to increase both blood clearance and hepatocyte yield (Moon et al., 1985). At the end of the perfusion, the liver was washed with HEPES buffer, and the hepatocyes were dissociated by using a sterile pipet and forceps. The hepatocytes were passed sequentially through sterile 500, 250, 150, and 75 gauge Nitex screening (Tetko, Inc., Elmsford, NY). The cell suspension was then centrifuged for 2 minutes at 35 x g. The pellet was resuspended in culture medium and centrifuged for 2 minutes at 35 x g. The final pellet was resuspended in culture medium. Viability was determined by trypan blue exclusion test, and only preparations with 95% viability were utilized.

Selection of Doses

The effect of vehicle control and doses were determined from a preliminary screening assay. Those doses that induced a response (increase in FACO activity) without causing severe cytotoxicity (<85% viability, as judged by trypan blue exclusion) were chosen for the final experiment.

Culture Conditions

Hepatocytes were seeded at 1×10^6 viable cells per 5 ml of culture medium in 60-mm petri dishes and incubated at 15°C in 100% air. After 5-6 hours, treatment was commenced by replacing the culture medium with medium containing test compound dissolved in dimethyl sulfoxide (DMSO, final concentration 0.4% v/v). Every 24 hours thereafter, the medium was removed, and the cultures were treated again. Four culture dishes per treatment level were used. Vehicle control cultures were exposed to 0.4% DMSO (v/v).

As an indicator of retained function and hepatic differentiation, lactate dehydrogenase (LDH) leakage due to cytochrome P-450 activation by acetaminophen (Parker et al., 1981; Kocal et al., 1988) was assayed using a Sigma LDH assay kit at 25°C (Wroblewski and LaDue, 1955). Trypan blue exclusion was also used as an indicator of viability, which remained at 85 ± 5% throughout the experiment.

Acyl-CoA Oxidase Activity

At the end of 48 hours, cell monolayers were detached from culture dishes by means of a rubber policeman and centrifuged 4 minutes at 35 × g. Supernatant was discarded and pellet resuspended in 10% sucrose, 3 mM imidazole (SI) buffer. In order to solubilize the cells, Triton X-100 was added at a final concentration of 1%. Suspensions sat on ice for 10 minutes followed by centrifugation for 10 minutes at 6,000 × g. Supernatant was saved for analysis.

Fatty acyl-CoA oxidase (FACO) activity was measured spectrophotometrically (Small et al., 1985). Activity was quantified as nanomoles DCF oxidized/min/mg protein at 30°C. Protein was determined by the BCA Protein Assay (Pierce Chemical Co., Rockford, IL).

Peroxisomal Bifunctional Enzyme

Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gels (Laemmli, 1970), stained with Gelcode color silver stain (Pierce Chemical Co., kit #24597), and scanned by a MicroScan 1000 gel scanning system (Technology Resources, Inc., Nashville, TN). Densitometric results are reported in terms of percent protein present in the band containing PBE to total protein in the sample gel lane.

Statistical Analysis

Data were analyzed by ANOVA and Dunnett's test for multiple comparisons between treated groups and controls (Dunnett, 1955). Multiple regression analysis was performed to detect any linear trends.

RESULTS

LDH Assay

The results obtained in the LDH assay showed that hepatic metabolic activity was retained up to 48 hours. The acetaminophen-treated cultures released 48.0% LDH, which was comparable to the 48.9% reported by Kocal et al. (1988).

FACO Activity

All three agents produced dose-related increased in FACO activity, although statistically significant increases were only seen with ciprofibrate and clofibric acid (Table 1). No statistical differences were seen between the untreated controls and the vehicle controls. The highest FACO activity (2.5-fold over vehicle control) was seen at 2.75 mM clofibric acid. The decrease in activity at the highest dose corresponded to an increase in cytotoxicity as judged by trypan blue

Table 1. Effect of Gemfibrozil (Gem), Ciprofibrate (Cipro), and Clofibric Acid (Clof) on Peroxisomal Acyl-CoA Oxidase Activity (FACO) and Induction of PBE in Primary Cultures of Rainbow Trout Hepatocytes

Treatment	FACO[a]	% Vehicle Control	PBE[b]	% Vehicle Control
Vehicle Control	2.11 ± 0.27	100	2.83 ± 0.13	100
0.25 mM Gem	1.99 ± 0.48	94	2.23 ± 0.11	79
0.50 mM Gem	2.07 ± 0.20	98	2.40 ± 0.10	85
0.75 mM Gem	2.37 ± 0.27	112	3.00 ± 0.19	106
1.00 mM Gem	2.52 ± 0.36	119	2.63 ± 0.15	93
1.25 mM Gem	2.66 ± 0.33	126	2.80 ± 0.30	99
Vehicle Control	2.23 ± 0.14	100	2.70 ± 0.23	100
0.25 mM Cipro	2.49 ± 0.57	112	2.63 ± 0.18	97
0.50 mM Cipro	2.70 ± 0.57	121	2.98 ± 0.11	110
0.75 mM Cipro	2.49 ± 0.35	112	3.10 ± 0.30	115
1.00 mM Cipro	3.32 ± 0.26	149[c]	3.13 ± 0.29	116
Vehicle Control	1.08 ± 0.17	100	4.40 ± 0.05	100
2.25 mM Clof	1.56 ± 0.16	146	4.78 ± 0.46	109
2.50 mM Clof	1.91 ± 0.20	177	3.83 ± 0.38	87
2.75 mM Clof	2.66 ± 0.38	247[c]	5.18 ± 0.43	118
3.00 mM Clof	2.30 ± 0.48	213[c]	6.08 ± 0.56	138[c]

[a]Values = mean ± SE nanomoles of DCF oxidized/min/mg protein, N = 2-4 plates.
[b]Values = mean ± SE relative percentage protein, N = 2-4 plates.
[c]Statistically significant from respective control, $p < 0.05$.

staining (data not provided). Testing for a linear trend of the data revealed that the gemfibrozil data was not significant ($p = 0.0822$, $r^2 = 0.1310$), whereas the regression models for the ciprofibrate ($p = 0.0447$, $r^2 = 0.2060$) and clofibric acid ($p = 0.0018$, $r^2 = 0.378$) results were significant.

PBE Analysis

The densitometric analysis of PBE (Table 1) revealed modest increases in the relative percentages of PBE protein. Positive correlations (gemfibrozil = 0.9186, ciprofibrate = 0.9955, clofibric acid = 0.9945) were obtained with all three agents at increasing doses.

DISCUSSION

Treatment of rainbow trout hepatocytes with gemfibrozil, ciprofibrate, and clofibric acid induced peroxisomal β-oxidation as measured by increased acyl-CoA oxidase activity at 48 hours. This is correlated with an increase in the relative percent of PBE protein as determined by SDS-PAGE, supporting the conclusion that the hypolipidemic drugs induced a pleiotropic response in rainbow trout hepatocytes similar to that seen in mammalian models. Furthermore, the increases in FACO activity seen in this experiment are of the same order of magnitude as those seen *in vivo* in the trout (Yang, 1989; Yang et al., 1990). Increases in FACO activity of 1.4- and 1.8-fold over control were seen in trout following treatment with gemfibrozil (152 mg/kg/day for 2 weeks) or clofibrate (75 mg/kg every other day for 3 or 4 weeks), and a 1.8-fold increase was found using ciprofibrate (35 mg/kg every other day for 2 or 3 weeks). The direct cytotoxicity of the hypolipidemic drugs in culture prevented the use of the *in vivo* dose levels.

The large variation in the results may be due to interindividual variation between fish (Sellenger and Hazel, 1982; Klaunig, 1984). This high amount of variability is similar to that seen in rodent *in vitro* studies (Gray et al., 1983; Mitchell et al., 1984). It is also possible that intrahepatic factors, which could be retained in cell culture, were primarily responsible for the observed variability (Lake et al., 1986).

It appears from the present results and from previous *in vivo* work (Yang, 1989; Yang et al., 1990) that the rainbow trout is a weak responder to peroxisome-proliferating stimuli. This limited response is similar to that seen in mammals such as marmosets (Holloway et al., 1982), monkeys, dogs (Foxworthy et al., 1990), and humans (Stott, 1988), which are all weak responders when compared with the rodent (Reddy et al., 1973; Gray et al., 1983; Mitchell et al., 1984; Elcombe and Mitchell, 1986; Foxworthy and Eacho, 1986; Eacho et al., 1989; Foxworthy et al., 1990).

Finally, although this *in vitro* system was used to investigate peroxisome proliferation, it is not limited to this application and provides a necessary complement to mammalian cell culture techniques.

CONCLUSION

Treatment of primary cultures of rainbow trout hepatocytes with the peroxisome proliferators gemfibrozil, ciprofibrate, and clofibric acid induced peroxisomal β-oxidation as measured by increased acyl-CoA oxidase activity and PBE quantity at 48 hours.

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DIETARY REFINEMENTS IN A SENSITIVE FISH LIVER TUMOR MODEL: COMPARISON OF HEPATIC NEOPLASM FREQUENCY IN MEDAKA FED A PURIFIED CASEIN-BASED OR CONVENTIONAL DIET

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ABSTRACT

A recently developed purified casein-based diet (PC diet) for maintenance of medaka (*Oryzias latipes*) was used in comparison studies with a commonly employed commercial flake diet to determine suitability of the purified ration for use in carcinogenicity studies. From day 1 after hatch, fish were fed either the PC diet or a commercial flake ration supplemented with 2 days of *Artemia* sp. each week (F/A diet). At 21 days of age, both dietary groups of fish were exposed separately to aqueous solutions of 350 ppm (nominal) of diethylnitrosamine (DEN) for 48 hours. This regimen has been shown to yield tumors of the liver after a period of latency usually extending for 6 to 9 months. The use of this design afforded an opportunity to compare two dietary regimens over a period of time that would include initiation, promotion, and progression of hepatic neoplasms. Foci of cellular alteration were first seen after month 1. After month 3, initial neoplasms were observed, and hepatic tumors were found in animals fed each diet. At 259 days after initiation of exposure, and after one of the groups had shown a 50% incidence of tumors, all remaining fish were sampled and their livers processed for histopathology. Fish fed the F/A diet had a higher incidence of hepatocellular foci (58 basophilic, 54 eosinophilic, and 23 clear cells) than did fish fed the PC diet (30, 39, and 15, respectively). Also, incidence of hepatocellular neoplasms was higher (73 versus 47) in the former than in the latter. The PC diet yielded sufficient tumors for statistical analysis and, given its interbatch consistency, is recommended for further use. Growth of DEN-exposed fish was similar regardless of the diet fed. Depression of growth over the course of the study indicated that DEN, and not the diets, was the major factor responsible for weight differences.

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INTRODUCTION

The medaka (*Oryzias latipes*), orange-red or golden variety, is a small aquarium fish that is receiving increasing attention as a vertebrate test organism for rapid and thorough *in vivo* screening of suspect carcinogens (Braunbeck et al., in press; Laurén et al., 1990). In addition, because many of the important commercial fish species are annual breeders and their eggs and early life stages are only available for brief periods of time, medaka are finding use as surrogate monitors of environmental pollution in aquatic field surveys (Fabacher et al., 1991; Marty et al., 1990). Medaka are tolerant to a wide range of environmental conditions, including salinities and temperature, and their small size and rapid maturation support uncomplicated breeding and maintenance of large numbers in laboratories (Aoki and Matsudaira, 1977; Egami et al., 1981; Kyono and Egami, 1977). For the careful analysis of primary neoplasms and their metastatic sites, the small size of the medaka is providing a cost-effective means for screening all important visceral organs. Given the potential importance of medaka as a vertebrate model, the need exists for a nutritionally adequate and consistent experimental diet for this species (Bailey et al., 1984; Consensus Committee, 1984). Our laboratory has developed a purified casein-based diet (PC diet) for the medaka (DeKoven et al., 1992). The overall nutritional adequacy of the PC diet for medaka was evaluated and compared with three diets. The PC diet supported good survival, growth, reproduction, and normal histology. This diet provides a standardized, nutritionally adequate and consistent alternative to undefined conventional diets and is less likely to contain the range of xenobiotics possible in whole, live food (DeKoven et al., 1992). The following investigation was undertaken to determine the suitability of the PC diet for use during carcinogenesis studies. However, since mammalian carcinogenesis studies have shown that diet is one of the major causes of variation in tumor promotion studies (Pitot et al., 1989), it is entirely possible that the time to tumor as well as the tumor frequency will vary as a function of the diet. To determine whether the PC diet is suitable as a maintenance ration for medaka during carcinogenesis bioassay, we exposed young medaka (21 days after hatch) to an aqueous solution of 350 ppm of diethylnitrosamine (DEN) for 48 hours. Two groups of fish (cohorts from a group of medaka maintained in our colony) were used. Beginning with the day of hatch, each of the fish scheduled for use in this study were fed either the PC diet or a conventional ration currently used by other groups. The latter included a flake food (TetraMin®) from a commercial supplier to which *Artemia* sp. nauplii were supplemented by 2 days feeding per week. Fish from the respective diets were returned to their original diet after exposure. In this report, we detail our findings comparing tumor frequency in medaka fed the PC diet with those fed the flake plus *Artemia* (F/A) diet.

MATERIALS AND METHODS

Fish

Broodstock of golden variety medaka were maintained at 25°C under a 16L:8D photoperiod. Eggs from several females were collected, mechanically separated by digital manipulation of egg masses, and pooled and incubated in modified (no methylene blue), aerated, embryo-rearing media (Kirchen and West, 1976; Rugh, 1962) at 25 ± 1°C. Medium was replaced daily, at which time any white eggs with presumptively dead embryos were removed. Hatch took place at 9 to 10 days after fertilization. All fish were reared in a recirculation aquarium system using reconstituted water prepared following EPA guidelines (Horning and Weber, 1985) for moderately hard water. Reconstituted water was prepared in batches of 500 gallons using as a feed water produced by reverse osmosis. Water temperature was maintained at 25 ± 1°C. Ammonia and nitrite levels were monitored weekly and maintained at less than 0.1 ppm. Nitrates, pH, conductivity, and hardness were monitored weekly. Dissolved oxygen was maintained at or near saturation.

Newly hatched larvae with normal swimbladder inflation were randomly sorted and equally distributed among aquaria. To reduce bias in selection of fish, a 10-inch wide net was used to trap and concentrate approximately 100 fish. From this pool, and while the fish remained immersed, individuals were collected with a glass beaker and randomly assigned, in series, to the aquaria. The process was repeated serially until the desired number of fish was obtained for each aquarium. An assistant was given four identically sized, sealed envelopes of uniform appearance and asked to place one on each aquarium. A card, inside each envelope, designated the diet for fish within that aquarium. Fish were fed to slight excess twice daily, and tanks were siphoned to remove excess food and feces.

At 21 days of age, normal and healthy juveniles, of uniform size (500 fish per treatment), were selected as described above. In addition, 100 fish were selected from each diet for the control group at this time. Abnormal or weak fish were discarded. Selected fish, in labeled containers corresponding to diet group, were then transported to our exposure facility.

Exposure Design

Medaka from each diet group scheduled for exposure to DEN were placed in 10-liter glass aquaria (density = 50 fish/L) and exposed for 48 hours to an aqueous bath of 350 ppm DEN. Actual concentrations of DEN in exposure water were determined at 0, 12, and 24 hours. After this exposure, aquaria contents were replaced with fresh reconstituted water plus DEN (350 ppm). Similarly, DEN concentration was assayed immediately after mixing in the aquaria and at 36 and

48 hours. Except for DEN, control fish were subjected to the same conditions as the exposed fish and were placed in a glass aquarium with 2 liters of reconstituted water (density = 50 fish/L). The fish were then transferred to clean reconstituted water, and feeding with their respective previous diets was initiated. A brief duration was selected in order to ensure homogeneity of DEN concentration within aquaria. This regimen has been used in our laboratory to induce hepatocellular carcinoma in medaka. Aquarium water was tested for residual DEN. When none was detected, fish were transferred to the recirculating system in our rearing facility. Exposed fish were then placed in two 20-gallon aquaria, 250 fish per aquarium. Control fish were placed in one 10-gallon aquarium. Fish were allowed to grow out under the same conditions as before, including their previous diet regimens. Selection of rack and placement within racks for the four aquaria containing DEN-exposed fish and the two aquaria for control fish was done randomly using sealed envelopes as described above.

Quantification of DEN

Quantification of DEN was accomplished by spectrophotometric analysis at a wavelength of 230 nm (International Agency for Research on Cancer [IARC], 1982), of water taken directly from the aquaria. Standards were prepared with reconstituted water and ranged from 0.1 to 10 *ppm*. By direct comparison with gas chromatography, our previous work had shown that the spectrophotometric method has proven to be reliable and faster.

Diet

Particle sizes of the formulated rations were increased as the fish grew. From hatch to 4 weeks, fish were fed formulated rations ranging in particle diameter from 100 to 250 μm. After 4 weeks, the fish were fed particle sizes ranging in diameter from 250 to 850 μm. Newly hatched brine shrimp were separated from unhatched and empty cysts and rinsed with distilled water prior to being fed to fish (flake/*Artemia* [F/A] group only). The F/A group was fed flaked food 5 days per week and brine shrimp nauplii 2 days (Tuesday and Friday) each week. Fish in the other two aquaria were fed only the PC diet (DeKoven et al., 1992).

Sampling

Each month, fish were randomly sampled (10 per replicate, total of 20 per treatment-exposed fish; 4 per control group) for wet weight determinations and general histology. Collected fish were anesthetized in MS-222, placed in Bouin's fixative for 48 hours, dehydrated in graded alcohol solutions, and processed for paraffin embedment. Sections of paraffin-embedded

material were cut at 6 μm and stained with hematoxylin and eosin (H&E). Histologic analysis was performed to enumerate tinctorially altered foci, adenoma, cholangioma, hepatocellular carcinoma, cholangiocellular carcinoma, and mixed cell (both hepatocytes and biliary epithelial cells) tumors. The above histologic methods closely followed procedures that have been used in this lab in previous studies (Hinton et al., 1987; Laurén et al., 1990).

Statistical Analysis

Differences in mortality, frequency of tumors, and frequency of cellular alterations between the two diets were evaluated with Fisher's exact test (Sokal and Rohlf, 1981). Fish in the two replicate aquaria for each diet were pooled before the analysis was conducted. Fisher's exact test also was used to evaluate differences between sexes with regard to foci and tumors. Fish were pooled across diets for this analysis. Differences in growth (wet weight) between the two diets at termination were evaluated using student's *t* test (Sokal and Rohlf, 1981). Relationships between the frequencies of the different types of cellular alterations and tumor frequency was evaluated by Principal Components Analysis (PCA) (Sokal and Rohlf, 1981). Table 1 presents key features of the medaka classification system for foci and neoplasms, the principal lesions of this study. Only those lesions visible with H&E staining were enumerated.

RESULTS

DEN Concentrations

Actual mean aqueous exposure concentration was 250.8 ppm (Table 2) and 257.4 ppm for PC- and F/A diet-fed exposure groups of medaka. Means were determined from individual spectrophotometric assays at 12, 24, 36, and 48 hours. Statistical analysis (student's *t* test) revealed no significant differences between exposure concentrations ($p \leq 0.05$).

Growth

Data on growth of the fish are summarized in Table 3. Control fish fed the PC diet averaged 412 mg wet weight, and those fed the F/A diet averaged 510 mg wet weight. These differences were statistically significant ($p < 0.05$). Fish in the DEN treatments averaged 349 and 310 mg wet weight, respectively, for the PC and F/A diets. These weights were different from each other and also different from their respective controls.

Table 1. Classification of Liver Alterations in Hematoxylin and Eosin-Stained Liver Sections of Medaka Exposed to Diethylnitrosamine.

Foci of Cellular Alteration	Description
General Features	
Size	Varies from small collection of cells to large lesions occupying 30% of liver sectional area. Often multiple. Occasionally mixed characteristics.
Border	Distinct. Cells not surrounded by capsule. Shares architecture of surrounding parenchyma. One hepatic tubule may continue from adjacent (not involved parenchyma) into the focus.
Architecture	Same as surrounding parenchyma.
Cytology	Nuclei usually normal but may be enlarged. Cytoplasm usually normal, except for tinctorial properties.
Mitotic Figures	Data incomplete. Most foci do not reveal enhanced mitotic figures.
Specific Categories	
1. Basophilic	Normal-to-small hepatocytes with marked accentuation of cytoplasmic basophilia. Mitotic figures sometimes encountered.
2. Eosinophilic	Individual hepatocytes vary in size. Some are quite large. Abnormally large nuclei may be present. Eosinophilia predominates over cytoplasm. Typical hepatocytes contain homogeneous pink cytoplasm.
3. Clear Cell	Cells of clear cell foci reveal little to no staining. Companion serial sections are positive for glycogen (PAS^a method). Clear white nature of cytoplasm distinguishes these from the other two foci. Margin of clear space is irregular unlike vacuolated cells.

Table 1. Classification of Liver Alterations in Hematoxylin and Eosin-Stained Liver Sections of Medaka Exposed to Diethylnitrosamine (continued).

Foci of Cellular Alteration	Description
Neoplasms	
1. Hepatocellular Adenoma	Cells may stain basophilic, eosinophilic, or a mixture. Lesion presents as a distinct nodular mass with well-defined margin. Component cells are arranged as tubules. Number of cells in an individual tubular profile usually increased.
2. Hepatocellular Carcinoma	No clear margin present. Growth extensions into adjacent tissue are usually seen. Gradations from minimal deviation (well-differentiated) to maximal deviation (poorly differentiated) are seen. Cells may appear as sheets. Nuclei are typically altered and often frequent in number.
3. Cholangioma (Biliary Adenoma)	Enlarged and hyperplastic profiles of tubular epithelium. Nuclei show piling up and slight gradation from normal appearance. Lesion is confined within basal lamina of biliary ducts/ductules.
4. Cholangiocarcinoma	Cells are anaplastic. Abundant nuclear and cytoplasmic atypia. Growth by extension into liver parenchyma and/or into intestine or head kidney. Mass may dissect along hepatic veins into pericardium.
5. Other Tumors	
A. Mixed	Carcinomas of both hepatocellular and cholangiocellular components. Both portions show anaplastic features.
B. Spindle Cell	Component cells are attenuated and arranged as strata. Tumor is believed to be of perisinusoidal fat-storing (Ito) cell origin.

[a]Periodic Acid Schiff (PAS) reagent method for glycogen with diastase as a histochemical control.

Table 2. Actual Concentrations of Diethylnitrosamine (DEN) During Exposure

Time	DEN Exposure to Fish PC[a] Diet + DEN		DEN Exposure to Fish F/A[a] Diet + DEN		DEN Exposure to Fish Control PC and F/A Diet	
	Actual Conc[b] (ppm)	Nominal Conc (ppm)	Actual Conc (ppm)	Nominal Conc (ppm)	Actual Conc (ppm)	Nominal Conc (ppm)
0 hr	267	350	266.5	350	0	0
12 hr	248	350	255	350	0	0
24 hr	237	350	250.5	350	0	0
24 hr (new stock)	259	350	247.5	350	0	0
36 hr	239	350	267	350	0	0
48 hr	255	350	258	350	0	0
Average =	250.8 11.7[c]		257.4 8.1[c]			
24 hr postexp[d]	0		0		0	0
48 hr postexp	0		0		0	0

[a]Diets are described in the text: PC, purified casein based (DeKoven et al., 1992); F/A, commercial flake diet supplemented on 2 days/week with *Artemia* sp. nauplii.
[b]Samples were run in duplicate, values are mean of two samples. Conc, concentration.
[c]Values were not significantly different by student's *t* test ($p \leq 0.01$).
[d]Postexp, postexposure.

Table 3. Wet Weights of control and Diethylnitrosamine- (DEN)-Exposed Medaka Fed the Two Different Diets[a] for 280 Days

Treatment	Wet Weight (mg)	
	$\bar{x}$ + S.D.	*n*
F/A Control	510 ± 115[1]	56
PC Control	412 ± 161[2]	42
F/A + DEN	310 ± 90[3]	198
PC + DEN	349 ± 103[4]	168

[a]Diets: PC, purified casein based (DeKoven et al., 1992); F/A, commercial flake diet supplemented on 2 days/week with *Artemia* sp. nauplii.
[b]Treatments with different superscripts are different ($p < 0.05$).

As Figure 1 shows, control groups showed greater growth than their respective exposed groups throughout the exposure period and throughout the 259 additional days. Furthermore, the clustering of fish from the two exposed diet treatments further suggests that it was DEN and not the particular diet that was primarily responsible for the altered growth pattern. When compared to controls fed F/A diet, controls fed PC diet showed a trend toward greater growth during the 90- to 259-day period (Fig. 1).

Mortality

Two DEN-exposed fish, one from each diet group, died during the 48-hour exposure. No control deaths were recorded during that 48-hour period (Table 4a). Control mortality throughout the 259 days was 9% (PC) and 7% (F/A). Subsequently, 135 fish (27%) died in the group fed PC diet and exposed to DEN, and apparent lower mortality (98 fish, 20%) was seen in the group fed F/A diet and exposed to DEN (Table 4b). The histogram (Fig. 2) presents actual numbers of dead fish in individual grow-out aquaria. We observed no tank-specific effect. Mortality data demonstrate that fish fed either diet showed DEN-induced toxicity.

Histopathology

Control hepatic alterations were minimal throughout the study. After the initial 48 hours of the test, controls showed no lesions. After 1 month, a single PC diet-fed control fish showed focal[2] perivascular necrosis and spongiotic change resembling spongiosis hepatis. No lesions were seen in the F/A diet-fed control fish at 1 month. At 2 months, a single F/A diet-fed fish showed a single vacuolated focus. However, since we encountered vacuolated foci in both controls and treated fish at relatively high frequency, these alterations were not considered DEN specific. After 2 months, PC diet-fed control fish showed spongiotic change (spongiosis hepatis). After 3 months, PC diet-fed controls showed spongiotic change (3 of 4 medaka examined). F/A diet-fed controls showed no lesions at end of month 3. In the sampling after month 4, one F/A-fed control showed spongiosis hepatis.[3] The above changes have been illustrated previously

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[2]Foci of cellular alteration, as used herein, apply to eosinophilic, basophilic, and clear cell changes in medaka hepatocytes. The use of the term "focal" to designate a small, microscopic, histopathologic lesion is a different usage from foci of cellular alteration as applied to neoplasia. Therefore "focal spongiotic change" or "focal necrosis" designate different lesions than those of carcinogen-associated, neoplastic initiation and promotion.

[3]Spongiosis hepatis has been reported in carcinogen-exposed rats (Bannasch et al., 1981) and in medaka (Hinton et al., 1984*a*) and *Cyprinodon variegatus* (Couch, 1991). The lesion has also been reported at low incidence in control fish after several years.

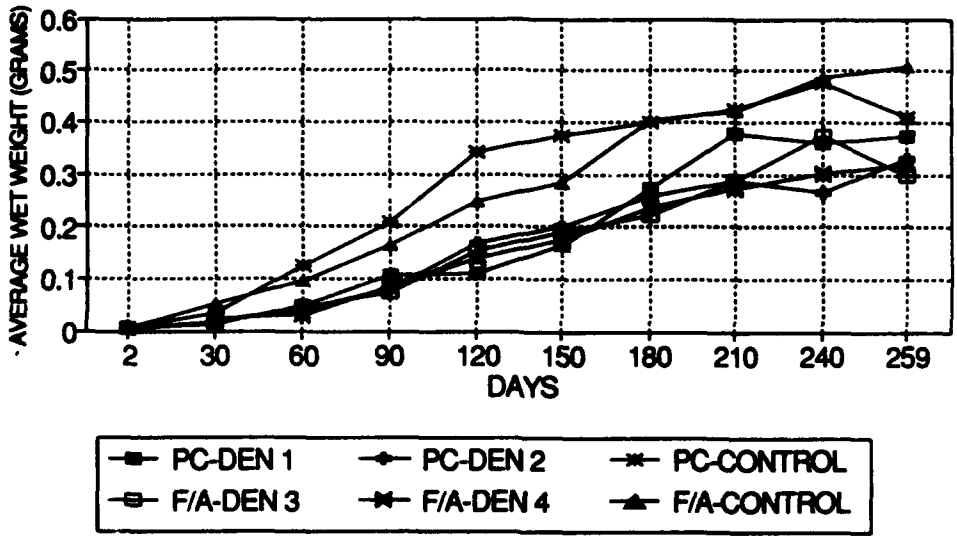


Figure 1. Effect of diet on individual wet weight of diethylnitrosamine-exposed medaka and controls fed purified casein (PC) diet or flake diet supplemented with *Artemia* nauplii (F/A diet).

(Laurén et al., 1990). Corresponding PC diet-fed controls were lesion-free. Except for the single eosinophilic focus (in PC diet-fed control at termination of study), all control livers from months 5 through 8 were free of tinctorially altered foci and neoplasms.

Alterations encountered after medaka were exposed to DEN included an early hepatotoxicity primarily directed at hepatocytes. Lesions of an early toxic nature characterized all DEN-exposed fish of this study. These alterations have been described in detail at both the light and electron microscopic levels (Braunbeck et al., in press; Laurén et al., 1990).

The foci and neoplasms of this study first appeared after 1 and 3 months, respectively. Regardless of the diet, DEN-exposed fish developed foci (eosinophilic, basophilic, and clear cell) and neoplasms, further supporting our earlier studies with this compound in medaka (Hinton, 1989; Hinton et al., 1988*b*; Hinton et al., 1985; Hinton et al., 1988*a*; Hinton et al., in press). Monthly samplings were conducted, and foci (Table 5) and neoplasms (Table 6) were enumerated in two, non-overlapping sections oriented to provide maximum surface area. Foci preceded neoplasms, suggesting similarity in progression between rodent (Farber, 1976; Pitot, 1983) and medaka (Hinton et al., 1988*b*) hepatocarcinogenesis. Of the medaka foci, basophilic and eosinophilic were generally regarded as the most relevant to tumorigenesis. The initial tumors were found in month 3 sampling, and neoplasms were found in both diet groups (Table 6).

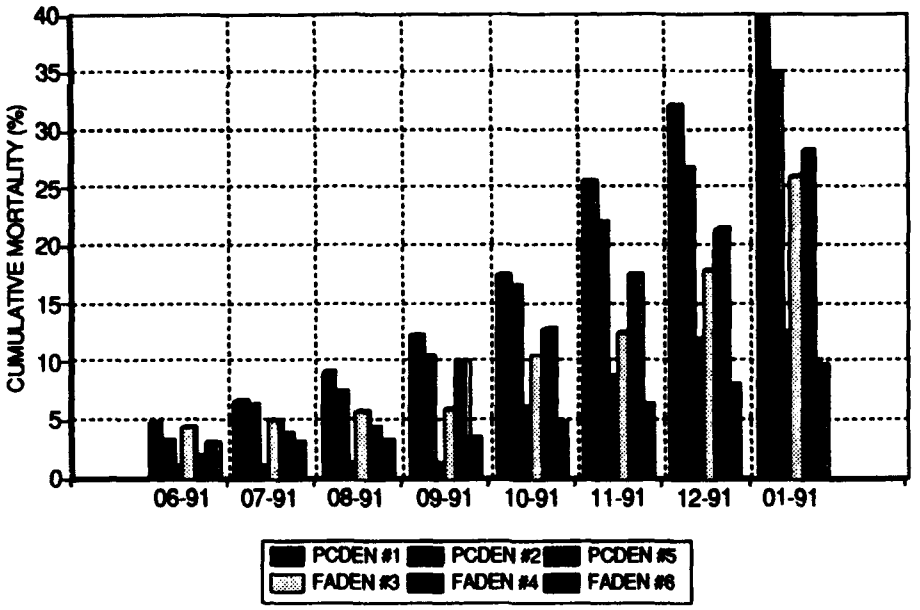
Examples from the monthly histologic examinations are included to illustrate features of foci and neoplasms. A basophilic focus found in a liver section from a PC-fed, DEN-treated, female

Table 4a. Mortality in Diethylnitrosamine- (DEN)-Exposed and Control Medaka

	Mortality, Fish Fed PC Diet		Mortality, Fish Fed F/A Diet	
Date,	Exposed	Control	Exposed	Control
04/29/91, *n*	0	0	0	0
04/30/91, *n*	1	0	1	0
05/01/91, *n*	0	0	0	0
	0.19% (N = 500)	0% (N = 100)	0.19% (N = 500)	0% (N = 100)

Table 4b. Monthly Mortality During Recovery From Diethylnitrosamine (DEN) Exposure

	Mortality, Fish Fed PC Diet			Mortality, Fish Fed F/A Diet		
Date	Exposed DEN #1	Exposed DEN #2	Control DEN #5	Exposed DEN #3	Exposed DEN #4	Control DEN #6
05/91, *n*	12	8	1	11	5	3
06/91, *n*	4	7	0	1	4	0
07/91, *n*	5	2	0	1	1	0
08/91, *n*	6	6	0	2	12	0
09/91, *n*	10	12	4	7	5	1
10/91, *n*	14	9	2	3	8	1
11/91, *n*	10	7	2	9	6	1
12/91, *n*	11	12	0	13	10	1
TOTAL	72	63	9	47	51	7
	27% (N = 500)		9% (N = 100)	20% (N = 500)		7% (N = 100)



	PCDEN #1	PCDEN #2	PCDEN #5	FADEN #3	FADEN #4	FADEN #6
06-91	4.8	3.2	1.0	4.4	2.0	3.0
07-91	6.7	6.2	1.0	5.0	3.8	3.1
08-91	9.1	7.4	1.1	5.6	4.3	3.3
09-91	12.3	10/4	1.1	5.9	10.0	3,4
10-91	17.6	16.7	6.0	10.5	12.9	4.8
11-91	25.5	22.0	8.8	12.5	17.5	6.2
12-91	32.1	26.8	11.8	17.9	21.6	7.9
01-91	40.0	35.0	12.5	26.1	28.3	9.7

Figure 2. Cumulative mortality of medaka in each individual aquarium after diethynitrosamine (DEN) exposure.

medaka at 6 months after onset of exposure is shown in Figure 3. The major difference between cells within the focus and their counterparts in the "noninvolved liver" is related to the staining within the cytoplasm. The architectural arrangement of surrounding liver and focus is nearly identical with the latter, perhaps showing slight enhancement of the tubular pattern (Fig. 3).

Figure 4 illustrates feaures of an eosinophilic focus. This particular focus was seen in the liver section of a female medaka fed the F/A diet for 6 months. Component hepatocytes of eosinophilic foci differ appreciably in size (Fig. 4).

Features of a clear cell focus from the liver of a female medaka at 5 months after the onset of exposure are seen in Figures 5 and 6. This fish was fed the PC diet. Cells of clear cell foci show the least staining over cytoplasm in H&E stains. They are followed by normal, glycogen-enriched cells and then by the cells of eosinophilic and basophilic foci. Abrupt margins where

Table 5. Number of Basophilic (B), Eosinophilic (E), and Clear Cell Foci (C) in Serial Monthly Samplings

Month	Number of Foci PC + DEN	Control	Number of Foci F/A + DEN	Control
1	B-1; C-4	0	B-5	0
2	E-2; C-2	0	E-3; C-2	0
3	B-2; C-4	0	B-1; C-2	0
4	B-5; E-3; C-9	0	B-1; E-4	0
5	E-1; C-3	0	E-6; C-2	0
6	B-3; E-5; C-1	0	E-2	0
7	B-8; E-7; C-2	0	B-4; E-4; C-14	0
8	B-8; E-2; C-5	0	B-11; E-13	0

Table 6. Effect of Diet on Frequency of Liver Neoplasms in Medaka (*Oryzias latipes*) Exposed to 350 ppm Diethylnitrosamine for 48 Hours

Months after Initiation	1	2	3	4	5	6	7	8
PC Diet[a]	0/20[b]	0/20	4/20	1/20	2/20	1/20	2/20	7/20
F/A Diet[a]	0/20	0/20	2/20	2/20	5/20	3/20	7/20	6/20
Control								
PC diet only	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
F/A diet only	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

[a]Diets: PC, purified casein based. Fed from day 1 of hatch through day 21 and daily after 48-hour bath exposure. F/A, Tetramin flake diet 5 days/week supplemented with *Artemia* nauplii on 2 days/week. Fed from day 1 of hatch through day 21 and daily after 48-hour bath exposure.

[b]Tumor-bearing medaka per number exposed. Neoplasms included adenoma, cholangioma, hepatocellular carcinoma, cholangiocellular carcinoma, mixed hepato- and cholangio-cellular carcinoma, and spindle cell tumor.

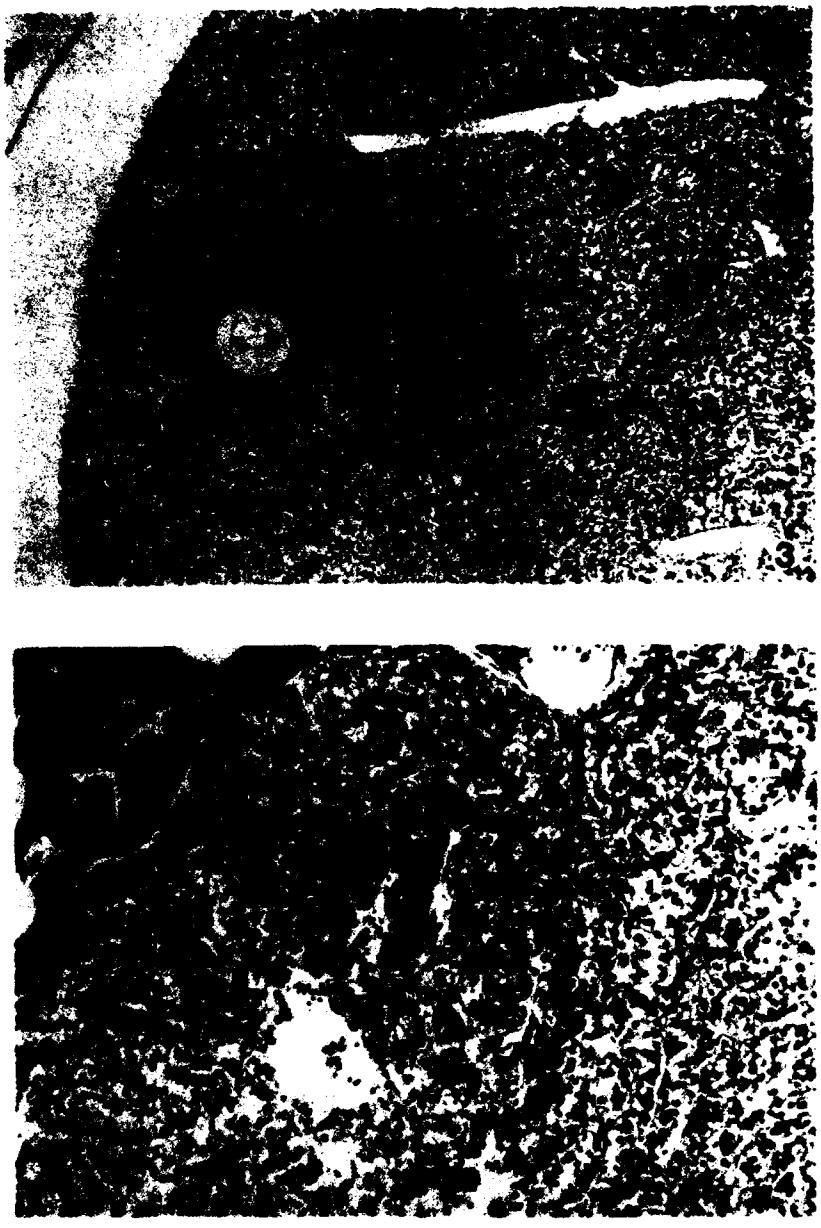


Figure 3. (top) This basophilic focus, of approximate lobular dimension, (325 μm diameter) contains dark-staining basophilic hepatocytes. Note similarity of cellular dimensions in focus and in surrounding hepatocytes. Tubular architectural pattern of hepatic parenchyma is enhanced in this focus. Female medaka fed the flake/*Artemia* diet and exposed for 48 hours to 350 ppm diethylnitrosamine (DEN). Fish was fixed at 6 months after onset of exposure. Hematoxylin and eosin stain, ×112.

Figure 4. (bottom) Eosinophilic focus deep within liver section shows some enlarged hepatocytes and others of near normal dimensions. Component cells of focus illustrate two variants, the granular eosinophilic cells (large arrows) and the eosinophilic cell with glycogen remnants (small arrows). The majority of the cells are hypertrophic, and multiple nucleated forms are seen. Female medaka fed the purified casein diet before and after a 48-hour bath exposure to 350 ppm DEN. Lesion was detected at 6 months after onset of exposure. Hematoxylin and eosin stain, ×225.

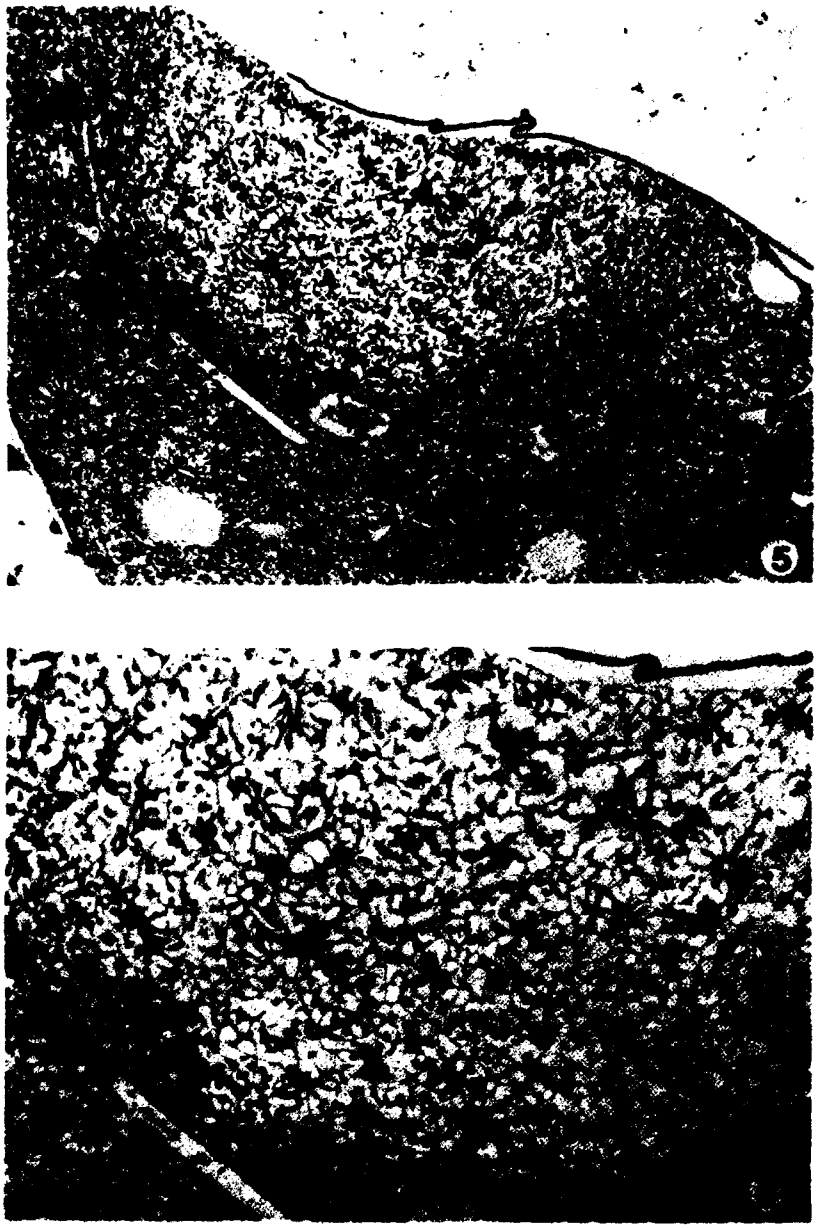


Figure 5. (top) Clear cell focus (arrows) at margin of liver section. Cells of foci appear larger than the surrounding cells. Histochemistry in prior companion studies shows glycogen in "clear" areas. Margin is indicated by arrows. This lesion appeared in the liver of a female medaka fed the purified casein diet. Fish was exposed for 48 hours to 350 ppm diethylnitrosamine (DEN), and lesion was detected at 5 months after initiation of exposure. Hematoxylin and eosin stain, ×112.

Figure 6. (bottom) Higher magnification view of the focus illustrated in Figure 10. Cells of focus are larger and stain less than adjacent cells at bottom of field (arrows). Within individual clear cells, observe absence of smooth, rounded margins characteristic of fat vacuoles. A light gray material (C) surrounds clear areas within clear cells. This represents remaining elements in cytoplasm which take up stain. See legend for Figure 10 or text for details of diet and exposure. Hematoxylin and eosin stain, ×225.

cells with focal-staining characteristics abut on cells with normal features characterize most foci (Figs. 3, 4, 5, 6).

A vacuolated focus is shown in Figure 7. This lesion was seen in the liver of a PC-fed, female fish at 7 months after the onset of exposure. By contrast with Figures 5 and 6, the large vacuoles of smooth outer contour and eccentric nuclei differ from the appearance of clear cells. Vacuolated cells are regarded as fat-filled hepatocytes. No diet-specific differences among a single category of foci were encountered.

Histopathologic analysis of a male medaka exposed to DEN for 48 hours (350 ppm) and fed the F/A diet revealed the presence of a cholangioma at 6 months after onset of exposure (Fig. 8). Architecture of the biliary passageways retained a differentiated state. However, the profiles of ducts are numerous in this lesion. In addition, the fairly typical nuclei are beginning to "pile up" in multiple rows. There is no evidence that the neoplasm has invaded the adjacent parenchyma.

A cholangiocarcinoma is shown in Figure 9. This neoplasm was found in the liver of a male medaka fed the F/A diet. The neoplasm was encountered at 4 months after the onset of exposure to DEN (48-hour bath at 350 ppm concentration). By contrast with Figure 11 (below), the cholangiocarcinoma has invaded the adjacent parenchyma. Both ductular and broad tubular or trabecular patterns are indicated. Numerous mitotic figures are present (Fig. 9).

A mixed hepato- and cholangio-cellular tumor is shown in Figure 10. This neoplasm was detected in the liver of a female medaka at 3 months after the onset of exposure to DEN. This particular neoplasm was predominantly cholangiocellular with hepatic parenchymal tubules between ductular elements. Elements of a solid hepatocellular carcinoma are shown in Figures 11 and 12. In the low magnification view (Fig. 11), the solid features and basophilic staining contrast with the remainder of the liver. Under higher magnification, nuclear pleomorphism and transformation of tubules into broad sheets of tumor cells are apparent (Fig. 12). This tumor was detected at 7 months after onset of exposure and was in the liver of a male medaka fed the F/A diet.

At 7 months after onset of exposure, a female medaka fed the F/A diet was shown to have developed a large hepatocellular carcinoma (Figs. 13 and 14). This lesion occupied the majority of the liver section and had a large necrotic component in its center (Figs. 13 and 14). Tumor also contained foci of spongiosis hepatis (Fig. 14).

Frequency of foci and neoplasms in the terminal (259-day) sampling are provided in Table 7. By design, the experiment was terminated when the monthly frequency indicated that 50% of the animals in either DEN group showed tumors. The data on cellular alterations and tumors in the

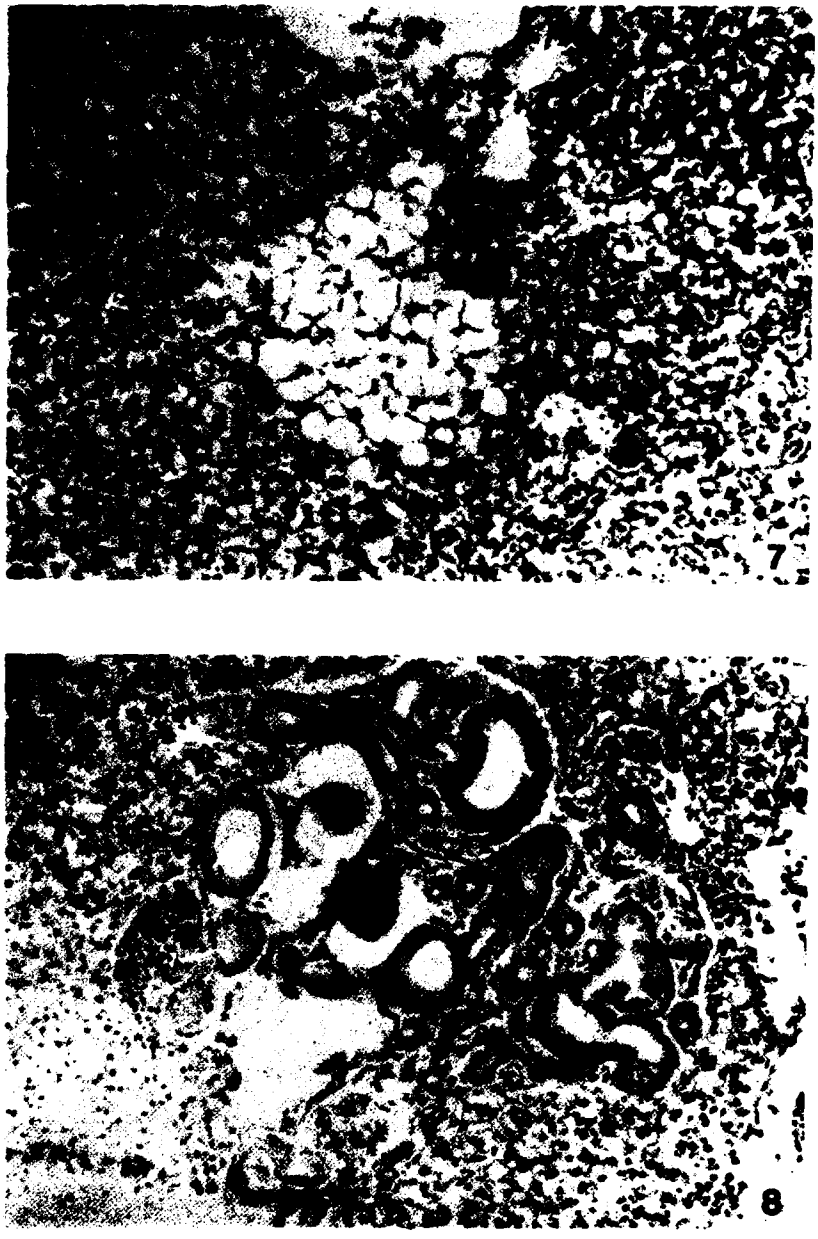


Figure 7. (top) Vacuolated focus reveals large vacuolar profiles with some confluence. Compare with Figs. 10 and 11 to observe differences in vacuolated and clear cell foci. Nuclei of cells within this focus show peripheral displacement. This type of focus is occasionally encountered in control fish but more often in fish exposed to diethylnitrosamine (DEN). This lesion was detected at 7 months after onset of exposure (48-hour bath, 350 ppm DEN). Fish was a female and was fed the purified casein diet. Hematoxylin and eosin stain, ×225.

Figure 8. (bottom) Cholangioma involving intrahepatic bile ducts. Columnar to cuboidal epithelial cells continue to form mural elements of biliary passageways; however, "piling up" of nuclei is seen. Early stages of nuclear atypia are indicated by large elongated nuclei (arrows). All epithelial cells continue to appear surrounded by their basal laminae, and no invasion of parenchyma is apparent. Male medaka fed the flake/*Artemia* diet and sampled at 6 months after onset of a 48-hour bath exposure to 350 ppm DEN. Hematoxylin and eosin stain, ×225.

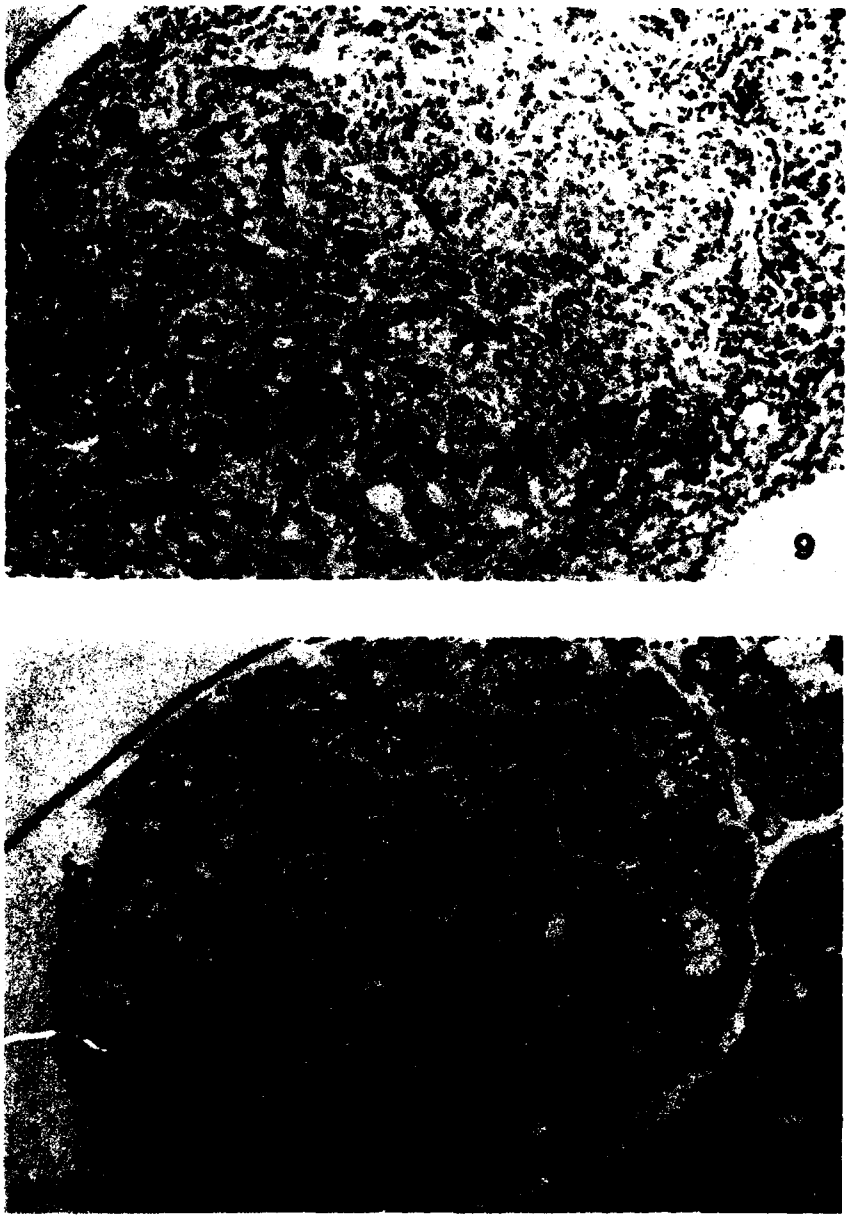


Figure 9. (top) Cholangiocarcinoma. Compare with Figure 13. Cells of carcinoma reveal a much higher incidence of pleomorphic nuclei. Cellular pattern is less like a duct and more solid, trabecular. Note the retention of duct-like structure at bottom of lesion. Cells within middle of lesion have proliferated until they appear as continuous sheets. At top left of field, the lesion shows invasion of adjacent hepatic parenchyma. Arrows point to mitotic figures. Male medaka fed flake/*Artemia* diet before and after a 48-hour bath exposure to 350 ppm diethylnitrosamine (DEN). Fish was sampled 4 months after onset of exposure. Hematoxylin and eosin stain, ×225.

Figure 10. (bottom) Mixed hepato- and cholangio-cellular carcinoma. Large, spherical lesion contains both ductule- and duct-resembling elements (cholangiocellular component). However, cells at the bottom of lesion and between ductlike structures resemble hepatocytes (arrows). Female medaka fed the purified casein diet before and after a 48-hour bath exposure to 350 ppm DEN. Fish was sampled at 3 months after onset of exposure. Hematoxylin and eosin stain, ×225.

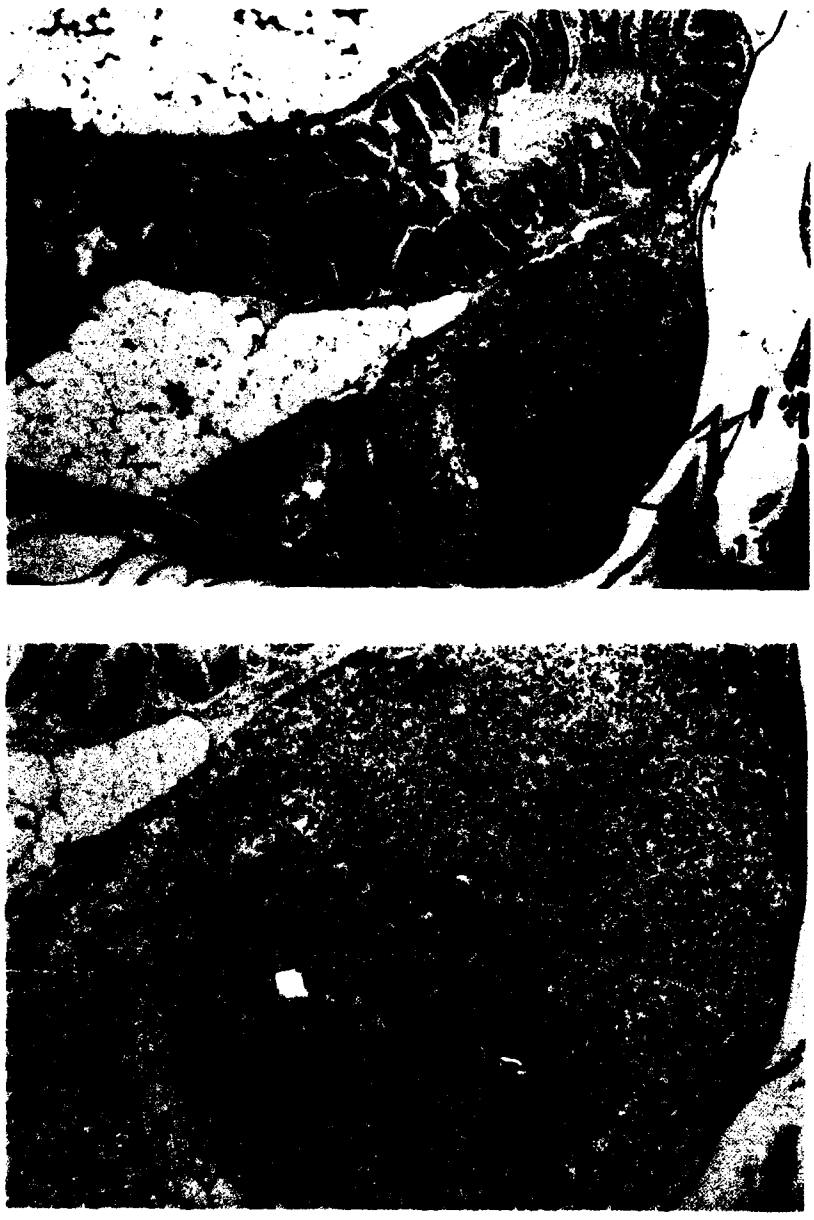


Figure 11. (top) Solid, trabecular pattern of hepatocellular carcinoma. Tumor occupies approximately 40% of section area. Note spongiotic lesions at left corner of liver section. Intestine (I) is at top of field and surrounded by fat-laden mesentery. Body wall is at bottom and right of field. Male medaka fed flake/*Artemia* diet prior to and after a 48-hour bath exposure to 350 ppm diethylnitrosamine (DEN). Sampling and fixation was at 7 months after onset of exposure. Hematoxylin and eosin stain, ×45.

Figure 12. (bottom) Enlarged view of hepatocellular carcinoma in Fig. 16. Superficial examination suggests solid sheet of cells. However, finer analysis shows enlarged and hypercellular hepatic tubules forming trabeculae, which are compressed together. Extension of the lesion into adjacent liver parenchyma is shown (arrows). Conditions of exposure and time of sampling are given in legend to Figure 16. Hematoxylin and eosin stain, ×112.

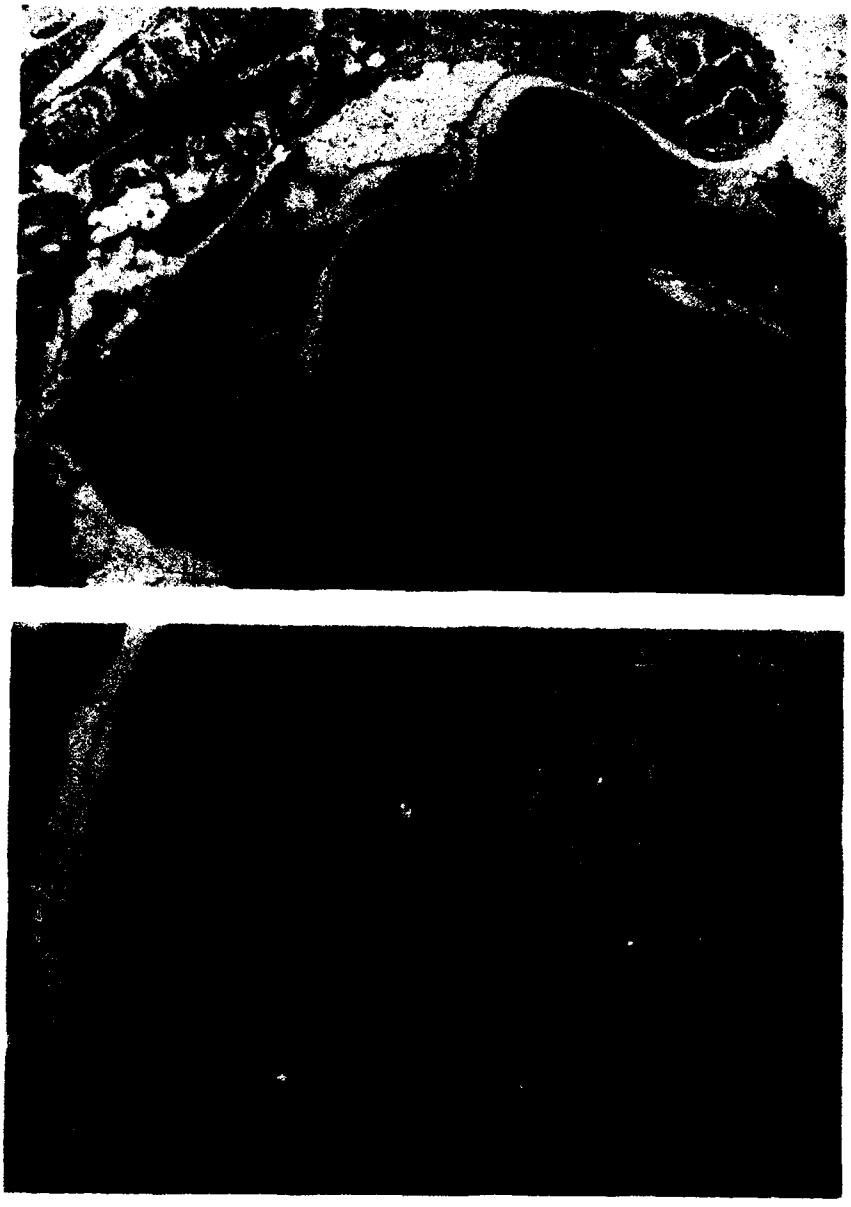


Figure 13. (top) Extremely large hepatocellular carcinoma occupying majority of liver section shows a central area of necrosis (N) and spongiosis hepatis (S). H, heart; E, esophagus; P, pharynx; I, intestinal bulb. Female medaka sampled at 7 months after onset of exposure to a bath of 350 ppm diethylnitrosamine (DEN) for 48 hours. Fish was fed flake/*Artemia* diet. Hematoxylin and eosin stain, ×45.

Figure 14. (bottom) Enlarged view of hepatocellular carcinoma shown in Figure 18. Note nuclear pleomorphism in tumor trabeculae. Necrotic areas (arrows) contrast with spongiosis hepatis (SH). Diet, sex, time of sampling, and conditions of exposure are in legend to Figure 18. Hematoxylin and eosin stain, ×112.

terminal sampling are presented in Table 7 for the DEN-exposed fish and the controls. An average of 26.7% of the fish fed the PC diet exhibited tumors at termination, compared with an average of 36.9% of fish fed the F/A diet. Statistical analysis of the tumor frequencies associated with the different diets indicates that they were different ($p < 0.05$). Significantly higher frequencies of basophilic and eosinophilic foci also were associated with the F/A diet. Conversely, the incidence of clear cell foci did not appear to be affected by the diet; incidences of 8.5 and 11.6% clear cell foci were noted in fish fed the PC and F/A diets, respectively.

Table 7. Focal Cellular Alterations and Tumors in Diethylnitrosamine- (DEN)-Exposed and Control Fish at 259 Days After Onset of Exposure

Number of Alterations by Exposure and Diet[a]	Basophilic Focus	Eosinophilic Focus	Clear Cell Focus	No. of Tumors
DEN-exposed fish				
PC ($n = 176$), n	30*	39*	15	47*
F/A ($n = 198$), n	58	54	23	73
Control				
PC ($n = 100$), n	0	1	0	0
F/A ($n = 100$), n	0	0	0	0

[a]Diets: PC, purified casein based (DeKoven et al., 1992); F/A, commercial flake diet supplemented with *Artemia* sp. nauplii.
*Significantly different between diets; $p < 0.05$.

A comparison of the frequencies of cellular alterations with the frequencies of tumors associated with the respective diets suggested that the frequencies of basophilic and eosinophilic foci might be more closely related to the observed frequencies of tumors than clear cell foci (see Table 7).

The correlation matrix for the PCA shown in Table 8 provides further insight into the potential relationship between different cellular alterations and tumors. Clearly, the frequency of eosinophilic foci was most closely related to the frequency of tumors. The correlation coefficient of 0.92 suggests that over 80% of the variation in tumor frequency can be explained on the basis of this one variable. The next highest correlation coefficient, 0.62, is associated with basophilic foci. However, this parameter explains only 38% of the variation in tumor frequency.

The distribution of foci and tumors among male and female fish is summarized in Table 9. The data, pooled across diets, show that tumors and all three types of foci were represented in

Table 8. Correlation Matrix of Cellular Alterations and Tumors in Diethylnitrosamine-(DEN)-Exposed and Control Fish at 259 Days After Onset of Exposure

	Tumors	Basophilic Focus	Eosinophilic Focus	Clear Cell Focus
Tumors	1			
Basophilic Focus	0.62	1		
Eosinophilic Focus	0.92	0.33	1	
Clear Cell Focus	0.26	0.57	0.28	1

*Significantly different between the two replicates; $p > 0.05$.

Table 9. Distribution of Foci and Tumors in Male and Female Medaka After 259 Days

Sex	Clear Cell	Basophilic	Eosinophilic	Tumors
Males (n = 184)	21	35	53	57
Females (n = 190)	17	53	40	63

both sexes. There were no statistically significant differences between the sexes for any of categories of foci or tumors.

DISCUSSION

There are no published guidelines for nomenclature of fish, specifically medaka, hepatic neoplasms and associated lesions. Rather, a collection of various terms and lesion descriptions exists, usually as a small section in each of the original papers. Rodent bioassays have adopted uniform criteria by which alterations are classified (Boorman et al., 1990; Maronpot et al., 1986). The development of such criteria for medaka, while facilitating uniformity of bioassay results, must reflect the breadth and nature of histopathologic alterations. The classification scheme herein reflects the tubular architecture and biliary epithelial cell localization arrangement as described in our previous papers. One of the authors (D.E.H.) has contributed a chapter on normal morphology and early fish hepatic alterations encountered after laboratory exposure to

carcinogens (Hinton, in press, *a*). In addition, this laboratory has contributed to the understanding of histopathologic biomarker lesions (Hinton, in press, *b*; Hinton et al., 1992; Hinton and Couch, 1984; Hinton et al., 1987; Hinton and Laurén, 1990*a*; Hinton and Laurén, 1990*b*; Hinton et al., 1984*b*). In response to needs expressed at the 1991 Histopathologic Workshop on Liver Lesions of Fishes Exposed to Carcinogens, sponsored by the United States Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, Florida, and at the recent National Toxicology Program meeting on Fish Carcinogenic Models, this report describes and illustrates medaka liver histopathology associated with DEN-induced carcinogens.

Eosinophilic foci might be useful in predicting the eventual presence of tumors or, the corollary, exposure to a tumor-causing substance. However, 1 of 100 control organisms also exhibited such a focus, even though no tumors were evident. This suggests that there may be a low background incidence of this alteration or that a weak carcinogen was present in the diet. Assuming that the former explanation is more likely, the estimated background incidence of this alteration was 1%. In order to be reasonably certain ($p < 0.05$) that such alterations were an indication of carcinogenesis, at least 7 organisms out of a sample of 100 would have to exhibit this trait. Alternatively, since none of the controls exhibited basophilic or clear cell foci, any combination of these foci that totaled 4 or more in a sample of 100 could also be considered indicative of tumors or exposure to a tumor-causing agent.

Collectively, these results suggest that, based on potential differences in growth and frequencies of foci and tumors, qualitative differences may exist between the two diets. However, the PC diet did support the survival and growth of the experimental fish, and treated fish fed the PC diet also produced significant quantities of foci and tumors. Because the PC diet is a purified diet, it would be more nutritionally consistent and less likely to contribute xenobiotics. These advantages may be significant in separating the effects of initiating and promoting agents in aquatic toxicology.

One of the needs that is apparent from this investigation is the lack of details on mitotic activity within specific lesions. To explore this area, it may be possible to adapt techniques featuring a proliferating cell nuclear antigen that has been used in mammals. Plans have been made to try both this antigen and a previously used monoclonal antibody against bromodeoxyuridine (BrdU). A recent paper by Moore (1991) indicates that the BrdU antibody technique is useful in determining the proliferative potential of different hepatic lesions. This work was done in the liver of winter flounder (*Pseudopleuronectes americanus*).

Although we did not list them on our table of focal alterations, the vacuolated cell foci are of interest. These were seen at fairly high frequency in both control and DEN-exposed fish. These

may be more analogous to some of the foci seen in rodent liver, which are fairly frequently found in both controls and treated animals (Pitot et al., 1989).

The mortality data indicate that more fish being fed the PC diet died during the course of the investigation than did those fed the F/A diet. Statistical evaluation to see whether these differences are real or not is in process. However, the actual numbers of difference were 135 total mortalities over the 259-day period in fish fed the PC diet versus 98 mortalities in the fish fed F/A diet.

Determining statistically whether the time to tumor was different as a function of diet is confounded by the possibility of a tank effect. In order to address this question experimentally, a more elaborate design is needed, employing perhaps a smaller number of fish in individual tanks with a much larger number of total tanks devoted to the study. In this way, tank effects can be controlled for and then of fish within the total population of a tank can serve, when randomly selected, as the sampling for each serially determined duration.

The total tumors in mortalities are now being determined, and these will be statistically figured in to derive the final number of tumors in the total population of fish fed the PC diet versus the F/A diet. The technique for performing enzyme histochemical analysis of focal cellular alterations was described recently (Teh and Hinton, in press). With this technique, it is possible to obtain information from conventional stains and enzyme histochemical procedures on serial sections. It will be very interesting in later work to determine multiple markers for given focal alterations. In this way, it may be possible to see whether the eosinophilic focus continues to be that focus that is more akin to eventual tumor formation. Using a battery of enzyme phenotypes, it will be possible to compare eosinophilic and basophilic foci for their relationship to subsequent neoplasms. At least some of the populations of the resultant neoplasms show phenotypic alterations akin to those seen in earlier foci processed for enzyme histochemistry (Hinton et al., in press).

Although no differences were observed in the incidence of hepatocellular foci in tumors in male and female medaka after 259 days, it is possible that sex-related differences may be encountered using other DEN regimens. For example, unpublished studies from this laboratory indicate that male and female differences with respect to hepatic mixed function oxidase activity are not seen at day 21. However, when sexually mature fish are examined, activity for various MFO enzymes is higher in male than in female fish. If exposure were delayed until male and female sexual differences in cytochrome 450-associated enzymes were encountered and the tumor and hepatocellular foci incidences established, sex-related differences may arise. However, these remain to be confirmed by experiment.

The PC diet has proved adequate as a single ration for medaka. The consistency of batches of the open-formula, purified diet now makes it possible for us to pursue modulatory effects of diet and environment on fish liver carcinogenesis.

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DEVELOPMENT OF CARCINOGENESIS BIOASSAY MODELS: RESPONSE OF SMALL FISH SPECIES TO VARIOUS CLASSES OF CARCINOGENS

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ABSTRACT

Studies were conducted dealing with the development of small fish carcinogenesis bioassay models using the medaka (*Oryzias latipes*) and the guppy (*Poecilia reticulata*). The specific aim was to identify certain classes of carcinogens that would induce neoplastic lesions in those model species. Bioassays were conducted on the following compounds: the halogenated hydrocarbons 1,1,2,2-tetrachloroethane (TeCE), chlorodibromomethane (CDBM), and vinylidene chloride (VDC), the heavy metal cadmium (Cd), the aromatic amines 2-acetylaminofluorene (AAF) and 4-aminobiphenyl (ABP), the nitrogen-substituted polyaromatic hydrocarbon dibenzocarbazole (DBC), the industrial waste acrylonitrile (AN), and the pharmaceutical methapyrilene (MP). Mechanisms of ethylene dibromide metabolism were examined in medaka and of acetylaminofluorene in medaka and guppy. Other carcinogen bioassay factors such as the occurrence of background and rare carcinogen-induced tumors were examined in both species.

INTRODUCTION

A measure of the usefulness of a carcinogenesis model is the range of compounds that induce neoplastic lesions in the model. This project was designed to facilitate the development of small fish carcinogenesis bioassay models, specifically the guppy and medaka, by identifying the specific chemicals representing classes of carcinogens that induce neoplasia in those species. This project expanded the classes of potential carcinogens, especially halogenated hydrocarbons, that were active in fish models. A strong carcinogenic response to short-chain halogenated hydrocarbons, such as ethylene dibromide and vinylidene chloride, contrasted with a lack of carcinogenicity of 1,1,2,2-tetrachloroethane and chlorodibromomethane. Also, the model carcinogenic aromatic amine, 2-acetylaminofluorene, was weakly carcinogenic in the fish models under the conditions of these tests.

Additional goals were to gain insight on carcinogenic mechanisms in the two species and to examine factors that affect the interpretation of the results of small fish carcinogenesis bioassays such as the occurrence of background and rare carcinogen-induced tumors. Confirmation of the diagnosis and documentation of the occurrence of background neoplasms are important in developing carcinogenesis bioassay models. In this project, considerable emphasis was placed on using this laboratory's large and developing histopathological data base to examine rarely occurring neoplasms and to distinguish between those that are spontaneous (background) and those that are probably carcinogen induced. Background neoplasms that have been analyzed in the medaka, which appears far more susceptible than the guppy to the development of background neoplasms, included thymic lymphoma, acinar cell carcinoma of the exocrine pancreas, germ cell neoplasms, and hepatocellular proliferative lesions. In the guppy, hepatocellular proliferative lesions and a single case of adenocarcinoma of the retinal pigment epithelium were analyzed.

MATERIALS AND METHODS

Table 1 summarizes the bioassays that were conducted in this project. They include: the halogenated hydrocarbons 1,1,2,2-tetrachloroethane (TeCE), chlorodibromomethane (CDBM), and vinylidene chloride (VDC), the heavy metal cadmium (Cd), the aromatic amines 2-acetylaminofluorene (AAF) and 4-aminobiphenyl (ABP), the nitrogen-substituted polyaromatic hydrocarbon dibenzocarbazole (DBC), the industrial waste acrylonitrile (AN), and the pharmaceutical methapyrilene (MP). Exposures of TeCE, CDBM, and VDC were conducted under flow-through conditions. For AAF, ABP, DBC, and MP exposures were conducted under static or static renewal conditions. Carcinogenicity of Cd and AN were examined using both intraperitoneal injection and static exposures in separate tests. Exposures to examine the carcinogenicity of ethylene dibromide (1,2-dibromoethane; DBE) were conducted under another project. In the present project, results were analyzed and biochemical studies of DBE carcinogenesis were undertaken in medaka. Studies on the metabolism of AAF related to carcinogenesis were conducted in medaka and guppy. Approaches to those studies are described with each individual study.

Table 1. Summary of Small Fish Carcinogenicity Bioassays

Test Compound	Species	Type Exposure
Tetrachloroethane	Medaka	Flow-through
Tetrachloroethane	Guppy	Flow-through
Vinylidene chloride	Medaka	Flow-through
Vinylidene chloride	Guppy	Flow-through
Chlorodibromomethane	Medaka	Flow-through
Chlorodibromomethane	Guppy	Flow-through
Cadmium	Medaka	Static/multiple
Acetylaminofluorene	Medaka	Static/multiple
Acetylaminofluorene	Guppy	Static/multiple
Cadmium	Medaka	IP injection
Cadmium	Guppy	IP injection
Acrylonitrile	Medaka	IP injection
Dibenzocarbazole	Medaka	Static/multiple
Methapyrilene	Guppy	Static/multiple
Methapyrilene	Medaka	Static/multiple
Acrylonitrile	Guppy	Static/multiple
Acrylonitrile	Medaka	Static/multiple
Aminobiphenyl	Medaka	Static/multiple
Aminobiphenyl	Medaka	Static/multiple
Aminobiphenyl	Guppy	Static/multiple

STUDY SUMMARIES

1. **Absence of carcinogenic effects in guppy and medaka following exposure to the halogenated hydrocarbons chlorodibromomethane and tetrachloroethane.**

1,1,2,2-Tetrachloroethane (TeCE) is a solvent used in cleaning processes and in the manufacture of paints, varnishes, and rust removers. Chlorodibromomethane (CDBM) occurs in municipal drinking waters as a result of the interaction of organic materials with byproducts of chlorination. TeCE and CDBM were shown to be carcinogenic when administered by gavage to

B6C3F1 mice but were not carcinogenic when administered to Fischer 344 rats. The compounds cause hepatic adenomas and carcinomas in female mice and adenomas only in male mice (Haseman et al., 1984). Because TeCE and CDBM are contaminants of ground supplies of drinking water, they are considered potential health threats and are on EPA's Priority List of Drinking Water Contaminants (U.S. EPA, 1988).

Carcinogenesis bioassays were conducted with TeCE and CDBM against the medaka and the guppy in flow-through exposures. After exposure to TeCE or CDBM for 3 months (concentrations of 4-10 mg/L intermittently or continuously), fish were allowed to grow-out in clean water for additional periods of 3, 6, or 9 months. Histopathological examination of specimens exposed to TeCE or CDBM did not reveal carcinogenic effects in the test fishes.

2. Carcinogenic effects of 1,2-dibromoethane on the medaka and changes in glutathione *S*-transferase activities.

1,2-Dibromoethane (DBE; ethylene dibromide) is a halogenated aliphatic hydrocarbon that has been used as a pesticide and gasoline additive and is of concern to humans because of potential industrial and environmental exposures (Brown, 1984; Hanson, 1984). In rodents, DBE induces neoplasms mainly at the site of exposure when administered chronically by gavage or inhalation (Weisburger, 1977; Olson et al., 1973; Wong et al., 1982). Although neoplastic lesions of the liver have been induced by DBE exposure (Wong et al., 1982; Moslen, 1984), the liver and other internal organs appear less sensitive than directly exposed tissues. In rodents, DBE has an unusual carcinogenic mechanism that utilizes what is normally a detoxification pathway, conjugation with glutathione, to form an electrophilic episulfonium ion species that alkylates DNA and initiates carcinogenesis (Guengerich et al., 1987).

The carcinogenicity of DBE was examined in the medaka. Medaka were 6 to 12 days old at the beginning of the exposures. DBE was administered to the fish in the ambient water continuously for 103 days in the low concentration group, 79 days in an intermediate concentration group, and intermittently once each week for 24 hours for 103 days in a high concentration group. Mean measured DBE concentrations in the ambient water were 0.133 mg/l, 6.20 mg/l, and 18.58 mg/l in the low, intermediate, and high concentration groups, respectively. The test incorporated two control groups, one of which was held inside and one outside the exposure apparatus. Samples were taken for histopathological examination at 24, 36, and 58 weeks from the beginning of exposure. The incidence of hepatic neoplastic lesions for both control groups from all three sampling periods was 0.33% (1/299). DBE was clearly carcinogenic to medaka in the intermediate and high concentration groups, causing neoplastic lesions of the

hepatic parenchyma, biliary ducts, and gall bladder. Hepatocellular lesions included persistent foci of cellular alteration, adenomas, and carcinomas. Biliary neoplasms were diagnosed as cholangiomas and cholangiocarcinomas. Neoplasms of the gall bladder epithelium appeared to be papillary adenomas and adenocarcinomas. Lesions similar to those seen in the gall bladder also occurred within the liver and probably arose from biliary ducts near their entrance into the gall bladder. An increased incidence of thymic lymphoma occurred in DBE-exposed specimens but probably was not related to DBE exposure. Medaka exposed to 1 ppm DBE for 2 to 5 weeks had elevated hepatic glutathione *S*-transferase activities, indicating induction of the pathway that results in formation of the reactive metabolite of DBE in mammalian models. Furthermore, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of hepatic cytosolic fractions of DBE-exposed medaka showed a pronounced increase in a band at 26,000 daltons, the expected position for glutathione *S*-transferase.

3. Induction of hepatic neoplasia in guppy and medaka by vinylidene chloride.

Vinylidene chloride (1,1-dichloroethylene; VC), an air and drinking water contaminant (U.S. EPA, 1982, 1986), is a monomer used to produce polyvinylchloride resins and plastic food wraps (Quast et al., 1986). Its potential for workplace and consumer exposure and its structural similarity to other compounds of environmental interest such as vinyl chloride, trichlorethylene, 1,2-dichloroethane, 1,2-dibromoethane, and tetrachloroethylene make VC a likely candidate for carcinogen bioassay. Recently, the International Agency for Research on Cancer has reevaluated VC and concluded that VC, along with trichloroethylene, belongs to the Group 3 animal carcinogens, meaning that there is limited evidence for the carcinogenicity of those compounds to animals (Van Duuren, 1989). The term "limited evidence" is applied because whereas 2-year bioassays of Fischer 344 rats and B6C3F1 mice did not result in significant increases in tumors of dosed compared with control animals (see Chu and Milman, 1981), an inhalation study on Swiss mice resulted in increased incidences of kidney tumors in the male mice (Maltoni et al., 1977*b*; Maltoni, 1977).

Carcinogenesis bioassays were conducted with VC using the medaka and the guppy in flow-through exposures. After fish were exposed to VC for 3 months to concentration of about 20-30 mg/L intermittently or continuously, they were allowed to grow-out in clean water for additional periods of 3, 6, or 9 months. Histopathological examination of specimens indicated that this compound was hepatocarcinogenic to both species. Although the incidences are in the range of 15-20%, which is considered a moderate carcinogenic response in these models, the lesions were histologically well advanced and would likely affect the health of the organisms.

4. Studies on the carcinogenicity and metabolism of the aromatic amine 2-acetylaminofluorene in the medaka and guppy.

The aromatic amines are a class of chemicals that include the carcinogens benzidine and aniline as well as 2-acetylaminofluorene (2-acetamidofluorene; *N*-2-fluorenylacetamide; 2-AAF). Although the carcinogenicity of 2-AAF in rodents is well known and it is widely used as a model carcinogen in initiation-promotion tests, its carcinogenicity has not been comprehensively tested in a fish model. Compared with some other commonly studied carcinogens such as diethylnitrosamine, methylazoxymethanol acetate, and several polycyclic aromatic hydrocarbons, few aromatic amines have been widely tested for carcinogenicity in small fish species. Some studies have indicated that aromatic amines are weakly to moderately carcinogenic in small fishes. For example, aminoazotoluene and 2-AAF induced liver neoplasms in medaka (Hatanaka et al., 1982) and guppies (Sato et al., 1973).

2-Acetylaminofluorene has been used as a model carcinogen to study mechanisms of initiation and promotion in rodents. For 2-AAF to be carcinogenic, it must be N-hydroxylated by a cytochrome P-450-dependent, microsomal-bound enzyme (Weisburger, 1989). Ring hydroxylation by another P-450 enzyme appears to be a detoxification step. These processes are not well understood in fish models although recent studies have shown that rainbow trout microsomes are capable of N-hydroxylating aniline and 4-chloroaniline (Dady et al., 1991). In this study, the metabolism of 2-AAF was examined in medaka and guppy, and it was found that the guppy was able to produce more of the carcinogenic metabolite of 2-AAF than was the medaka. Correspondingly, in parallel carcinogenicity studies of 2-AAF in the two species, the guppy developed hepatic neoplastic lesions sooner and in higher incidences than did the medaka.

5. Studies on the effects of cadmium on medaka and guppy.

The toxic heavy metal cadmium is widely distributed in nature and affects humans through occupational exposures such as those from smelters, through food consumption mainly in the form of contaminated seafood, and through tobacco use (Kazantzis, 1987). Absorbed cadmium is eventually bound to a low-molecular-weight, metal-binding protein, metallothionein. Metallothionein-bound cadmium accumulates mainly in kidney proximal tubular cells. Cadmium is associated with unusual patterns of carcinogenesis. Following subcutaneous injection in rats, it induces injection-site sarcomas (mainly fibrosarcomas and rhabdomyosarcomas) as well as reproductive organ neoplasms in males (Haddow et al., 1961; Kazantzis and Hanbury, 1966; Lucis et al., 1972). In bioassays, however, cadmium administered in drinking water, in the diet, or by gavage was not carcinogenic in rats (Loser, 1980).

Medaka were exposed for various periods of time to waterborne cadmium at near toxic levels. The rationale was that this type of exposure would combine skin contact exposure, epithelial (gill) uptake exposure, and possible enteric exposure through consumption of the cadmium-containing water. Cadmium exposure was not associated with increased carcinogenesis in medaka in this bioassay. Similarly, when the study was repeated using intraperitoneal injection as the route of exposure for both medaka and guppy, cadmium also was not carcinogenic.

6. Studies on the effects of acrylonitrile on medaka and guppy.

Acrylonitrile is a bulk industrial chemical used primarily in the synthesis of acrylic and modacrylyic fibers for clothing and home furnishing and for the production of various resins. Acrylonitrile is also used as an absorbent, an anti-stall additive to gasoline, a fumigant for stored tobacco, for flour milling and bakery food processing equipment, and in pesticides. As an industrial chemical, acrylonitrile is widely produced, transported, and utilized in many countries. According to IARC (1979), acrylonitrile not only occurs in work places but also in the air near industrial production and processing sites, in rivers as effluent from chemical and manufacturing plants, and thus as trace amounts in drinking water. Acrylonitrile has also been found as a contaminant of fish and shellfish, and in food treated with acrylonitrile-containing fumigants. Acrylonitrile also occurs in food containers and packaging materials, and it may contaminate the stored materials.

Epidemiological studies of workers exposed to acrylonitrile showed excesses in colon and lung cancers (Finklea, 1977). Results of toxicological and metabolic studies, however, suggest that acrylonitrile does not fit the criteria of a typical carcinogen. It is mutagenic in some assays (Rabello-Gay and Ahmed, 1980; Milvy and Wolff, 1977) and appears to bind covalently with nucleic acids (see Farooqui and Ahmed, 1983). In rats, acute exposure to acrylonitrile has been shown to cause adrenocortical necrosis (Szabo et al., 1982) but not hepatotoxicity (Silver et al., 1982). Several bioassays to determine the carcinogenicity of acrylonitrile have been conducted on rodents. Maltoni et al. (1977*a*) exposed Sprague-Dawley rats to acrylonitrile by inhalation (up to 60 ppm, 5 days weekly, 52 weeks) and detected an increase in different types of tumors, most noticeably gliomas in the brain. In a parallel study in which acrylonitrile was administered by stomach tube (olive oil carrier, 5 mg/kg, once daily, 3 times weekly, 52 weeks), no carcinogenic effects were detected. Gallagher et al. (1988) exposed male Sprague-Dawley rats to up to 500 ppm acrylonitrile in drinking water for 2 years and observed that tumors of Zymbal's gland occurred in a dose-related fashion. A trend toward the development of forestomach papillomas was noted in rats receiving the highest concentration of acrylonitrile. Fischer 344 rats administered

acrylonitrile in the drinking water for 12 to 18 months developed primary brain tumors that were difficult to classify (Bigner et al., 1986). In this project, studies were conducted to determine whether multiple short-term (pulse) waterborne exposures to acrylonitrile induced neoplastic lesions in medaka and guppies. Medaka and guppies were exposed to approximately 35 ppm acrylonitrile for 24 hours once a week for up to four exposures. Histologic examination of specimens at 24 weeks after the initial exposure revealed no neoplastic lesions. At the present time, therefore, acrylonitrile does not show evidence of carcinogenicity under the exposure and evaluation conditions of this study.

7. **Studies on the effects of methapyrilene in the medaka and guppy.**

Methapyrilene is an antihistaminic drug that was widely used as an over-the-counter sleep aid before it was shown to be hepatocarcinogenic to rats (Connors, 1984). In rodent carcinogenicity studies, methapyrilene incorporated into the diet and administered over 64 weeks induced hepatic neoplasms (hepatocellular carcinomas and cholangiocarcinomas) in nearly 100% of the exposed rats, and about one-half of them developed metastatic lesions (Lijinsky et al., 1980). Methapyrilene represents a class of carcinogens that possesses methylamino and dimethylamino groups that can be metabolized to carcinogenic intermediates. That class incudes both carcinogenic and noncarcinogenic analgesics, some antipsychotic drugs, diazepam, and methylene blue (Connors, 1984).

Methapyrilene is no longer manufactured. Dr. W. Lijinsky (BRI-Basic Research Program, Chemical Carcinogenesis Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland) supplied the compound for these tests.

Medaka and guppies were exposed to a single nominal concentration (about 2 mg/L methapyrilene), determined not to be lethally toxic in preliminary range-finding tests. Specimens were administered methapyrilene in one, two, or four 24-hour exposures at 1 week intervals. Several methapyrilene-exposed medaka examined 1 week postexposure showed severe gill necrosis, which might account for some of the unexpected toxicity of this compound. Examination of 70 specimens from the high-exposure (× 4) group at 24 weeks postexposure revealed no hepatocellular neoplastic lesions or any other type neoplasm. Methapyrilene showed no evidence of carcinogenicity in the medaka under the conditions of this test.

Some methapyrilene-exposed guppies, examined histologically at 3 weeks postexposure revealed lesions that suggested hepatotoxic effects. Histopathological examination of 70 specimens from the high-exposure (× 4) group, however, did not reveal any hepatocellular neoplastic lesions or any other type of neoplasm. Under the conditions of this test, methapyrilene did not show

evidence of carcinogenicity to the guppy. The early hepatotoxic effects, especially in the guppy, however, suggest that hepatocarcinogenesis may develop at some later time period.

8. Studies on the effects of dibenzocarbazole on the medaka.

7H-Dibenzo[*c,g*]carbazole (DBC), an N-heterocyclic compound, is an environmental pollutant that is associated with combustion-related processes. It is found in cigarette smoke, soots and tars, wood smoke, diesel exhaust, and synthetic fuel material. DBC is a potent carcinogen in mouse tissues and, compared with other carcinogenic polynuclear aromatic hydrocarbons such as benzo[*a*]pyrene, is hepatocarcinogenic in mice (Schurdak and Randerath, 1989). A variety of N-heterocyclic compounds have been found in industrially polluted sediments in the Puget Sound, Washington, and have been implicated in hepatocarcinogenesis in bottom-dwelling English sole associated with the contaminated sediments (Malins et al., 1982). Although N-heterocyclic analogues of PAH occur in concentrations 1 to 3 orders of magnitude less than that of benzo[*a*]pyrene, their apparently high carcinogenic potency coupled with their expected increase in the environment make them important compounds for study (Santodonato et al., 1981; IARC, 1972). In this study, a carcinogenesis bioassay was conducted with DBC on the medaka. Although the study failed to demonstrate DBC carcinogenicity in the medaka, methodological approaches were established to examine this kind of highly insoluble environmental polycyclic aromatic compound in small fish carcinogenesis systems.

9. Studies on the effects of 4-aminobiphenyl on the medaka and guppy.

4-Aminobiphenyl (4-ABP) is an aromatic amine that has been shown to cause bladder cancer in humans. Production of the carcinogenic metabolite depends on N-hydroxylation, a process that occurs in both medaka and guppies as described above with another aromatic amine, 2-acetylaminofluorene. Additional studies with this compound were planned to further examine the carcinogenicity of aromatic amines in small fish models, as well as to determine whether carcinogenicity of this compound, which affects only the kidney in mammals, would be expressed in another site in the fish models.

Medaka and guppies were administered 4-ABP for 24 hours for one, two, or four times at weekly intervals. No evidence was detected of exposure-related toxicity in specimens examined at the end of their exposure periods. Also, it did not appear that 4-aminobiphenyl induced neoplastic lesions in the medaka or guppy examined 24 weeks after the beginning of the exposure periods.

10. Spontaneous hepatic proliferative lesions in designated control specimens of medaka and guppy from carcinogenicity tests at the Gulf Coast Research Laboratory.

Small fish species are useful as carcinogenesis bioassay models because of their low incidence of spontaneous neoplasms (Dawe and Couch, 1984). Incidences of spontaneous neoplasms in control specimens at the Gulf Coast Research Laboratory of medaka and guppies support this view. Several studies on spontaneous neoplasms from those two species at the laboratory already have been published. In medaka, they include reports on thymic (lymphoblastic) lymphoma (Battalora et al., 1991) and pancreatic acinar cell carcinoma (Hawkins et al., 1991), and an adenocarcinoma of the retinal pigment epithelium in a guppy (Fournie et al., 1992). The laboratory's histologic archives also contain a few examples of what seem to be proliferative lesions in guppy swim bladders. Summaries of studies on proliferative lesions in the liver of medaka and guppies and on germ cell neoplasms in medaka are presented below.

Few spontaneous neoplasms of the liver, the most frequent site of neoplasia following carcinogen exposure, or from other organs in medaka (Masahito et al., 1989) or guppy been reported. In this study, the incidences of hepatocellular proliferative lesions (altered foci, adenomas and carcinomas) and biliary proliferative lesions (cholangiomas and cholangiocarcinomas) are reported in control specimens of medaka and guppy from carcinogenesis bioassays. Although the diagnostic criteria used here have been applied in other studies on the carcinogenic responses of small fish to several carcinogenic compounds (Hawkins et al., 1988, 1989, 1990), a conventional scheme of diagnosis and nomenclature has not been firmly established and accepted for neoplastic lesions in small fish.

Data represent approximately 4,000 medaka and 1,000 guppies used as designated control specimens in carcinogenesis bioassays of various compounds. Diagnoses were made from hematoxylin- and eosin-stained sections of specimens examined at approximately 24, 36, and 52 weeks of age for the presence of hepatocellular lesions including foci of cellular (staining) alteration (altered foci), adenoma, and carcinoma and biliary lesions including cholangioma and cholangiocarcinoma. Incidences of the hepatocellular lesions were low for both species. For example, the highest incidence recorded for medaka was 0.16% (3/1877) for altered foci from the 36-week sample and for guppy was 1.3% for carcinomas in the 36-week sample. Similarly, biliary neoplastic lesions were also low, with none being diagnosed in either species. By comparison, in Fischer 344 rats used in carcinogenesis bioassays, the spontaneous incidence of foci of cellular alteration is about 80% by 9 months and 100% by 15 months (Maronpot et al., 1989).

This study substantiates the low rate of occurrence of altered foci in control medaka and guppies. The low number of bioassay-relevant spontaneous neoplastic lesions in control medaka and guppy enhances the sensitivity of the species to weakly carcinogenic test compounds and heightens the statistical power of the small fish carcinogenesis tests. The lack of sanctioned diagnostic criteria for hepatocellular proliferative lesions in small fish, however, impedes the development of studies aimed at estimating carcinogen potency or defining dose-response curves.

11. Background neoplasms in medaka: germ cell neoplasms.

Neoplasms of reproductive tissues, especially those involving germ cells (spermatogonia and oogonia), are rare in fishes (Leatherland et al., in press). Spermatocytic seminoma is a type of seminoma that is characterized by stages of spermatogenesis. Germ cell neoplasms have been diagnosed in fishes. These include seminomas and a single case of dysgerminoma in largemouth bass (*Micropterus salmoides*) and Japanese dace (*Tribolodon hakonensis*) and a single case of dysgerminoma in a largemouth bass (Masahito et al., 1984*b*). Single cases of spermatocytic seminoma have been reported in African lungfishes (*Protopterus aethiopicus*) (Masahito et al., 1984*a*), *P. annectens* (Nigrelli and Jakowska, 1953), and *P. dolloi* (Hubbard and Fletcher, 1985) and a pike eel (*Muraenesox cinereus*) (Honma, 1976, cited in Leatherland et al., in press). Down and Leatherland (1989) reported numerous cases of proliferative conditions in reproductive elements that they considered to be seminomas and spermatocytic seminomas in carp-goldfish hybrids.

From these studies, 24 cases of germ cell neoplasms were identified from approximately 10,000 specimens of medaka used in carcinogenesis tests. The neoplasms resembled spermatocytic seminomas and occurred in both female and male specimens. Histologically, components of the neoplasms resembled the cellular stages of spermatogenesis and included primary spermatocytes, secondary spermatocytes, and spermatids but not mature spermatozoa. Many of the neoplasms, even ones in males, contained scattered cells that resembled oocytes. The occurrence of the neoplasms appeared related to age but not to chemical exposure as they occurred in both control specimens and in specimens exposed to a variety of carcinogenic and noncarcinogenic chemicals. The fact that multiple cases of the neoplasm occurred in specimens from the same aquarium or in specimens from a particular study suggests that cultural, including viral, or genetic factors may affect the development of the lesions.

SUMMARY AND CONCLUSIONS

These studies provide information that supports the use of small fish species as carcinogenesis bioassay models. Purported attributes of low background rates of neoplasia, high sensitivity to certain known carcinogens, and ease of experimental manipulation were corroborated. Vinylidene chloride was shown to be carcinogenic in both the medaka and guppy, causing hepatic neoplasms in both species. Exposure to acetylaminofluorene resulted in the induction of hepatic neoplasms in both species but in higher incidences in the guppy than in the medaka. In studies of the metabolism of AAF by liver microsomes, the enhanced carcinogenicity of AAF in the guppy was related to the ability of the guppy to produce more of the N-hydroxylated metabolite, the carcinogenic metabolite, than the medaka which produced more of the ring-hydroxylated metabolites that result in detoxification. Studies on the hepatic metabolism of ethylene dibromide in the medaka suggested that the carcinogenic mechanism of this compound is similar to that in rodents and may depend on a phase II (detoxification) pathway to exert its carcinogenicity. Although the other compounds were negative in these tests in this laboratory, it can only be stated that these compounds showed no evidence of carcinogenicity in the fish models under the conditions of the tests rather than that these compounds should be considered noncarcinogenic in the test species.

Using the large histopathological data base developed in this project, these studies have shown the occurrence of spontaneous (background) neoplasms for the medaka and guppy. The rate of spontaneous hepatic neoplasms in designated control specimens was confirmed to be low, far less than one percent, in specimens examined at the critical sampling times of 24, 36, and 52 weeks. Other background neoplastic lesions that were identified in the more than 14,000 medaka examined histologically included thymic (lymphoblastic) lymphoma (about 25 cases), pancreatic acinar cell carcinoma (7 cases), and germ cell neoplasms (24 cases). Nonhepatic neoplasms occurring in the guppy from over 12,000 specimens include only a few individual cases, including an adenocarcinoma of the retinal pigment epithelium and a pigment cell neoplasm, and a few cases of swim bladder epithelial neoplasms.

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A SMALL FISH MODEL FOR ASSESSING CARCINOGENIC RISK AT LOW CARCINOGEN CONCENTRATIONS: PRELIMINARY INVESTIGATIONS

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ABSTRACT

Understanding the dose-response curve at low carcinogen concentrations is important for estimating practical risks of exposure to carcinogenic compounds. Describing that curve, however, is difficult using traditional test models because of the expense, time, and facilities required to produce and maintain the extremely large numbers of specimens required. At our laboratory, studies are in progress to examine the carcinogenic response in a small fish, the medaka (*Oryzias latipes*), exposed to low concentrations of *N*-nitrosodiethylamine (DEN, CAS 55-18-5). Results are being analyzed from preliminary studies designed to establish a dose-response curve for DEN-induced lesions at incidences from more than 20% to about 1%. In two preliminary studies, one initiated with 6-day-old fish and the other with 52-day-old fish, medaka were exposed under flow-through conditions to 0, 2.5, 5.0, 10, and 20 mg/liter DEN for 28 days, removed to clean water, and sampled for histopathological examination at 2 weeks and 1, 2, 4, 6, and 9 months after initiation of exposure. The results of the preliminary studies will be used to design a 30,000+ specimen study that will attempt to establish the carcinogen dose-response curve at and below the 1% incidence level.

INTRODUCTION

In conventional animal models, empirical determination of the dose-response curve at low carcinogen concentrations is generally precluded by the requirement for an exceptionally large number of animals. Instead, risk at low carcinogen doses is estimated through linear extrapolation of effects expressed at high doses, a procedure that may inaccurately predict risk.

The medaka, *Oryzias latipes*, is a small freshwater killifish native to areas of Japan, Taiwan, and southeastern Asia. Studies conducted at several laboratories over about the past 15 years indicate that the medaka could be used in large-scale, low-dose carcinogenicity bioassays. Here, we present a summary of preliminary studies with the medaka designed to assess the effect of dose and age at exposure on the carcinogenic response within the approximately 1% to 10% incidence range and to examine neoplasm induction and progression. The results of these studies will be used to design a definitive study to define the carcinogenic dose-response curve at and below the

1% incidence level. In this presentation we describe the experimental design of the preliminary investigations, present some preliminary findings, and discuss some aspects of the definitive studies.

APPROACH

Test Animal

The test animal for these studies was the medaka, *Oryzias latipes*. The medaka offers several advantages as a test animal including ease of breeding and maintaining in large numbers under laboratory conditions; sensitivity to a large number of known rodent carcinogens; low spontaneous tumor rate in all potential target organs; short latency to tumor formation; and apparent lack of difference in carcinogenic responsiveness between sexes (Masahito et al., 1988, 1989). Medaka brood stock were originally obtained from Carolina Biological about 12 years ago and have been continuously cultured at the Gulf Coast Research Laboratory since that time.

Test Chemical

The test chemical was *N*-nitrosodiethylamine (diethylnitrosamine, DEN, CAS 55-18-5). DEN was chosen because it is available in pure form; is a known carcinogen and produces characteristic tumors in medaka; is readily acceptable to the medaka; is stable in the exposure medium; and is easily quantified chemically. The DEN administered in these studies was provided by the Midwest Research Institute.

Experimental Design

Exposure and Grow-out

For the preliminary studies, two exposures were conducted, one initiated with 6-day-old medaka and the other with 52-day-old medaka. The experimental design for these studies is summarized in Figure 1. Fish were exposed for 28 days to nominal DEN concentrations of 0.0, 2.5, 5.0, 10, and 20 mg/liter under intermittent-flow conditions at 27 ± 1°C. Each treatment included four replicates of 150 fish each. To achieve the desired DEN test concentrations, a water partitioner delivered unamended well water to the exposure system, while simultaneously activating a series of Hamilton PLD-II precision liquid dispensing pumps that provided appropriate volumes of a DEN stock solution to each treatment. Following the exposure period, fish were transferred to aquaria containing toxicant-free well water and maintained under flow-through conditions for up to 8 months to allow for tumor development.

Histopathology

Samples of 100 fish per treatment were randomly taken for histopathological analysis at 2 weeks (during exposure) and at 1, 2, 4, 6, and 9 months post-initiation of exposure. Histopathological approaches are summarized in Figure 2. Sampled fish were anesthetized by immersion in tricaine methanesulfonate, fixed in Lillie's fixative, and embedded individually in paraffin blocks. Each fish specimen was cut through the midsagittal and midplane in 5-μm sections and stained with hematoxylin and eosin for pathological assessment. The liver, the target organ for DEN in medaka, served as the focus of pathological evaluations, but other organs were included in pathological descriptions.

RESULTS AND DISCUSSION

Exposure Concentrations

For the young (6-day-old medaka) fish test, mean measured DEN concentrations in the exposure groups were 2.43, 5.05, 10.1, and 20.8 mg/liter, with coefficients of variation ranging from 4.1% to 5.7%, as indicated in Figure 3. During the older (52-day-old medaka) fish test, mean measured DEN concentrations were 2.47, 4.71, 10.6, and 18.6 mg/liter. Measured concentrations and coefficients of variation are shown in Figure 4.

DEN Waste Treatment

A DEN effluent treatment strategy was devised specifically for this study. As the effluent stream exited the exposure chamber, it was amended with urea and pumped first through a series of three ultraviolet sterilizers, then through two primary activated charcoal filters, and finally through secondary charcoal filters. Passage through the UV system disrupted the DEN molecule, and some of the breakdown products were scavenged by the urea to prevent recombination. DEN concentration was reduced from about 7.5 mg/liter in the effluent stream to about 0.25 mg/liter, resulting in an approximate 97% destruction of the parent DEN. Passage through the initial charcoal filters resulted in an additional 10-fold reduction in DEN effluent concentration. We did not identify any DEN breakdown product(s).

Mortality

Distribution of fish specimens and mortality data are summarized in Table 1 for the young (6-day-old) fish study and in Table 2 for the older (52-day-old) fish study. Individual fish specimens were counted three times during the study: (1) at initial introduction into the exposure system, (2) at the time of transfer of fish from the exposure system into the grow-out system, and

EXPOSURE TYPE	28-day intermittent flow-through
TOXICANT DELIVERY	Water partitioner; Hamilton PLD-II dispensing pumps
NOMINAL CONCENTRATIONS	Control, 2.5, 5, 10, 20 mg/L with 4 replicates of each
NO. OF FISH AT EXPOSURE INITIATION	150/replicate 600/treatment
DILUTION WATER	Non-chlorinated well water
TEMPERATURE	27 ± 1°C
PHOTOPERIOD	16-h light : 8-h dark
FEEDING	Artemia nauplii; high-protein flake food
POST-EXPOSURE	8 months in flow-through grow-out tanks

Figure 1. Summary of Exposure/Grow-out

SAMPLING EVENTS	Time post-initiation of exposure: 2 weeks and 1, 2, 4, 6, and 9 months
NO. OF FISH SAMPLED PER SAMPLING EVENT	25/replicate 100/treatment
PROCESSING	Fixed in Lillie's. Individually embedded in paraffin blocks
SECTIONING	Cut through the mid-lateral and median plane in 5-μ sections. Stained with hematoxylin and eosin.
PATHOLOGICAL EVALUATION	Target organ is liver. Other organs included in descriptions.

Figure 2. Summary of Histopathology Approach

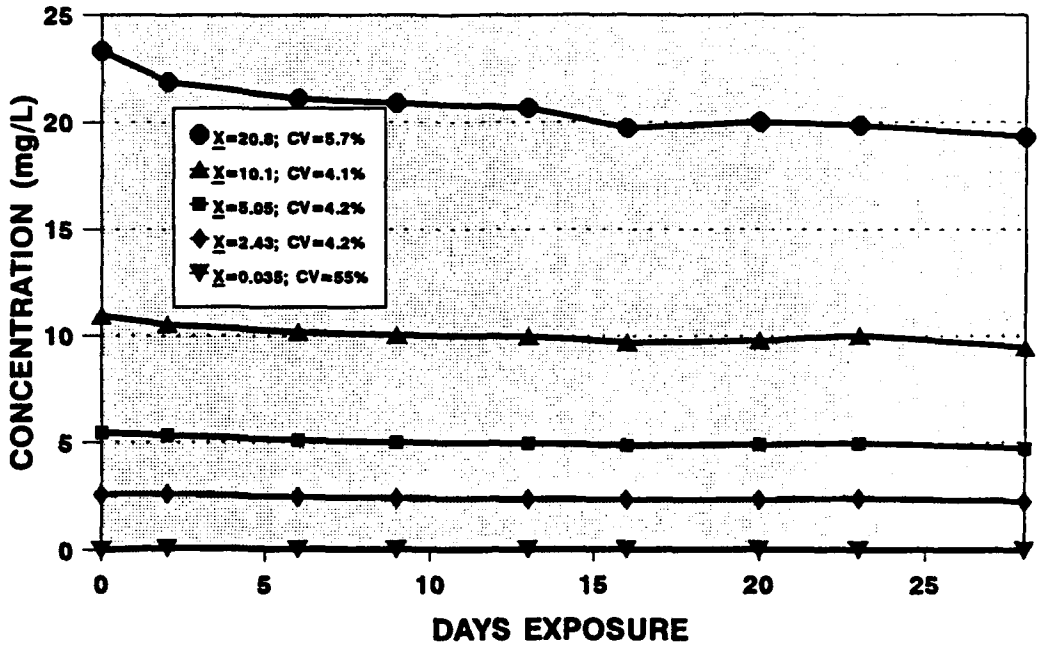

Figure 3. Measured DEN Concentrations FT-74/6-Day-Old Medaka

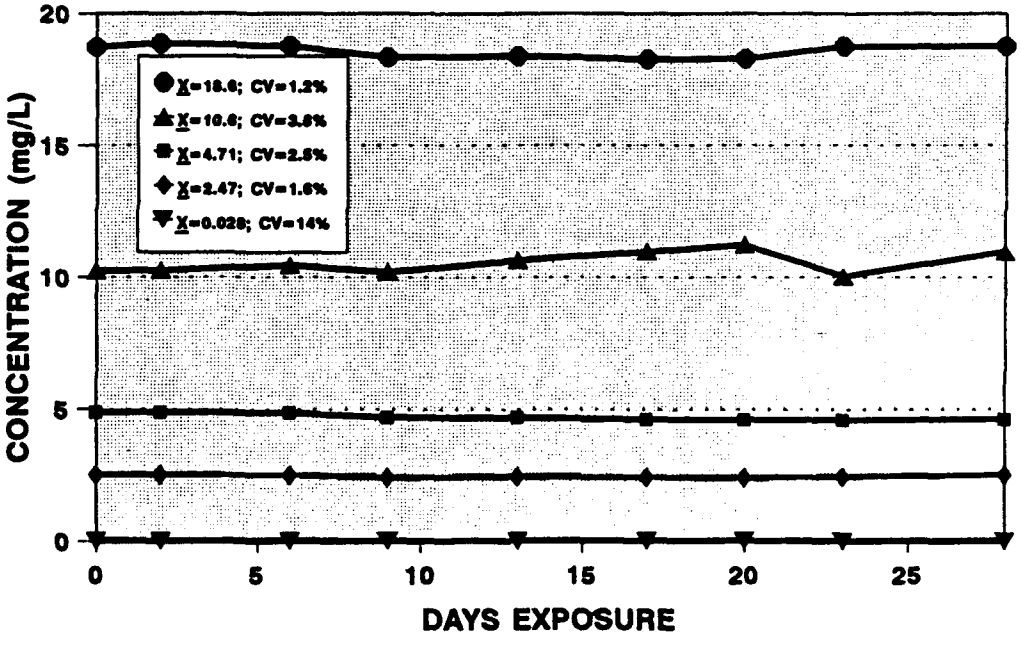

Figure 4. Measured DEN Concentrations FT-74/52-Day-Old Medaka

Table 1. FT-74/ DEN ; 6-day-old Medaka: Fate of Fish During Exposure and Grow-out

TREATMENT	Replicate	EXPOSURE						GROW-OUT								
		Tank #	N at Exposure Day 0	2-wk sample	4-wk sample	Dead Removals	Unaccounted for	Tank #	N at Growout Day 1	2-mo sample	4-mo sample	6-mo sample	9-mo sample	Dead Removals	Sampled Moribund Specimens	Unaccounted for
Control	A	2	150	25	25	4	-6	35	90	25	25	25	13	2	0	0
Control	B	13	150	25	25	4	-3	21	93	30[a]	25	25	11	2	0	0
Control	C	7	150	25	25	1	0	54	99	25	25	25	23	0	0	-1
Control	D	20	150	25	25	0	-4	13	96	30[a]	25	25	14	1	1	0
2.5ppm	A	12	150	25	25	7	-3	18	90	25	25	25	11	2	0	-2
2.5ppm	B	5	150	25	25	2	-4	48	94	25	25	25	19	0	0	0
2.5ppm	C	9	150	25	25	3	-5	9	92	25	25	25	17	1	0	+1
2.5ppm	D	19	150	25	25	1	-1	49	98	25	25	25	21	1	0	-1
5.0ppm	A	1	150	25	25	3	-5	42	92	25	25	25	14	2	0	-1
5.0ppm	B	4	150	25	25	2	-6	62	92	25	25	25	15	1	0	-1
5.0ppm	C	6	150	25	25	1	0	16	99	25	25	25	20	3	0	-1
5.0ppm	D	18	150	25	25	2	-5	17	93	25	25	25	18	0	0	0
10ppm	A	11	150	25	25	1	-2	14	97	25	25	25	14	6	1	-1
10ppm	B	15	150	25	25	2	-5	32	93	25	25	25	16	1	0	-1
10ppm	C	16	150	25	25	2	-3	58	95	25	25	25	14	4	0	-2
10ppm	D	17	150	25	25	2	-5	41	93	25	25	25	16	2	0	0
20ppm	A	3	150	25	25	2	-2	25	96	25	25	25	13	7	0	-1
20ppm	B	14	150	25	25	2	-2	11	98	25	25	25	18	4	0	-1
20ppm	C	8	150	25	25	5	-3	33	92	25	25	25	7	8	1	-1
20ppm	D	10	150	25	25	2	-5	60	93	25	25	25	16	2	0	-1[b]

[a] Sampling error

[b] This tank had an extra fish from FT-75/Tank 61.

Table 2. FT-75/ DEN / 52-day-old Medaka: Fate of Fish During Exposure and Grow-out

TREATMENT	Replicate	EXPOSURE						GROW-OUT								
		Tank #	N at Exposure Day 0	2-wk sample	4-wk sample	Dead Removals	Unaccounted for	Tank #	N at Growout Day 1	2-mo sample	4-mo sample	6-mo sample	9-mo sample	Dead Removals	Sampled Moribund Specimens	Unaccounted for
Control	A	1	150	25	25	1	0	39	99	25	25	25	24	0	0	0
Control	B	4	150	25	25	1	0	1	99	25	25	25	24	0	0	0
Control	C	15	150	25	25	0	0	52	100	25	25	25	25	0	0	0
Control	D	17	150	25	25	0	0	30	100	25	25	25	22	0	0	-3
2.5ppm	A	10	150	25	25	2	0	61	98	25	25	25	19	2	0	-2
2.5ppm	B	16	150	25	25	0	-1	6	99	25	25	25	23	1	0	0
2.5ppm	C	3	150	25	25	0	-1	43	99	25	25	25	23	1	0	0
2.5ppm	D	6	150	25	25	1	0	29	99	25	25	25	24	0	0	0
5.0ppm	A	11	150	25	25	0	0	7	100	25	25	25	23	2	0	0
5.0ppm	B	9	150	25	25	0	0	37	100	25	25	25	23	2	0	0
5.0ppm	C	5	150	25	25	0	0	63	100	25	25	25	23	2	0	0
5.0ppm	D	7	150	25	25	0	-5	26	95	25	25	25	17	2	1	0
10ppm	A	19	150	25	25	0	0	40	100	25	25	25	23	2	0	0
10ppm	B	12	150	25	25	0	0	56	100	25	25	25	22	1	0	-2
10ppm	C	14	150	25	25	1	-1	3	98	25	25	25	21	1	0	-1
10ppm	D	8	150	25	25	0	0	23	100	25	25	25	22	3	0	0
20ppm	A	18	150	25	25	1	-1	12	98	25	25	25	17	4	0	-2
20ppm	B	2	150	25	25	0	+1	20	101	25	25	25	23	3	0	0
20ppm	C	13	150	25	25	0	-5	57	95	25	25	25	19	1	0	0
20ppm	D	20	150	25	25	0	-1	34	99	25	25	25	20	1	0	-2[a]

[a] One fish from this tank lost to FT-74/Tank 60 during sampling.

(3) at the 9-month sample when all fish were removed from the grow-out aquaria. For the young fish test, cursory examination of data suggested that mortality patterns were not related to exposure group during the exposure phase, but were during the grow-out phase. Although mortalities were much less frequent in the older fish test, this pattern seemed to hold. In the young fish test, the number of specimens unaccounted for during the exposure period was much higher than for the older fish test. This disparity was likely related to the fact that 6-day-old medaka were very small and difficult to detect in tanks containing up to 150 specimens. Dead fish typically autolyze rapidly in warm water, and many of the small, dead specimens probably deteriorated between the daily observation periods. Some of the specimens in the young fish test died during the sampling from all of the tanks at 2 weeks postexposure initiation. Four moribund specimens, three from the young fish test and one from the older fish test, were accessioned and processed for histological evaluation.

Histopathological Observations

Histopathological analyses revealed that (1) few neoplasms occurred in control specimens and (2) carcinogenic target effects of DEN were limited to the liver. For the most part, we found that our processing, sectioning, and staining protocols were adequate. However, several problems arose that need to be corrected for subsequent studies. One sample of specimens in the young fish test, 4-month grow-out, were part of a batch involved in a tissue processor malfunction that prolonged their residence in xylene substitute. The effects were apparent mainly as severe fragmentation in sections of the liver. Nevertheless, we attempted to give a diagnosis for each of these and all other specimens, regardless of technical quality. Another condition that affected the liver was cracking, which probably resulted from overexposure to alcohol or to molten paraffin. We adapted our processing schedule, and this did not seem to be a problem in later samples.

Neoplastic Lesions

These preliminary studies reconfirmed the low rate of spontaneous (background) neoplastic lesions in medaka. From a total of 553 controls from both studies at 3 sampling times, only 5 neoplasms were found. In the young fish study, only one neoplasm was encountered, a case of thymic lymphoma in a specimen from the 6-month sample. The other four cases occurred in the 9-month sample of the older fish test. The lesions included a single case of esophageal carcinoma, a case of a lesion tentatively diagnosed as a mesothelioma of the peritoneum, and two cases of hepatocellular carcinoma.

Test-related increases occurred in the incidences of several types of hepatic neoplasms, including hepatocellular adenomas, hepatocellular carcinomas, biliary neoplasms, and hepatic hemangiopericytoma. These increases were related to (1) age at initiation of exposure; (2) DEN exposure concentration; and (3) time postexposure. Histopathological diagnoses and incidence levels are discussed in general terms here because the diagnoses are currently being reevaluated to refine the design of the definitive study.

The young fish exhibited higher incidences and shorter latencies for each type of DEN-induced neoplasm. Neoplasm incidences clearly increased with exposure concentration and time postexposure. At this time, we have not analyzed for sex-related neoplasm incidences.

The most sensitive dose-response was the induction of combined hepatocellular adenomas and carcinomas. With the exception of the 2.5 and 5.0 mg/liter DEN in the 4-month sample in both the young and the older fish tests, each successive exposure concentration caused an increase in combined incidences of adenomas and carcinomas. Incidences were as high as 85% in young fish exposed to 20 mg/liter DEN and sampled at 9 months.

Concentration- and time-related increases were also identified for biliary neoplasms (combined cholangiomas and cholangiocarcinomas) and for hepatic hemangiopericytoma. Although hepatic hemangiopericytoma has been reported in several small fish species, usually following exposure to high (probably cytotoxic) DEN concentrations, little is known about the progression of these lesions. We included what we believed were early stages of the lesion that appeared as mesenchymal proliferations. The decreasing incidences of hemangiopericytoma in the 9-month sample possibly suggests that we are dealing with two separate lesions, one a well-differentiated pericytoma, the other a poorly differentiated mesenchymal lesion.

Diagnostic Criteria for Hepatocellular Proliferative Lesions

A classification scheme for fish hepatic neoplasms is not yet available. Currently, most researchers apply diagnostic criteria developed for rodent neoplasms. We recognized three types of proliferative lesions originating from hepatocytes in the medaka, including the altered focus, hepatocellular adenoma, and hepatocellular carcinoma. A lesion that we tentatively denoted as an eosinophilic focus appeared more degenerative than proliferative and consisted of clustered hypertrophied hepatocytes containing bright eosinophilic material.

Altered foci were uniformly round lesions recognized by their increased staining, usually basophilic but sometimes eosinophilic, relative to surrounding tissues. The border of the altered focus tended to blend with the surrounding normal tissue at the periphery of the lesion with little or no disruption of parenchymal pattern in the focus itself. Cells of the foci were well

differentiated in form and retained their ability to store glycogen or fat similar to that of surrounding normal hepatocytes. Foci contained few mitotic figures and little cellular pleomorphism. In contrast with adenomas and carcinomas, foci appeared to contain other normal hepatic cellular constituents such as perisinusoidal cells and biliary elements, other than altered hepatocytes.

Like foci, adenomas were uniformly round. The determining criterion for distinguishing adenoma from focus was the distinct border that separated the adenoma from surrounding normal tissue, suggesting clonal expansion of the lesion. The parenchymal pattern in this lesion was thus distinct from that of surrounding tissue, but still usually only two cells thick. Some adenomas compressed adjacent tissues. Mitotic figures were occasionally observed and hepatocytes in adenomas tended to be pleomorphic, either larger or smaller than normal hepatocytes. Most lesions diagnosed as adenoma were basophilic. Hepatocytes of adenomas did not store glycogen or lipid to the same extent as normal tissues. However, two types of lesions categorized as adenomas were characterized by cells containing (1) large eosinophilic deposits and (2) small clear vacuoles resembling ground glass cells. Adenomas appeared to be composed principally of hepatocytes and were devoid of perisinusoidal cells or biliary profiles.

Hepatocellular carcinomas were usually basophilic, and lesions often occupied 25% or more of a liver section. In some specimens, carcinomas occupied the entire plane of section, making it difficult to distinguish well-differentiated carcinomas from normal liver. The borders of carcinomas were usually irregular, but the lesions were distinct from surrounding normal tissues. Cells at the periphery of carcinomas invaded or compressed adjacent normal tissues. In well-differentiated lesions in which parenchymal patterns were evident, hepatic cords were thickened with stacked neoplastic hepatocytes. The neoplastic hepatocytes of carcinomas stored no fat and contained no glycogen vacuoles. Some lesions diagnosed as carcinoma, however, were characterized by cells containing large eosinophilic vacuoles. Mitoses were easy to find in many carcinomas, especially the more anaplastic lesions. In contrast with well-differentiated lesions, cells and nuclei in anaplastic carcinomas were highly pleomorphic. Although most carcinomas usually exhibited a single cellular pattern, some lesions contained areas of dedifferentiated cells, possibly constituting "carcinoma within carcinoma." In high-dose specimens, it was not uncommon to find several types of carcinomas in a single specimen in the same plane of section. Whereas hepatocellular carcinomas generally consisted solely of cells that appeared to be hepatocytic in origin, some advanced lesions were juxtaposed to areas resembling cholangiocellular neoplasms, suggesting that the neoplastic hepatocytes have the capacity to differentiate into biliary

cells. Although there was little evidence of metastasis, in one case it appeared that a carcinoma had metastasized to the wall of the branchial chamber.

Pre- and Nonneoplastic Lesions

Correlates of three types of putative preneoplastic lesions in the liver were recognized, including basophilic foci, eosinophilic foci, and ground glass cell foci. Included in the counts of eosinophilic and ground glass foci were the occurrences of individual and clusters of the cell types that made up those foci. This was not the case for basophilic foci, which were always recognized as discrete lesions. Although not yet statistically analyzed, the incidence of each of these lesions generally appeared treatment-related for both tests. Incidences in controls were low, with only a few cases of eosinophilic foci/cells noted. Hepatic cytotoxicity/degeneration (both tests) and hepatic cell vacuolation (older fish test) also appeared to be increased by treatment. Other conditions appeared to be related more to age than to treatment, such as spongiosis hepatis, CNS vacuolation, and gill telangiectasis.

General Objectives

Some of the principal objectives of the preliminary phase of these studies were (1) to increase production of medaka fry without using methylene blue; (2) to develop procedures to deliver DEN to the test fish, to analytically measure waterborne DEN, and to develop methods to destroy residual DEN before discarding the effluent; (3) to develop appropriate histopathological protocols for sampling, processing, and examining large numbers of specimens; and (4) to determine DEN-induced tumor progression, the appropriate age of medaka for exposure, and the general shape of the tumorigenic dose-response curve.

Before beginning these studies, our culture facility was capable of producing the numbers of medaka necessary for large-scale dose-response studies. Our present production capacity of up to 25,000 medaka per day is more than sufficient for the planned large-scale study that currently is projected to be conducted in five replicates of about 6,700 animals each. A key objective was met in this study when we were able to eliminate the use of methylene blue as fungicide in our hatching solution. Although this compound is a very effective fungicide, it is considered by some to be mutagenic or, at least, structurally related to some known mutagens, and therefore unacceptable for use in a carcinogenicity test.

This study demonstrated that consistent DEN concentrations can be delivered to test organisms for extended periods of time. Furthermore, methodology was developed that eliminated DEN from the effluent and allowed the compound to be disposed of safely. We are

therefore confident that this approach can be used effectively in the large-scale, multireplicate study to follow.

DEN-Induced Hepatic Neoplasia

N-nitroso compounds have been the most widely used class of carcinogens to examine mechanisms of carcinogenesis in small fish species. This class of compounds has caused liver neoplasia in every species in which it has been tested and esophageal and intestinal neoplasia in some cases (Metcalfe, 1989). Nevertheless, our studies generated a considerable amount of new information on the dose-dependent initiation and progression of DEN-induced lesions and the age-related sensitivity of medaka to DEN. Khudoley (1984) demonstrated the hepatocarcinogenic effects of several nitrosamines, including dimethylnitrosamine, diethylnitrosamine, and nitrosomorpholine in zebra fish and guppies. In the topminnows, *Poeciliopsis* spp., tumorigenic sensitivity, as indicated by type of hepatic neoplasm and by incidence, is related to species and strain (Shultz and Schultz, 1982*a*,*b*; 1988). This differential induction of tumorigenesis among genotypes was shown to be related in part to the ability of sensitive species and strains to take the first steps toward metabolically activating DEN to its carcinogenic metabolites (Kaplan et al., 1991).

From literature analyses, DEN effects were expected to be mainly in the liver and to include various toxic effects, carcinogen-specific toxic effects, development of foci of staining alteration, hepatocellular adenomas, hepatocellular carcinomas, cholangiocellular proliferation, cholangiomas, and cholangiocarcinomas. No clear patterns of extrahepatic carcinogenesis have been established in DEN-exposed small fish species. The present studies confirmed the primary hepatocarcinogenicity of DEN in medaka over a wide range of exposure concentrations and two age groups. Our studies with other carcinogens tested against medaka indicate that this species is prone to develop an array of extrahepatic neoplasms including tumors of renal, neural, and soft-tissue origin after challenge with potent compounds such as methylazoxymethanol acetate and dimethylbenzanthracene.

Hepatic neoplasia induced by DEN in medaka appears to progress in stages (Bunton, 1990; Lauren et al., 1990; Hinton et al., 1988) somewhat similar to those described for rodent hepatic neoplasia. Protocols for DEN hepatocarcinogenicity vary, but generally involve exposure to 15-100 ppm DEN in ambient water for about 8 weeks followed by an additional 8 weeks or longer for tumor development (see Hinton et al., 1985). Hepatic neoplasia induced by DEN in medaka can be enhanced by several factors, including partial hepatectomy preceding exposure (Kyono-Hamaguchi, 1984), increased environmental temperature during exposure and grow-out phases

(Kyono-Hamaguchi, 1984), and increased exposure concentration, duration of exposure, and duration of grow-out (Ishikawa and Takayama, 1979). Exposure concentration of DEN also affects the type of hepatic neoplasm induced. The modifying conditions also apply for other nitrosamines in other small fish species (Khudoley, 1984). Usually, DEN causes a spectrum of hepatocellular neoplastic lesions, including foci of cellular alteration, adenoma, and carcinoma. Cholangiocellular lesions sometimes occur frequently, as in genotypes of *Poeciliopsis* (Schultz and Schultz, 1988), but usually less frequently than hepatocellular neoplasms. High exposure concentrations and extended grow-out periods often result in the development of various kinds of sarcomas in medaka liver (Bunton, 1990). The transplantability of DEN-induced hepatic neoplasms, hepatocellular carcinoma, and hemangiopericytoma, was demonstrated in *Poeciliopsis* spp. (Schultz and Schultz, 1985).

Diagnostic criteria for the stages of hepatocellular neoplasia in small fish have not been formalized. This issue is critical in dose-response studies in which hepatocellular adenomas and carcinomas are combined to establish the neoplastic response in a specific cell lineage. In this study our principal objective was to be consistent in applying diagnostic criteria. Although not counted as a neoplasm for developing the dose-response data, the basophilic altered focus appeared to play a role in the progression of hepatocarcinogenesis as opposed to the typical eosinophilic focus, which appeared to be a toxicity-induced, end-stage lesion. Evidence of clonal expansion was the principal diagnostic feature that separated diagnoses of basophilic focus and adenoma. This evidence resided mainly in the features of the lesion border; the border of the altered focus blended into the surrounding parenchyma, whereas that of the adenoma distinctly delineated the lesion from the surrounding tissue. Clinical features such as compression were given less weight in diagnosis of adenoma.

In fish, the lesion described as the altered focus is biologically distinct from that typically seen in rodents. Foci of both animals are characterized by altered patterns of enzyme expression. In some rodent carcinogenesis models, the spontaneous incidence of foci of cellular alteration is 80% by 9 months and 100% by 15 months (Maronpot et al., 1989); altered foci are rarely seen in control medaka (or other fish species). Altered foci in both fish and rodents increase following exposure to hepatocarcinogenic compounds. However, the rodent foci regress following cessation of exposure, whereas the fish foci persist and continue to grow. Although ample justification thus exists to classify fish altered foci as neoplasms and to incorporate them in the total neoplastic response, we have chosen to include only adenomas and carcinomas in our counts.

Nevertheless, diagnostic criteria are clearer for distinguishing altered foci and adenoma than for distinguishing adenoma from well-differentiated carcinoma. The fact that well-differentiated

carcinoma can so closely resemble adenoma argues for the progression of specifically initiated lesions rather than for the progression of stages in which one stage develops from another. Several observations from this study suggest that the three apparent stages of hepatic neoplasia are not developmentally linked, although their occurrence is certainly a function of exposure concentration, length of exposure, time postexposure, and age at exposure. Although each lesion was not easily categorized, rarely did we observe what could be considered transitional stages. That is, we did not often note what could be considered lesions within lesions. Indeed, the 4-month samples exhibited a complete array of hepatic neoplasms with the same characteristics as similar lesions seen in later samples, only smaller in size.

Nonhepatocellular Neoplasms

A lesion diagnosed as a hemangiopericytoma was the only nonhepatocellular neoplasm that appeared to be DEN-induced. The advanced stages of these lesions, which demonstrated well-developed swirling patterns of neoplastic cells, were limited to the liver and occurred primarily in specimens from the young fish test exposed to high DEN concentrations. In the incidence data for this lesion we also included poorly differentiated mesenchymal proliferative lesions that occurred mainly in early samples. We considered these lesions to be early stages of hemangiopericytoma. The data, however, suggest that this might be a separate lesion, even a toxic response that is resolved in later samples. A reanalysis of the DEN-induced hepatic mesenchymal lesions will have to take place before this information is published.

Toxic Lesions

Few toxic lesions that could be related to test conditions were noted. As with the neoplastic lesions, toxic lesions were almost exclusively limited to the liver. This will greatly simplify histopathologic evaluation of the sample sizes in the large-scale study that will follow. The principal toxic lesions included spongiosis hepatis, clear hepatic cysts (these two lesions may be one and the same), and various manifestations of hepatocellular cytotoxicity including vacuolation and the occurrence of eosinophilic and other inclusions. Analysis of early (2 and 4 weeks) samples from these studies might reveal that some of these toxic lesions predict the subsequent occurrence of neoplastic lesions.

Definitive Test Conditions

The present studies were designed so that we could gain experience in conducting large-scale carcinogenesis studies and to help establish conditions for a dose-response test that will examine

the curve as far below the 1% incidence level as statistical considerations will allow. The preliminary studies have accomplished those objectives, and, depending on statistical analyses of data following review, several test scenarios based on specimen age-at-exposure, DEN exposure concentrations, and duration of grow-out could be constructed. Preliminary statistical analyses of 6- and 9-month data showed that all four data sets (2 age groups x 2 sampling times) exhibited significant dose effects on the incidences of neoplastic lesions. A clear increase was seen in the incidence of liver lesions from around the 5 mg/liter level and above. The incidence among older fishes (52-day at the beginning of the exposure) was lower than that of the younger (6-day-old) fishes, and the incidences were higher at 9 months than at 6 months. Also, the data did not appear to indicate significant tank (replicate)-to-tank variation.

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DEVELOPMENT OF THE ZEBRA DANIO MODEL: CARCINOGENESIS AND GENE TRANSFER STUDIES

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ABSTRACT

The overall goal of this project is to systematically establish the projected strengths of the zebrafish (*Brachydanio rerio*) as a highly desirable small fish model for carcinogenesis studies. The evaluation of the zebrafish model will focus on five aspects of carcinogenesis studies. Objective 1 is to establish the dose-responsive sensitivity of zebrafish to representative carcinogens from six different classes. To date, only one study of diethylnitrosamine carcinogenesis in zebrafish has been reported, so sensitivity to a broader range of carcinogens must be demonstrated. Objective 2 is to focus on the pathology of induced neoplasms, identifying target organs, tumor types, and transplantability of induced tumors into cloned, homozygous diploid fish. Objective 3 is to investigate interactions between carcinogens and modulators, using inhibition and/or promotion protocols. Objective 4 is to explore carcinogen metabolism by phase 1 and 2 enzymes, and the resultant DNA binding and DNA repair. Such studies will allow comparisons of the results in zebrafish and medaka to those in other fish and mammalian carcinogenesis models. Last, objective 5 is to use the techniques of molecular biology in order to analyze the role of oncogenes in the carcinogenic process and to create transgenic lines of zebrafish expressing characteristics that influence the carcinogenic process. The last-mentioned experiments aim to create monitoring strains that respond to a broader range and to lower doses of environmental carcinogens that are of interest to the Army and others.

INTRODUCTION

The traditional rodent bioassays for detection of suspect chemical carcinogens are so costly ($0.6-$1.0 million per chemical) and slow (2 years) that only a few hundred of the many thousands of industrial chemicals have been tested. The possibility for markedly increased testing is minimal, and the potential for unraveling complex environmental mixtures is even less encouraging. Although short-term screening assays such as the Ames bacterial mutation assay (McCann and Ames, 1977) are mechanistically informative, they do not show cancer as the end

point, nor can any *in vitro* test mimic the fundamentally important physiological and pharmacokinetic parameters that often determine whether a compound will cause cancer in whole animals. Thus, there is a continuing need to develop alternative vertebrate models to supplement the use of rodent systems for identifying and understanding the action of agents that cause cancer. Species that may be useful as both laboratory models and field monitors are of special interest.

Fish have received increased attention as useful alternative cancer models. Fish are especially appropriate species for testing and monitoring carcinogenic hazards in our surface and groundwater supplies, which are susceptible to contamination by toxic chemicals in industrial dump sites, munitions manufacturing and testing sites, fuel depots, and other facilities that release potentially hazardous chemicals into the biosphere. Over the past two decades, our laboratory has developed the rainbow trout as the most widely recognized fish model for carcinogenesis research. Many desirable attributes of this model have been established, including high sensitivity, low cost, a relatively large data base, well-characterized histopathology, established husbandry requirements, a body size range from milligrams to kilograms, and a useful nonmammalian comparative status.

While the trout will continue to be highly useful, it has limited use as a field monitor, and it has certain other disadvantages that may be offset by the use of other fish with different biological features. Genetic studies in trout are severely restricted because of a 2- to 3-year maturation period; feeding costs that rise with age; and duration of tumor studies, which require at least 9 months and can be initiated twice per year at most using photoperiod control of spawning. By comparison, many aquarium fish reach maturity at 2 to 3 months, spawn regularly, develop tumors somewhat more rapidly, and have negligible food costs. The Japanese medaka (*Oryzias latipes*) is being widely developed as one such model. It is responsive to several carcinogens (Ishikawa et al., 1975; Aoki and Matsudaira, 1977; Hawkins et al., 1988), adapts to a wide range of water conditions, and has economical space and rearing costs. However, as with trout, no single species can respond to every environmental insult. Thus, in the development of fish models as sentinels to detect waterborne carcinogenic hazards *in situ*, reliance on a single species may yield aberrant false-positive or false-negative data. Ideally, a second aquarium species would complement the medaka by responding to a wider range of carcinogens and environmental modulators, and would provide its own unique strengths to enhance the overall goal of detecting carcinogenic hazards and understanding the mechanisms underlying the observed results. In the final analysis, mechanistic information, rather than dose-response data, can be most readily extrapolated to predict human risk.

Among the several aquarium species being developed (e.g., guppy, platyfish, topminnow), we believe the zebrafish or danio (*Brachydanio rerio*), a small cyprinid fish, holds exceptional promise. Of greatest interest and potential utility is the unique and superb history of genetics and developmental biology that has been amassed for zebrafish. Completely homozygous cloned lines have been derived (Streisinger et al., 1981) that could be of great use in cancer research. The embryos are transparent and allow a view of the effects of environmental toxins on embryonic development (Hanneman et al., 1988). Albino strains are available as a convenient recessive color marker. Embryos are so small that direct microinjection may permit testing of rare suspect compounds at the picogram level. Studies in the danio on gene transfer for the introduction, propagation, and expression of foreign genes have been successful, and danio have been among the most successful of all fish models (Stuart et al., 1988). Danio should readily adapt to studies of genes of interest in cancer research.

In 1966, the danio was the first aquarium species in which chemically induced tumors were demonstrated (Stanton, 1965), so the species is known to be carcinogen-responsive. Nevertheless, this species has received little further attention for carcinogenesis research. The aims of these experiments are to greatly expand the data base of carcinogen classes tested in the zebrafish, to fully characterize neoplastic responses in this species, and to understand the responses in terms of procarcinogen metabolism, DNA adduction, and DNA repair. Complementary studies conducted with medaka will allow comparison between the two species. In addition, investigations of the molecular biology of carcinogen response in the danio will be undertaken, and gene transfer methods will be applied to introduce into this species genes of specific interest to the cancer process. These studies are aimed at increasing the range, sensitivity, and power of the model. Many of these carcinogenesis procedures are well established in this laboratory with the trout and should be readily transferred to aquarium species. In turn, gene transfer may be more readily developed first in the danio, and later transferred to trout, medaka, and other fish.

METHODS AND RESULTS

Objectives 1 and 2

Lethal Concentration 50% (LC_{50}) Study

Because little is known about the response of zebrafish to carcinogen doses, a preliminary study was undertaken to investigate the effect of aflatoxin B_1 (AFB_1), administered by gill uptake, on adult zebrafish. The results of histopathology studies have been recently completed, subsequent to the contract effective date, and are reported here. Because of the limited aqueous solubility of numerous carcinogens of interest, variations in dosing were achieved by controling

the time of exposure to a fixed concentration of carcinogen, which may or may not provide a linear response. This experiment also was designed to test this hypothesis for future reference.

Duplicate groups of ten 3-month-old zebrafish were exposed to 1 ppm AFB_1 (in ethanol carrier) in a 500-ml tank, containing water at 27°C for the indicated time period (Table 1). During the experiment, the beakers containing fish were covered with opaque paper to limit photodegradation of aflatoxin. At the end of the exposure period, fish were rinsed, transferred to separate 10-gallon aquaria of clean system water for grow-out. Fish deaths were recorded over the next 7 days. All deaths occurred within 6 days of exposure. Survivors were examined 12 months after exposure and were prepared for histological examination as defined in the research proposal.

Table 1. Exposure Protocol for Zebrafish LC_{50} Study

Group	Exposure (hrs)	Ethanol (%)	AFB_1 Concentration (ppm)
Control	24	0	0
Carrier	24	0.5	0
AFB_1-1	1	0.5	1
AFB_1-4	4	0.5	1
AFB_1-24	24	0.5	1

AFB_1, Aflatoxin B_1.

Exposure by gill uptake to 1 ppm AFB_1 for longer than 1 hour produced a clear increase in mortality over that due to experimental stresses alone. Mortality rates in the 4- and 24-hour exposure groups were 35% and 90%, respectively. The mortality rates in the control, carrier control, and 1-hour exposure groups were each 10%. However, these deaths were entirely confined to the second duplicate groups which, at the end of exposure, were transferred to water ~3°C cooler (24°C) than that of the first duplicate group. Consequently, their deaths may be attributed primarily to thermal stress. A plot of the combined percent mortality for both duplicate groups versus the logarithm of exposure time in hours produces a linear model (correlation coefficient = 0.9903) of the form:

$$\% \text{ mortality} = (0.586)(\log \text{exposure}) + 0.063$$

Thus, the LC_{50} value for adult zebrafish exposed by gill uptake to 1 ppm AFB_1 in aqueous solution at 27°C is 5.57 hours.

Only two fish, both females, survived the 24-hour exposure to AFB_1, and they began to exhibit lethargy and swollen abdomens by 7 months after exposure. Because of one fish's worsening condition, it was sacrificed 1 month before the planned termination of the experiment. This fish was found to have a massive, mixed hepatocholangiocellular carcinoma, with virtually no normal hepatic tissue remaining. Histopathologic examination of the remaining survivors at 12 months revealed the following: one small focus of neoplastic biliary cells was found in the liver of 1 of the 10 fish from the 1-hour exposure, and a focus of neoplastic biliary cells and a basophilic focus were seen in 2 separate fish of the 6 fish exposed for 4 hours. No liver lesions were seen in the other 24-hour-exposed fish.

Water Bath Exposures

Juvenile zebrafish were exposed to several carcinogens by high-dose, short-term static water exposures. With each carcinogen, several doses were used, in order to identify lethal concentrations, as well as to identify at least three tolerated doses with which to establish a dose-response curve after a 6- to 9-month grow-out period. In cases where the doses used were too high, exposures were repeated using lower doses. Fry were exposed at 14 to 21 days of age post-hatch at a water temperature of 27°C. Exposure chambers were constructed of 2½-inch, polyvinylchloride (PVC) pipe with an outer diameter (OD) of 61 mm and a fine mesh nylon cloth tied over one end. Within these chambers, the zebrafish fry were easily placed in beakers containing the carcinogenic solutions and were removed, rinsed, and placed in holding tanks.

Carcinogens used to date include methylnitronitrosoguanidine (MNNG), diethylnitrosamine (DEN), dimethylnitrosamine (DMN), and methylazoxymethanol acetate (MAMA). One hundred and fifty fry were exposed at each dose of each carcinogen (Table 2, a-d).

Because of the persistent mortalities in the MNNG groups, additional experiments will be required to test doses above and below 1 ppm. The dose-response curve appears to be extremely steep for this compound, so in future experiments the laboratory will use the following doses: 0.5, 1.0, 1.5, and 2.0 ppm. Also, zebrafish will be exposed to dimethylbenzanthracene (DMBA) and 4-aminobiphenyl in the near future. In addition, medaka will be exposed to a single appropriate dose of each carcinogen to compare the response in the two species.

A significant amount of time has been spent in learning how to perform necropsy of zebrafish, perfecting embedding, sectioning, and staining procedures and becoming familiar with

Table 2a. Fish Survival Rates and Doses for Diethylnitrosamine (DEN) (11/26/91)

Dose (ppm) 24-hr exposure	Mortalities at 24 hrs	Mortalities at 72 hrs	Survivors on 2/14/92
0	0	0	>100
500	8	1	>100
1,000	23	1	>100
1,500	39	0	≈100
2,000	113	0	6

Table 2b. Fish Survival Rates and Doses for Methylnitronitrosoguanidine (MNNG) (first exposure 11/26/91)

Dose (ppm) 16-hr exposure	Mortalities at 10 hrs	Mortalities at 72 hrs	Survivors on 2/14/92
0	0	1	>100
5	12	16	0
10	all	-	0
20	all	-	0
40	all	-	0

Table 2c. Fish Survival Rates and Doses for MNNG (second exposure, 11/27/91)

Dose (ppm) 24-hr exposure	Mortalities at 24 hrs	Survivors on 2/14/92
1	0	100
2.5	0	≈30
7.5	113	0

Table 2d. Fish Survival Rates and Doses for Methylazoxymethanol acetate (MAMA) (12/23/91)

Dose (ppm) 2-hr exposure	Mortalities at 2 hrs	Survivors on 2/14/92
6.25	0	>100
12.5	0	>100
25.0	0	>100
50.0	0	>100

the 30 (plus or minus) tissues routinely observed in tissue sections. Tissue accountability has been good, except for a few tissues: corpuscles of Stannius, pineal gland, pituitary gland, and urinary bladder. Chromaffin tissue (adrenal medulla) was not seen; however, in a literature review, it was stated that zebrafish do not have chromaffin cells (Laale, 1977). Most of the other problems with tissues involve direct midline sections, so once a marker is pinpointed for that location, those organs should appear with greater regularity. An interesting tissue adaptation was observed in the dorsal pharyngeal/esophageal region directly across from the pharyngeal teeth of this cyprinid fish. A distinct thick pad of stratified squamous epithelium was observed there, surrounded on either side by the simple columnar epithelium normally seen in that region This would appear to be a specialization that provides a wear-resistant epithelium against which the pharyngeal teeth can grind food. As in the medaka, the zebrafish does not have a true stomach. The esophagus empties directly into an intestinal region.

Dietary Exposure

Prior to the effective date of the contract, two preliminary feeding trials were initiated to assess the response of zebrafish to dietary AFB_1 and to aid in determining appropriate doses for future experiments. In the first study, four groups of eighty 9-week-old fry were fed Oregon Test Diet (OTD) or OTD containing 0.4, 1.6, or 6.4 ppm AFB_1 twice daily for 14 days. The diets were prepared according to established protocols and were loaded into short segments of PVC tubing, of which one end was open to accept a plunger and the other end was covered with a fine mesh screen. Feeding was accomplished by pushing approximately 1 mm of diet through the screen, scraping off small amounts with a spatula, and introducing the resulting fine pieces into the aquaria. Fish were fed slowly over a 10-minute period to ensure the exposure of each individual to nearly equivalent amounts of diet. Total diet consumed per day was approximately 1 gram per group. During exposure, fish were maintained in 10-gallon aquaria equipped with an external air source but without outside filtration. Following exposure, each group of fish was rinsed three times in clean system water and transferred to separate 29-gallon aquaria for grow out. No deaths due to acute toxicity were observed in any group over the course of exposure. These fish have been maintained on OTD according to procedures outlined in the research proposal. In an effort to establish the acute toxic dose of dietary administered AFB_1 in zebrafish, a second study was initiated with four groups of eighty 9-week-old fry fed OTD containing 0, 4, 16, or 64 ppm AFB_1. This experiment was conducted in exactly the same manner as outlined above. Very few deaths (five total) were observed in this trial, even though the aflatoxin doses used were three orders of magnitude higher than those used in similar experiments in trout.

At 7 months after exposure, a sample of five fish from each dose group were examined and prepared for histology. To date, histopathological examination of 30 zebrafish structures and tissues has revealed no evidence of tumor formation. Consequently, a termination date of 12 months after exposure was set for the remaining animals in these experiments (May and June 1992).

The apparent resistance of this species to dietary AFB_1 is not wholly unexpected and indicates that this route of exposure may not be optimum for this carcinogen in this animal. The maximum dosage employed in these trials (64 ppm) appeared to exceed the solubility limit of AFB_1 in the carrier, salmon oil, as evidenced by slight turbidity. Alternatively, a future goal would be to develop a different diet preparation capable of carrying this and other highly lipophilic carcinogens in higher doses. These decisions await the final outcome of this experiment later this year.

Objective 3

Until dose-response carcinogenicity studies are completed, no experiments will be conducted (objective 3) to investigate the effects of modulators of carcinogenesis in zebrafish.

Objective 4

The recent hiring of a postdoctoral biochemist will enable the initiation of these experiments in the near future.

Objective 5

The zebrafish is a popular model for studies of vertebrate development and toxicology. However, *in vitro* approaches with this organism have not been fully exploited, because cell culture systems have been unavailable. Methods were developed in this laboratory for the culture of cells from blastula-stage diploid and haploid zebrafish embryos, as well as cells from caudal and pelvic fin, gill, liver, and viscera of adult fish. The haploid embryo cells differentiated in culture to a pigmented phenotype and expressed, upon exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a protein immunologically and functionally similar to rainbow trout cytochrome P-450IA1. Zebrafish cultures were grown in a complex basal nutrient medium supplemented with insulin, trout embryo extract, and low concentrations of trout and fetal bovine serum because they could not be maintained in conventional culture medium containing a high concentration of mammalian serum. Using calcium phosphate-mediated transfection, a plasmid constructed for use in mammalian cells was introduced into zebrafish embryo cell cultures and expressed in a stable

manner. These results indicate that the transfection procedures used in mammalian systems also can be applied to zebrafish cell cultures, providing a means for *in vitro* alteration of the genotype and phenotype of the cells.

Zebrafish genomic DNA was using various restriction endonucleases. Southern blot analysis was carried out by the genomic digest. A digoxigenin-labeled probe, prepared by polymerase chain reaction (PCR), was used, which contains the conserved region IV of rainbow trout *p53* gene. Hybridization at 60°C revealed two specific bands that were visible on the EcoRI-genomic digest of zebrafish DNA.

Screening the zebrafish cDNA library (available from Department of Biochemistry and Biophysics, Oregon State University) is in progress, based on the procedures and probes used in the Southern blot analysis in searching for the *p53* tumor suppressor gene in zebrafish.

As outlined, a fish growth hormone gene is one transgene that may prove interesting because of the suspected influence of proliferative signals upon the neoplastic process. Dr. Dennis Powers (Hopkins Marine Station, Stanford University) gave this laboratory a plasmid containing the coho salmon growth hormone cDNA in a eukaryotic expression vector under the promotion of the Rous sarcoma virus long terminal repeat (RSV-LTR). However, the correct plasmid has not been recovered, even by glycogen precipitation, from the DNA solution sent to us. Other plasmids sent by Dr. Powers at the same time have performed well. A new shipment of this plasmid is expected.

One of the authors (DWB) has constructed a plasmid containing the prokaryotic β-galactosidase gene under the promotion of RSV-LTR and also containing the neomycin resistance gene. This plasmid holds promise as an easily assayable test vector for evaluating various eukaryotic promoters both *in vivo* and *in vitro*. Moreover, it may provide a suitable vector backbone for other transgenes of interest.

The suitability of electroporation as a method of introducing foreign DNA into early zebrafish embryos is being investigated. Electroporation may prove to be a much more convenient and efficient procedure for producing transgenic fish than DNA microinjection.

CONCLUSIONS

Because these projects are still in the early stages, few or no conclusions can be drawn at this time. Experiments are either in progress or will be repeated.

Zebrafish appear to be relatively resistant to dietary AFB_1. Planned AFB_1 metabolism studies may help explain this apparent resistance.

During the next year, other plans include an extension of efforts in the areas of all objectives, except objective 3. The recent addition of a new postdoctoral fellow should accelerate these efforts, particularly toward objective 4.

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EFFECTS OF PENTAMIDINE ON BIOCHEMICAL EVENTS IN HUMAN LIVER CELLS

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ABSTRACT

The present study was undertaken to determine the biochemical effects of pentamidine on an established human liver cell line (HEP-G2) and its drug-resistant subclone (HEP-G2DR). The results indicate a 5 μM concentration of the drug significantly reduced the level of protein synthesis, as well as the continued expression of albumin. Pentamidine also significantly inhibited DNA synthesis in HEP-G2 cells, but not HEP-G2DR cells, during the first 24 hours. There was no stimulation in the activity of various Phase I and Phase II drug-metabolizing enzymes following pentamidine treatment. Conversely, only the resistant cells showed a significant recovery in both protein and albumin synthesis after the drug was removed (reversal). The significant recovery in these biochemical parameters was observed during the initial 24-hour reversal period, whereas a recovery in albumin secretion was not obtained in the HEP-G2 cells even after 72 hours. These findings suggest that drug resistance plays a role in the mechanism of action of pentamidine, since biochemical modulation of both cell lines is brought about by the drug, but only the resistant cells appear to recover fully following the removal of pentamidine from the culture medium.

INTRODUCTION

Susceptible AIDS victims often manifest respiratory symptoms as a result of infectious pulmonary complications of HIV infection. Overall, the most common serious infection is *Pneumocystis carinii* pneumonia (PCP). Nearly three-quarters of patients with AIDS will have at least one episode of PCP during their lifetime (Kovacs, 1989). Other respiratory complications include infection by *Mycobacterium avium-intracellulare* complex (MAC), *M. tuberculosis*, *Cryptococcus* and *Candida* species. These diseases are referred to as opportunistic infections and are usually treated aggressively with a combination of drugs, vis, trimethoprim/sulfamethoxazole (co-trimoxazole) (Fischl et al. 1988), pentamidine (Drake, et al. 1985), dapsone, etc. Pentamidine isethionate is an aromatic diamidine developed as an anti-protozoal agent in the 1940's for treatment of illnesses such as African sleeping sickness and Indian kalaazar caused by

trypanosomes. It has been shown to be very effective in significantly reducing mortality and morbidity caused by PCP. While pentamidine produces severe adverse reactions in general, it has been shown to be less toxic to AIDS patients than is co-trimoxazole (Martin, et al., 1992).

Most of the published accounts on the mechanism of action of pentamidine deal with its effects on protozoa (Goa & Campoli-Richards, 1987), while little has been done to assess its effect on host tissues even though drug regimens usually involve a 2- to 3-week course for treatment, and up to 9 months for prophylaxis. Therefore, the goal of the present study was to gain a better understanding of the mechanism of action of pentamidine on the cells of the host. Two human liver cell lines were chosen to carry out this study; HEP-G2 the parenatal strain, and HEP-G2DR a drug-resistant strain derived from the G2 cell line. These two cell lines continue to express tissue-specific functions including the synthesis and secretion of albumin (Knowles, et al. 1980). The investigators used the cells to assess the continued expression of this differentiated tissue-specific function via immunoprecipitation of the newly synthesized radioactive product, as well as other macromolecular processes following drug treatment.

MATERIALS AND METHODS

Cell Culture

The human liver cell line (HEP-G2) was obtained from Dr. Edwin Ades at the Centers for Disease Control, Atlanta, Georgia. The drug-resistant subclone (HEP-G2DR) was also developed in Dr. Ades' laboratory by growing G2 cells over a period of time in increasing amounts of adriamycin (Sigma Chemical Co.) and subdividing the cells that survived once they reached a maintenance dose of the drug. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (J.R.B. Biosciences) supplemented with 10% fetal bovine serum (Hyclone) and with an antibiotic/antimycotic solution consisting of penicillin, streptomycin, and amphotericin B (Sigma Chemical Co.). Resistant cell cultures were routinely maintained in adriamycin (1.25×10^{-8} M). The cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. For most of the kinetic studies, the cells were seeded in 60 mm^2 dishes and generally allowed to acclimatize overnight prior to experimental manipulations. Experimental cultures were treated with 1 μM or 5 μM pentamidine for various periods of time. The pentamidine stock solution (1,000x) was made up in distilled water and filter-sterilized just prior to its use. At the end of the treatment phase, the pentamidine-containing medium was removed and the attached cells washed with DMEM before the addition of fresh DMEM containing the radioactive isotopes.

Measurement of Albumin Secretion, Total Protein, and DNA Synthesis

To measure albumin secretion, [^{3}H]leucine (5 μCi/ml) was added to fresh culture medium and the cells were allowed to grow for the appropriate lengths of time. At the end of the labeling period, the medium was removed and aliquots were used to immunoprecipitate the radioactive albumin as previously described (Rosebrock et al., 1981). In brief, human albumin and rabbit antihuman albumin (ICN Immunobiologicals) were used to establish a quantitative immunoprecipitation curve. From this, the researchers were able to determine that 0.1 ml of antibody and 20 μg of albumin gave an antibody excess that was sufficient to precipitate all of the labeled albumin contained in the 0.5-ml aliquots of culture medium tested, and to produce antigen/antibody pellets large enough to wash and analyze accurately. To determine the total cellular protein present on the culture dishes, as well as the amount of protein synthesized during the labeling period, the cell layers were washed (3×) with ice-cold physiological saline and scraped from the surface of the dishes into 10% trichloroacetic acid (TCA). The precipitates were incubated overnight at 4°C. The TCA pellets were collected by centrifugation (1,000 × g, 10 minutes), and washed three times with 5% TCA by centrifugation and resuspension. After the last wash, the pellets were solubilized in 1.0 ml of 1N NaOH and aliquots used for both protein determinations (Bio-Rad method) with bovine serum albumin used as a standard and liquid scintillation spectrometry.

For analysis of DNA synthesis, treated and control cells were given 1 μCi of [^{3}H]thymidine/ml DMEM for 24 hours. At the end of the labeling period, the medium was discarded and the cell layers were carefully washed three times to remove any unincorporated isotope. The cells were then precipitated with TCA, solubilized in NaOH, and used to assess the incorporation of thymidine and total protein as described above.

Radioisotopes, Chemicals, and Statistical Analysis

Radioactive isotopes were purchased from ICN Radiochemicals ([^{3}H]thymidine, specific activity [sp. act.] 68 Ci/mmol) and NEN Research Products ([^{3}H]leucine, sp. act. 156 Ci/mmol). All chemicals and reagents were of research grade. The pentamidine (Sigma Chemical Co.) was obtained as an isethionate salt. Statistical analysis of the data was accomplished with a two-tailed *t* test. Each experiment was done in triplicate and repeated at least twice.

CONCLUSIONS

Pentamidine has been shown to have a "cidal" role against protozoa, possibly via inhibition of nucleic acid and protein synthesis (Bornstein and Yarbro, 1970) or glucose metabolism (Pesanti

and Cox, 1981). While it is used widely in the treatment of protozoan infections in AIDS patients, little is known concerning its effects on host tissues. The results of our studies corroborate those reported for protozoa, but also suggest that there may be a different mechanism of action or of metabolizing pentamidine by sensitive and resistant cell lines derived from the same tissue. The concentrations of pentamidine used (1 and 5 μM) are in the range found in blood plasma following its administration to patients (Waalkes and DeVita, 1970). Specifically, the results indicate that protein synthesis in general, as well as tissue-specific functions such as albumin synthesis and secretion, are significantly affected by the drug. This is important to note, since these changes occur within the first 48 hours of use of a 5 μM concentration of pentamidine, whereas most drug treatment protocols call for a 14-day or longer treatment course.

There was also an effect on the rate of cell division, as measured by thymidine incorporation by drug-sensitive HEP-G2 cells. This alone may not be of major concern in a patient, because the liver is not an organ in which the parenchyma cells readily divide. On the other hand, pentamidine is reported not to undergo hepatic biotransformation in humans and is only slowly eliminated from the body because of its extensive binding to mammalian tissues (Waalkes et al., 1970). Thus the drug has the potential to affect most tissues of the body over this extended time frame. The cells were assayed for the activation of certain Phase I and Phase II drug-metabolizing enzymes following pentamidine treatment. Due to the lack of enzyme stimulation in this system, which responds to other known activators, the results also corroborate those reporting a lack of hepatic biotransformation of pentamidine (unpublished observations). While the use of pentamidine is targeted to lung infections, the manner in which it is eliminated, as well as its route of administration, also lend credence to the possible involvement of other organs. Furthermore, reports are now beginning to appear in the literature that document extrapulmonary *Pneumocystis carinii* infections in AIDS patients. These extrapulmonary sites include the liver, kidneys, and spleen (Cote et al., 1990; Witt et al., 1991). The liver was also a primary choice in which to study the effects of pentamidine, because of its overall role in drug detoxification and numerous other essential functions.

The slow release of pentamidine, as well as its initial effects, prompted the researchers to assess the reversibility of these altered processes once the drug was removed from the culture medium. In medium lacking pentamidine, the cells regained both their normal growth rate and morphology. The two cell lines significantly increased their rate of total protein synthesis, and the HEP-G2DR subclone regained its ability to express the tissue-specific function of albumin synthesis and secretion. Surprisingly, this was not the case for the parental HEP-G2 cells. These cells did not significantly increase their rate of albumin secretion even after 3 days of growth.

This suggests that drug sensitivity may play a role in the way these cells metabolize and eliminate pentamidine. This would not be surprising, because the HEP-G2DR cells were selected for their drug-resistant ability to grow in a low-maintenance dose of adriamycin. It has been shown that prolonged treatment with one drug can cause resistance to be established to that drug as well as to other structurally unrelated drugs (Cowan et al., 1986; Beck, 1987). This process is known as multidrug resistance, and it is not known whether these cells express this particular phenotype. It is known, however, that pentamidine increases the survival rate of immunodeficient individuals with *Pneumocystis carinii* pneumonia. Therefore, because this drug has been shown to be efficacious, it is important to know as much as possible about its mode of action, especially since it has been documented to produce severe side effects. A better understanding of these secondary toxic effects should lead to improved drug treatment regimens for immunosuppressed individuals suffering from repeated episodes involving protozoan infections.

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DEVELOPMENT AND USE OF TELEOSTS AS A MODEL FOR IMMUNOTOXICITY

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ABSTRACT

Several species of teleosts, including medaka (*Oryzias latipes*), have been studied with respect to neoplastic responses to known carcinogens (Hawkins et al., 1988). Little information, however, is available concerning the immunocompetence and the response of the immune system to environmental chemicals in the medaka. Initial studies were begun to characterize immune organ cellularity and immune function in medaka, with the goal of using the medaka as a predictive model of immunotoxicity of environmental contaminants in vertebrates. The research approach to achieve this goal is to adapt established procedures in the literature for mammalian and cold-water fish immunotoxicity assessment to one or more species of warm-water teleost.

INTRODUCTION

It has become increasingly evident over the last decade that the immune system is an important target organ for toxicity (Dean et al., 1982; Vos et al., 1989; U.S. Congress, 1991). Although immunotoxicology as a discrete field of study is advancing rapidly, systematic study of immunotoxicity is complicated by the complex nature of the immune system and the paucity of human exposure data available. Furthermore, currently no fully validated battery of short-term screening tests is available to detect and assess potential immunologic toxins (U.S. Congress, 1991; Luster et al., 1992). A "battery" of tests to assess immunocompetence is necessary due to the diverse and often overlapping mechanisms of defense utilized by the immune system in response to immunologic challenge. Immunotoxicity can be manifested when one or more systems are compromised. Conversely, suppression or hyperactivation of one immune function may have no adverse effect on overall host immunocompetence. Therefore, demonstration of *in vivo* adverse effects is critical in applied immunotoxicology research.

The goal of this proposed research is to develop and validate immunotoxicologic screening utilizing teleosts as the model system. Assays will be developed to assess potential immunotoxic effects on the two major categories of immune response: (1) acquired immunity, which is characterized by exposure/reexposure/recognition of antigen, and (2) nonspecific immunity,

which does not depend on these antigen-specific processes. Examples of assays to be developed, correlated, and validated to assess effects on acquired immunity are *in vitro* antibody production against T-cell dependent (lectins; bacterial antigen) or T-cell independent (lipopolysaccharide; DNP-Ficoll) antigens. Examples of determination of nonspecific immunity to be developed are assessment of macrophage and/or neutrophil activation via *in vitro* stimulation and quantitation of superoxide anion generation, hydrogen peroxide generation, and oxidant-dependent chemiluminescence (CL). Once baseline values for these parameters have been established, the effects of *in vivo* administration of known immunotoxicants on these parameters will be determined. Model immunotoxins for study will be selected in conjunction with a bacterial challenge to the test organism, to provide a valid *in vivo* demonstration of immunotoxicity. It should be noted that these are only examples of the types of assays to be developed; the assays that correlate well with immunotoxic potential of demonstrated immunotoxins will be further refined to improve assay efficiency and reproducibility and minimize costs.

MATERIALS AND METHODS

The teleost species and assays for *in vitro* assessment of immune function and *in vivo* assessment of immunotoxicity by toxin alteration to pathogenic challenge will be briefly described.

Fish

Three species of small, warm-water teleost are available for use in these studies, medaka (*Oryzias latipes*), zebrafish (*Brackydanio rerio*), or guppies (*Poecilia reticulata*). Initial studies will focus on the medaka. Adult fish will be obtained from in-house cultures or from certified laboratory breeders or other research facilities engaged in small fish breeding and culture. Fish will be housed in tanks at 25°C and fed nematodes, brine shrimp, and/or commercially available flake fish food.

Immune System Characterization

The immune organs of normal adults of varying age in the test species will be characterized histologically. Initially, spleen and head kidney cells, but not thymus, will be dissected out of fish for further characterization and utilization in *in vitro* assay development. Fish will be sacrificed by cervical transection, and the head kidneys and spleens will be dissected out and pooled into a small volume (1-2 ml) of phosphate-buffered saline (PBS) or other suitable medium or buffer. Tissue will be dispersed into a single-cell suspension in glass/glass homogenizers, and cell numbers

will be determined by hemacytometer counts. Microscope slides will be made from cells by using a cytocentrifuge, fixed, and then stained with Wright stain, standard hematoxylin and eosin, and stains for characteristic enzymatic activity: nonspecific esterase activity (primarily macrophages), myeloperoxidase activity (primarily neutrophic granulocytes), and acid phosphatase activity (primarily lymphocytes) (MacArthur and Fletcher, 1985). Initial population differentials will be performed in our laboratory with the aid of a fish histology atlas (Hibiya, 1982; Rowley, 1990). If necessary, population differentials will be confirmed by a certified veterinary pathologist.

Phagocytic Cell Function

Several functions contribute to the nonspecific immune response in fish. Phagocytic activity and release of bacteriocidal oxidants by cells such as macrophages and polymorphonuclear leukocytes are among the most important of these defenses. Phagocytic cell function will be determined *in vitro* in two areas: phagocytic activity and phagoctyic cell oxidant generation following *in vitro* stimulation. Phagocytic index (percentage of phagocytosing cells) and phagocytic capacity (number of particles phagocytosed/phagocytosing cell) will be measured (Mathews et al., 1990; Zelikoff et al., 1991). *In vitro* oxidant generation by physiologically stimulated macrophages will be measured using four different assays, specific for either superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2) using modifications of published procedures (Metcalf et al., 1986; Twerdok and Trush, 1988; Mathews et al., 1990). Specifically, assays to be adapted are (1) superoxide dismutase-inhibitable generation of superoxide measured by the reduction of cytochrome *c*, (2) superoxide dismutase-inhibitable generation of superoxide measured by the reduction of nitroblue tetrazolium (NBT), (3) H_2O_2 production measured by reaction with phenol red in the presence of horseradish peroxidase, and (4) oxidant-dependent generation of chemiluminescence in the presence of two chemilumigenic amplifiers, lucigenin (O_2^--dependent) or luminol (H_2O_2-dependent). Treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or autoclaved yeast particles will be used or adapted to stimulate fish phagoccytes *in vitro*. Quantities anticipated to stimulate the phagocyte oxidative burst will be in the range of 1-100 ng TPA per milliliter or a yeast particle:cell ratio of 10-20:1. Wherever possible, chromogenic assays will be modified for performance in 96-well microtiter plates to minimize tissue requirements.

***In Vitro* Antibody Production**

The presence of antibody-producing cells (APC) in the spleen following *in vivo* immunization will be assessed by modification of the passive hemolytic plaque assay (Anderson et

al., 1979; Anderson, 1990). In this procedure an immunogen of bacterial (e.g., *Yersinia ruckeri*) or protein origin will be administered by intraperitoneal (IP) injection or, preferably, bath exposure, and the appearance, plateau, and disappearance of splenic APC will be determined. Once baseline production of specific antibody has been determined, this response to an *in vivo* challenge will be assessed following exposure to demonstrated immunotoxins.

Bacterial LC_{50}

Little information is available regarding the sensitivity of small teleosts to bacterial pathogens. Therefore, we propose to test several species of bacteria, such as *Aeromonas hydrophilia*, *Edwardsiella tarda*, and *Yersinia ruckeri*, to screen for a suitable infectious challenge agent. Groups of 10 fish/dose will be exposed to live cultures of bacteria via IP injection (approximate range 100 - 10^3 organisms/fish) or bath exposure (approximate range 10^5 - 10^8 organisms/ml), and 96-hour LC_{50} values will be determined (Cipriano and Bertolini, 1988; Thuvander, 1989). The statistical method used for LC_{50} estimation and 95% confidence limits will depend on the number of groups with partial kills (Gelber et al., 1985; American Society for Testing and Materials, 1989). Factors used to select the bacterial challenge for subsequent toxicant selection will include ease of bacterial culture, ease of administration (bath versus IP injection), and slope of the dose-response curve.

Immunotoxicant Exposure

Model immunotoxins will be selected in conjunction with bacterial challenge. Toxins must demonstrate modulation of teleost immune response to infection when administered preceding an LC_{50} dose of pathogen. The expected effect on the system will be immune suppression expressed as increased susceptibility to the infectious challange. Toxins to test will include one or more of the following mammalian (Bayne and Levy, 1991; Luster et al., 1992) or teleost (Anderson et al., 1982; Thuvander, 1989) immunomodulatory chemicals: lead, cadmium, pentachlorophenol, corticosteroid, or cyclophosphamide.

SUMMARY

A systematic approach to the development of a teleost model for immunotoxicity was described. A battery of tests will be developed in a small, warm-water teleost, such as the medaka, for laboratory and/or field assessment of immunotoxicity of single compounds and/or complex mixtures such those found in groundwater. By using an integrated series of tests assessing both specific and nonspecific functions of the teleost immune system, we propose to

establish and validate a useful and predictive model for immunotoxic potential and risk of environmental contaminants.

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MODULATION OF OXYRADICAL GENERATION BY MEDAKA PHAGOCYTES FOLLOWING EXPOSURE TO ENVIRONMENTAL CHEMICALS

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ABSTRACT

Phagocytes isolated from the pronephros of the Japanese medaka (*Oryzias latipes*) actively secrete reactive oxygen intermediates (ROI), particularly after stimulation by ingestion of foreign particulates or after interaction with soluble mediators such as phorbol myristate acetate (PMA). Exposure of these cells to various aquatic environmental toxicants can produce dose-dependent suppression of ROI production; this activity occurs at sublethal xenobiotic concentrations. Cell-mediated ROI release was quantified as luminol-augmented chemiluminescence (CL) using a liquid scintillation counter adapted for single photon counting. Macrophage bactericidal and tumoricidal activities involve ROI cytotoxic mechanisms; therefore, alterations in CL activity can be used as a screen for immunomodulatory chemicals.

INTRODUCTION

As outlined in two previous research summary reports presented at U.S. Army Biomedical Research and Development Laboratory (BRDL) Annual Research Reviews, a number of methods to quantify reactive oxygen intermediate (ROI) production by macrophages from fish and shellfish have been developed in this laboratory. These include assays for superoxide anion (O_2^-) via ferricytochrome *c* reduction or nitroblue tetrazolium reduction, as well as hydrogen peroxide (H_2O_2) by oxidation of phenol red. Whereas these procedures may be carried out with medaka pronephros phagocytes, the most sensitive method for ROI production in our hands has been luminol-dependent chemiluminescence (CL). In this paper are described the responses of leukocyte CL after exposure to sublethal levels of a representative marine and estuarine pollutant pentachlorophenol (PCP). The presence of PCP in terrestrial and aquatic environments is ubiquitous, with an annual global production of about 50 million kg (Eisler, 1989). PCP has found widespread application as a herbicide, insecticide, antimicrobial agent, molluscicide, and wood preservative. It is a well-known uncoupler of mitochondrial oxidative phosphorylation and modulator of cytochrome P-450 (Arrhenius et al., 1977), which produces numerous physiological

effects including immunotoxicity. Analytical grade (pure) PCP (A-PCP) is reported to have little immunotoxicity in laboratory rodents, whereas technical grade PCP (T-PCP) shows immunosuppressive activity, probably due to the presence of contaminants such as 1,2,3,6,7,8-hexachlorodibenzo-*p*-dioxin (HCDD) (Holsapple et al., 1984, 1987; Kerkvliet et al., 1985*b*). However, exposure of the mollusc *Mercenaria* to analytical grade PCP produced an impairment in the ability of the phagocytic hemocytes to destroy bacteria *in vivo* or *in vitro* (Anderson et al., 1981; Anderson, 1988). In the current study with medaka, the effects of both A-PCP and T-PCP on phagocyte-mediated CL are considered.

Phagocytes play a significant role in the nonspecific immune capacity of fish by virtue of their ability to recognize and destroy various microbes. This antimicrobial activity is chiefly affected by certain intracellular hydrolytic enzymes and by reactive oxygen intermediates (ROI). Physiologically important ROI include the superoxide anion (O_2^-) and its dismutation product, hydrogen peroxide (H_2O_2). Both O_2^- and H_2O_2 have direct cytotoxic activities (Fridovich, 1988), and H_2O_2 in combination with myeloperoxide and chloride ions can form one of the most effective antibacterial systems of leukocytes (Klebanoff, 1968). In this study, phagocytically stimulated cellular chemiluminescence, as augmented by the presence of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), is measured as an indication of the production of cytotoxic intermediates. The oxidation of luminol by ROI produced during the respiratory burst results in the generation of an excited aminophthalate anion that relaxes to the ground state with the production of photons (Allen and Loose, 1976). The luminol-enhanced CL of phagocytizing human neutrophils was shown to be dependent on a myeloperoxidase-mediated reaction involving the generation of hypochlorous acid (DeChatelet et al., 1982). The involvement of myeloperoxidase in CL by medaka cells has yet to be documented.

Exposure of fish phagocytes to sublethal concentrations of certain environmental contaminants can produce various manifestations of immunomodulation, including impairment of CL. Exposure of trout cells to aluminum or to copper suppressed CL responsiveness to phagocytized bacteria (Elsasser et al., 1986). The phagocytosis-elicited CL response of kidney phagocytes from spot, *Leiostomus xanthrus*, collected from a river highly polluted with polycyclic aromatic hydrocarbons was negligible compared with the response in cells from spot living in a more pristine site (Warinner et al., 1988). Wishkovsky et al. (1989) reported that tributyltin (TBT) exposure *in vitro* inhibited CL responses to ingested zymosan in three species of estuarine fish. Rice and Weeks (1989) concurred that higher TBT doses (~500 μg/l) inhibited CL, but found stimulation of CL by 50 μg/l; the opposing effects of TBT probably result from alterations in calcium flux across the cell membrane induced by different concentrations of TBT. Peritoneal

macrophages from oyster toadfish (*Opsanus tau*) exposed *in vivo* to TBT showed a dose-dependent suppression of CL (Rice and Weeks, 1990). Clearly, the effects of TBT on CL are complex: at certain concentrations it stimulates or inhibits CL *in vitro*, at 50 μg/l it enhances phorbol myristate acetate (PMA)- and calcium ionophore (A23187)-stimulated CL but not zymosan-induced CL, and generally a dose-dependent inhibition of CL follows experimental exposure of fish *in vivo*.

METHODS

Fish

Commercially imported Japanese medaka were housed at ~21°C in laboratory aquaria (~20 fish per 10 gallon tank) supplied with aerated deep-well water. A photoperiod of 12 hours of light and 12 hours of dark was maintained. Adult fish of 6 to 12 months of age were used in the study; they were fed brine shrimp nauplii and flaked food. Conditions of fish maintenance were judged to be excellent, based on the low incidence of disease and mortality observed and on the high rate of fecundity.

Pronephros Cells

The fish were decapitated and the anterior kidneys were pooled in ~2 ml of medium (Leibovitz L15 medium containing 5% fetal calf serum and 3% antibiotic/antimycotic solution, all from Sigma Chemical Co.). The tissues were disrupted in ~4 ml of medium using a Dounce grinder. Tissue fragments and other debris were allowed to settle, and the overlying cell suspension was removed. It was necessary to pool head kidneys from more than 20 fish to obtain enough cells for a single experiment. Attempts to select particular cell types by density gradient centrifugation were not routinely performed because of the high degree of cell loss associated with the procedure. The cell preparations appeared to consist largely of macrophages, based on their ability to phagocytize zymosan or yeast cells and on positive staining for nonspecific esterase; contamination by erythrocytes was minimal. In order to prepare for subsequent assays, the cell suspension was counted in a standard hemocytometer, and the leukocytes were adjusted to 2×10^6/ml medium.

Chemiluminescence Assay

One-milliliter aliquots of the leukocyte suspension (2×10^6 cells) were introduced into conical centrifuge tubes as needed for control and experimental samples. The tubes were centrifuged (250 × g, 10 min, 21°C), the supernatants replaced by equal volumes of fresh medium (controls) or medium containing PCP (experimentals), and the cells resuspended. A concentrated

solution of T-PCP was initially prepared in a few microliters of ethanol. This was highly diluted with medium to reach the desired final PCP concentration prior to addition to the cells. The trace amounts of ethanol present in the final exposure media were determined to be nontoxic by trypan blue exclusion and by failure to influence leukocyte CL. Analytical grade PCP (as NaPCP) was directly soluble in the medium at the concentrations used in this work. The cell suspensions were then transferred to dark-adapted pony scintillation vials and incubated in total darkness for 1 or 20 hours at ~21°C. This and all subsequent steps were performed in a darkroom under dim red illumination. After the incubation period, 50 μl of filter-sterilized 1 mM luminol was added to each sample, and the tubes were counted at 1-minute intervals for about 5 minutes to establish the baseline, unstimulated level of CL. Then 100 μl of yeast suspension (autoclaved reconstituted lyophilized yeast cells, washed and readjusted to 8 mg/ml) was added to the samples and the resultant stimulated CL recorded for about 2 hours. CL activity was measured on a Packard Tri-Carb 1900CA liquid scintillation analyzer programmed for single photon counting.

The CL parameters quantified were: unstimulated levels, stimulated peak heights, and total stimulated CL (obtained by determining the area under the curve). In the case of the stimulated CL measurements, the values were corrected for CL activity of the unstimulated cells from the same pool (an aliquot of cells to which no yeast particles were added). Considerable variation in absolute CL activity, as measured by counts per minute, was observed between cell pools. However, if the data were expressed as percentages of control values, the relative effects of various PCP concentrations of leukocyte CL could be readily compared between cell pools. The statistical significance of the differences between the means of CL activities of control and experimental groups was determined by a two-tailed Student *t* test.

RESULTS

Approximately 95% of medaka pronephros cells remained viable after 1 hour of *in vitro* maintenance, and ~90% survived 20 hours in culture; viabilities were determined by trypan blue exclusion. Exposure to PCP produced dose-dependent lethality that was particularly evident in the 20-hour cultures. Under the conditions of this study, it was estimated that the 1-hour LC_{50} was >75 ppm, and the 20-hour $LC_{50} \approx 28$ ppm.

The kinetics of the phagocytically induced CL response were consistently different when elicited after 20 hours of culture, as compared with 1 hour of culture (Figure 1). Phagocytes from any given cell pool, when stimulated after 1 hour, produced a steadily rising CL response that tended to plateau and remain relatively constant for at least several hours. In contrast, aliquots

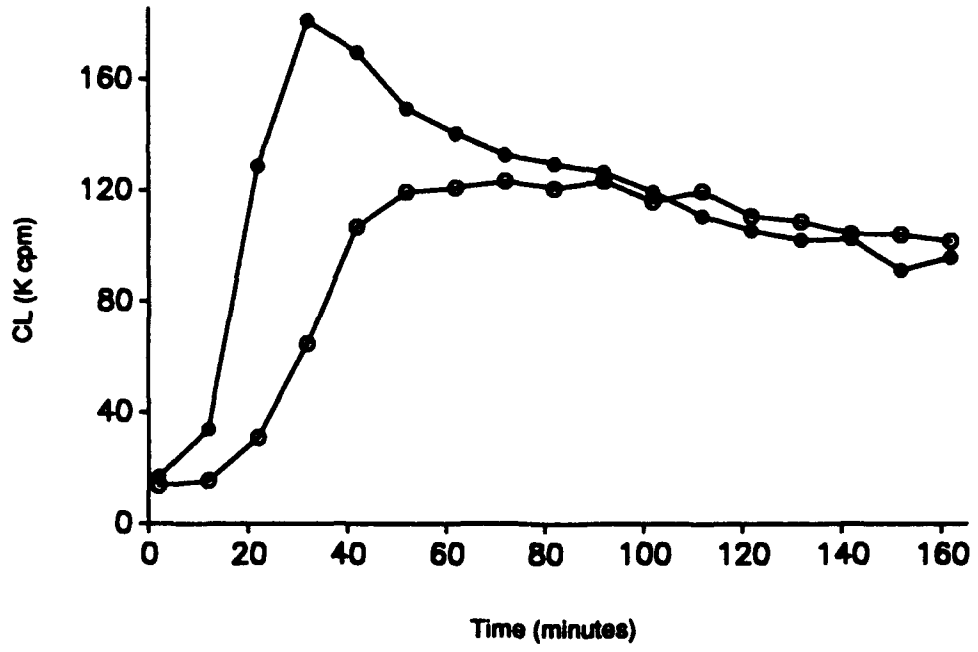

Figure 1. The chemiluminescent response induced by phagocytic stimulation in two aliquots from the same pool of unexposed pronephros cells after 1 hour of culture (open circles) and after 20 hours of culture (closed circles).

from the same pool, after incubation for 20 hours, responded with a more rapid CL activity increment, producing a clearly defined peak; subsequently the activity gradually decayed. On many occasions, the 20-hour CL peak and total CL value exceeded the comparable 1-hour values; however, this was not always observed.

The mean CL values from all cell pools examined in this study are summarized in Table 1. The CL parameters given are expressed as percentage of inhibition of untreated control cells after 1 and 20 hours and include unstimulated CL (background CL produced by cells in the absence of added yeast), stimulated CL peak (peak value minus background), and total stimulated CL (area under the curve minus background level). All three CL parameters were inhibited in a dose-dependent fashion by 1 or 20 hours of exposure to 5-20 ppm A-PCP. As might be expected, inhibition was more pronounced in the 20-hour groups, as was PCP-related loss of viability. However, even at the higher concentrations, the observed lethality could not totally account for the elevated levels of CL suppression. It appeared that the PCP effect on CL could easily be measured after a 1-hour exposure. The 1-hour test was shown to have about the same sensitivity as the 20-hour test without any measurable effects on cell viability.

Table 1. Inhibition of Pronephros Cells Chemiluminescence (CL) by Exposure to Analytical Grade Pentachlorophenol (PCP)

NaPCP (ppm)	Viability		Unstimulated CL		Stimulated CL Peak		Stimulated Total CL	
	1 hr	20 hr	1 hr	20 hr	1 hr[a]	20 hr	1 hr	20 hr
5.0	>95	>95	21.0 ± 15.0[b] (7)[c]	24.7 ± 14.4 (6)	11.9 ± 6.0	16.7 ± 8.3	9.2 ± 3.3	16.4 ± 10.2
10.0	>95	~94	14.6 ± 8.9 (5)	56.6 ± 18.8 (6)	29.7 ± 1.9	37.0 ± 8.3	23.5 ± 12.6	31.0 ± 15.5
15.0	>95	~81	31.8 ± 21.2 (7)	68.6 ± 11.5 (3)	42.6 ± 9.6	61.1 ± 9.0	46.8 ± 13.3	53.1 ± 15.2
20.0	>95	~65	93.0 (2)	97.0 ± 0.9 (3)	93.6	100.0	94.4	96.8 ± 2.0

[a]After 1 hour of culture, phagocytic stimulation produces a sustained plateau of CL activity rather than a well-defined peak; therefore, in this column the maximal CL level achieved after PCP exposure is compared with the maximal CL level of the unexposed control.

[b]Values are expressed as mean percent inhibition compared with unexposed control cell values ± SD.

[c]Number of cell pool samples in parentheses are the same in corresponding entries in the following columns of CL parameters.

Because PCP-mediated inhibition of stimulated peak CL and stimulated total CL were virtually identical (Table 1), only total CL data are presented in Table 2, which compares the effect of A-PCP with that of T-PCP. After 20 hours of exposure, T-PCP apparently caused more CL suppression than A-PCP at >5 ppm; this difference was significant at 15 ppm.

The PCP effect on CL was reversible, as shown in Table 3. In this experiment, pronephros cells were incubated for 1 hour in 25 ppm PCP, a treatment that resulted in >95% suppression of CL but no significant reduction in cell viability. The exposed cells were washed and resuspended in PCP-free medium; CL activity was about 70% restored by about 4 hours, and had returned to that of unexposed cells by about 24 hours.

DISCUSSION

Fish pronephros cells are essential accessory and effector cells in the immune system, and changes in their responsiveness are likely to have widespread repercussions in the host. Measurement of chemiluminescence serves as an important primary screening assay of immunomodulatory activities of environmental toxicants on mammalian macrophages (Tam and Hinsdill, 1990), and seems to find similar application to studies of fish immunotoxicology. In these studies, both A-PCP and T-PCP were shown to induce CL suppression in medaka leukocytes at sublethal concentrations. This effect was both dose-dependent and reversible. Previous studies with various species of fish have described inhibition of CL by metals (Elsasser et al., 1986; Wishkovsky et al., 1989) and polycyclic aromatic hydrocarbons (Warinner et al., 1988).

The immunotoxic effects of PCP have been studied in birds and mammals. Prescott et al. (1982) fed chickens a purified grade PCP, that contained no tetrachloro- or hexachloro-dibenzo-*p*-dioxins (TCDD, HCDD), 300 ng/g heptachlorodibenzo-*p*-dioxin (HpCDD) and 800 ng/g octachlorodibenzo-*p*-dioxin (OCDD); after 8 weeks, humoral and cellular immune responses were evaluated. Chickens receiving 2,500 ppm PCP showed decreased lectin-induced lymphoproliferation, lower white blood cell counts, decreased humoral response to bovine serum albumin, but normal levels of immunoglobulins IgM and IgG and normal humoral responses to sheep erythrocytes and Newcastle disease virus. Exposure of mice to T-PCP resulted in enhancement of Maloney sarcoma virus (MSV)-induced tumor susceptibility, inhibition of cytotoxic T-cell activity, slightly increased resistance to encephalomyocarditis virus (EMCV), and increased phagocytosis by peritoneal macrophages (Kerkvliet et al., 1982*b*). Macrophages play a primary role in resistance to EMCV infection, while T-cells are involved in the regression of MSV-induced sarcomas. No significant changes in immune function were induced by A-PCP administration. Mice fed T-PCP, but not A-PCP, showed depressed humoral immune responses

Table 2. Effect of 20 Hours of Exposure to Analytical or Technical Grade Pentachlorophenol (PCP) on Chemiluminescence (CL) Response to Phagocytic Stimulation

PCP (ppm)	Analytical Grade		Technical Grade		p
1.0	0	(2)	0	(2)	NS
5.0	16.4 ± 10.2	(6)	15.2 ± 4.5	(5)	NS
10.0	31.0 ± 15.5	(6)	42.5 ± 7.8	(5)	NS
15.0	53.1 ± 15.2	(3)	84.0 ± 6.9	(4)	<0.005
20.0	96.8 ± 2.0	(3)	100.0	(2)	NS

Data are expressed as mean percent inhibition of PCP-exposed cells compared with data from unexposed controls ± SD (N).

Table 3. Recovery of Chemiluminescence (CL) Response Following Removal of Pronephros Cells from Medium Containing 25 ppm Pentachlorophenol (PCP)

Hours in PCP-Free Medium	Total CL (% of control)	Cell Viability
0	<5.0	>95
3.0 - 5.5	71.3 ± 19.1 (3)	~95
21.0 - 26.0	94.4 ± 5.6 (3)	~90

Data are expressed as mean percent of unexposed control cells ± SD (N).

to both T-cell-dependent and T-cell-independent antigenic stimulation (Kerkvliet et al., 1982*a*). Rats fed 97% pure PCP had decreased antibody titers, decreased delayed-type hypersensitivity, and increased phagocytic activity by peritoneal macrophages (Exon and Koller, 1983).

To examine in greater depth the sensitivity of T-cells, natural killer (NK) cells, and macrophages to T-PCP, Kerkvliet et al. (1985*a*) studied their activities in mice following 8 weeks of dietary exposure. The only significant alteration in the parameters tested was reduced lymphoproliferative response in mixed lymphocyte culture, but not in response to mitogens. It was concluded that T-cells, NK cells, and macrophages were relatively resistant to T-PCP, in contrast to the marked sensitivity of elements of the humoral immune response. Several

contaminant fractions and purified isomers from T-PCP were analyzed for their humoral immunosuppressive effect (Kerkvliet et al., 1985*b*). The 1,2,3,6,7,8-hexachlorodioxin, 1,2,3,4,6,7,8-heptachlorodioxin, and 1,2,3,4,6,7,8-heptachlorofuran were all immunosuppressive; this and other evidence was presented for the role of toxic Ah-interactive dioxin and furan contaminants in T-PCP mediated immunotoxicity.

In light of the above, it is interesting that at lower doses the immunosuppressive effects of A-PCP on medaka phagocyte CL were virtually equivalent to those of T-PCP. The exact chemical composition of the T-PCP (Aldrich Chemical Company, Inc., lot 1907MH) was not known; however, the manufacturer determined that it was ~97% pure PCP, although 86% purity was indicated on the label. Because of the variation of percentage and identities of specific contaminants, it may be difficult to compare immunotoxic activities of T-PCPs used in various studies. We report that 15 ppm T-PCP caused significantly more CL suppression than 15 ppm A-PCP; this effect was not seen at lower PCP concentrations, higher levels of either T-PCP or A-PCP essentially abolished CL. Whether the slightly enhanced immunosuppressive effect of T-PCP was caused by contamination with dioxins and/or furans has yet to be determined.

It was evident that A-PCP (Aldrich Chemical Company, Inc., PCP sodium salt hydrate, >99% pure, lot 03512JM) caused dose-dependent suppression of CL by medaka phagocytes, despite its reported lack of effects on humoral or cellular immune parameters in mammals. Previously, A-PCP (Aldrich Chemical Company, Inc., lot 032487CC) was reported to cause decreased resistance to bacterial infection (Anderson et al., 1981), probably as a consequence of impaired haemocyte-mediated antibacterial capacity (Anderson, 1988), in the bivalve *Mercenaria mercenaria*. In this context, it is interesting to note that 2,4-dinitrophenol, another uncoupler of oxidative phosphorylation, has been reported to inhibit superoxide release and CL by alveolar macrophages (Miles et al., 1977; Castranova et al., 1987).

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STATUS AND FUTURE DEVELOPMENT OF FETAX

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ABSTRACT

Embryonic development is a weak link in the life cycle of most organisms. Many chemicals affect embryonic development at far lower concentrations than those that are toxic for adults, making it necessary to screen for developmental toxicants. FETAX is a 96-hour developmental toxicity test that utilizes the embryos of the South African clawed frog *Xenopus laevis*. The assay has applications for ecotoxicology and also for human health when an *in vitro* rat liver microsomal metabolic activation system (MAS) is used to simulate mammalian metabolism. The end points of the assay are the 96-hour LC_{50}, the 96-hour EC_{50} (malformation), and the minimum concentration that inhibits growth (MCIG). The teratogenicity of a compound is assessed by the teratogenic index (TI = 96-hour LC_{50}/96-hour EC_{50} [malformation]), the severity of malformations, and an inhibition of growth at concentrations <30% of the 96-hour LC_{50}. The state of development of FETAX as a screen and future research directions are addressed.

INTRODUCTION

The Need for Developmental Toxicity Testing

Developmental toxicity tests are designed to detect xenobiotic agents that affect embryonic development. Embryonic development can be considered a "weak link" in the life cycle of an organism. During this period unique cellular and molecular processes operate to generate a complex multicellular organism from a zygote. These processes are sensitive and easily perturbed by many chemicals. Developmental toxicants are chemicals that can exert their effects on embryos at concentrations lower than those required to affect adults or cause general cellular toxicity. For example, semicarbazide causes malformation in frog embryos at 1/3,000th the concentration required to kill embryos and affects embryonic growth at even lower concentrations (Schultz and Ranney, 1988). Tests of chronic exposure can be done for all phases of the life cycle, but take longer to run for vertebrates than the 4-day test presented here. Developmental toxicity tests can then be considered subchronic tests that approximately predict chronic effects in less time and at less cost. A developmental toxicant is defined as a chemical that causes deficit in an

embryo while a teratogen is more narrowly defined as causing only gross congenital malformation. Therefore, teratogens are a subset of developmental toxicants.

FETAX (Frog Embryo Teratogenesis Assay—*Xenopus*) is a 4-day whole-embryo developmental toxicity test that utilizes the embryos of the South African clawed frog, *Xenopus laevis*. FETAX was initially designed as an indicator of potential human developmental health hazards. The assay is well suited for testing complex mixtures and has been validated using chemicals known to be toxic to mammalian development. The assay also is applicable to ecotoxicology.

Uses of FETAX

Findings from FETAX data can be extrapolated to other species, because a conserved genetic program controls embryonic development. If differences such as metabolic activation and placentation are taken into account, it is even possible to extrapolate from the data for *Xenopus* to mammals. However, some features of the amphibian egg are unique and allow the use of this assay to find developmental toxicants that affect amphibians. Identifying these toxicants will aid studies designed to discover the reasons for the reported worldwide disappearance of amphibians even in pristine locations (Wake and Morowitz, 1990; Wake, 1991). This decline may be due in part to normal population fluctuations caused by climatological or anthropogenic factors (Pechmann et al., 1991). In at least one case, frog eggs failed to develop in pond water but developed normally when moved to the laboratory (Science Briefings, 1991). It is, therefore, possible that some of the decline may be due to pollution, and FETAX can be used to investigate the extent to which the decline is caused by environmental degradation.

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

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

MATERIALS AND METHODS

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

Conduct of FETAX") has been published that complements the ASTM guide. Both publications are available free from the author and provide a detailed description of FETAX.

Briefly, adult *Xenopus* are purchased from *Xenopus* I (Ann Arbor, MI). Male and female *Xenopus* are induced to breed by an injection of human chorionic gonadotropin into the dorsal lymph sac. The animals then mate normally and eggs are collected from the aquarium the following day. The jelly coat of the eggs is removed by treatment with L-cysteine, and normally cleaving blastulae are selected. Typically, a concentration series is constructed consisting of several concentrations plus controls. Two glass 60 x 15 mm Petri dishes each containing 25 embryos are used for each concentration tested. Therefore, each dead or malformed embryo represents a 2% response. The assay is a 4-day renewal ending with free-swimming larvae that have undergone all major phases of organogenesis. Exposure to the test material is continuous and dead embryos are removed on a daily basis to reduce microbial contamination. In addition, Aroclor 1254-induced and isoniazid-induced rat liver microsomes must also be used when screening mammalian developmental toxicants (Bantle et al., 1991). A balanced salt solution (FETAX solution) is used from mating onward in order to minimize variation caused by different diluent waters (Dawson and Bantle, 1987). Controls include a FETAX solution-only control, a positive control employing 6-aminonicotinamide, a positive metabolic activation system (MAS) control employing cyclophosphamide, and a solvent control (when a cosolvent is needed to solubilize the test material). At the conclusion of the 96-hour assay, mortality and gross congenital malformations are recorded, and the embryos are fixed in 3% formalin for length measurements. Head-tail length is also measured. A common darkroom enlarger is used to magnify the embryos ontc a digitizing pad. Sigma Scan software (Jandel Scientific, Corta Madera, CA) is then used to measure head-tail length, which is an indicator of growth. FETAX takes a single work week to perform and approximately 20 hours of technician labor.

CONCLUSIONS

Developmental Toxicity Assay Design

In designing a developmental toxicity assay, it is imperative to account for the normal molecular and cellular mechanisms that guide embryonic development. As mentioned earlier, a genetic program guides development and entails the sequential expression and repression of genes. Many of these genes are expressed for a short period only during a specific stage of embryonic development. Therefore, genotoxic agents are often developmentally toxic as well. Five cellular mechanisms operate in concert during development and each is critical in embryogenesis. These mechanisms are cell division, interaction (induction), migration, differentiation, and selective cell

death. The interruption of any of these mechanisms may cause abnormal development or even embryo death. This means that end points of any developmental toxicity assay must consider all of these mechanisms. FETAX end points are the 96-hour LC_{50}, the 96-hour EC_{50} (malformation), and the minimum concentration that inhibits growth (MCIG). These end points account for all important cell and molecular mechanisms, since the assay is based on the whole embryo and not on embryo parts or cultured cells.

Some developmental toxicants only affect certain stages of embryonic development. The drug thalidomide only exerts its devastating effect on normal limb development and stunting during a very short period. Treatment with thalidomide before or after this period results in little or no effect. Therefore, conditions in any assay must be designed to ensure exposure during all sensitive stages. Exposure is continuous in FETAX throughout the 4-day period of primary organogenesis thereby ensuring that all sensitive developmental stages are affected. During the 4-day exposure period, the embryo proceeds from a hollow blastula stage of a few hundred cells to a free-swimming larva that is ready to feed. All primary organogenesis is complete although limbs have not yet formed.

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

FETAX End Points and Assay Data

FETAX has three standard end points, and a TI ratio is calculated from two of the end points. Embryo death is measured by the 96-hour LC_{50}. Embryo malformations are recorded in live embryos and evaluated in terms of the 96-hour EC_{50} (for malformation). Standard concentration-response experiments are performed and curves constructed using probit analysis, which provides the appropriate LC_{50} or EC_{50} values with 95% confidence limits. The concentration-response curves are usually plotted together to show the separation between the two curves. The TI is found by dividing the 96-hour LC_{50} by the 96-hour EC_{50} (malformation). Values less than 1.5 indicate little developmental hazard, whereas values greater than 1.5 indicate

increasing developmental hazard. TI values exceeding 1,000 are rare, and developmental toxicants more commonly have TI's in the 10 to 200 range.

The MCIG is calculated by measuring the head-tail length of each embryo following the contour of the embryo. The embryos are fixed in 3.0% formalin prior to this procedure. Fixation does not seem to alter embryo length. Length data from each concentration set are compared to control length data using the *t* test for grouped observations. The lowest concentration set that inhibits growth at the $p = 0.05$ level of significance is the MCIG. The data are usually plotted as the percent of control versus the percent of the 96-hour LC_{50}. This makes it possible to compare results from different compounds.

The use of other end points is possible in FETAX as long as the required end points are reached. Additional end points such as pigmentation, locomotion, and hatchability are described in the ASTM guide to FETAX (Bantle and Sabourin, 1991). An EC_{50} can usually be obtained for all three. However, the determination of pigmentation and locomotion endpoints is very subjective, and much work needs to be done to make data collection objective.

Data Interpretation

Although additional end points exist, FETAX gives concentration-response data for mortality, malformation, and growth. These data can be compared with similar data on a molar basis using other pure chemicals to yield relative developmental toxicity. For example, saccharin has an LC_{50} of 18.4 mg/ml, whereas hydroxyurea has an LC_{50} of 1.8 mg/ml. Once these values are compared based on molarity, hydroxyurea is at least 10 times more toxic.

For assessing the developmental toxicity of complex mixtures, it must be remembered that any significant difference in malformations between the 100% concentration and controls represents some developmental toxicity and associated hazard. It is more difficult to assign the teratogenic hazard to a test material or pure compound. Recall that a teratogen is any agent to which an embryo is exposed that causes malformation in living offspring. In order to rank compounds according to their teratogenic hazard, Dumont and co-workers (Dumont et al., 1983) developed the teratogenic index or TI. This value is found by dividing the 96-hour LC_{50} by the 96-hour EC_{50} (malformation). Values <1.5 indicate little or no teratogenic hazard while values greater than 1.5 indicate increasing hazard. The TI is similar to the commonly used therapeutic index in pharmacology in that a specific effect (malformation) is compared to general toxicity (mortality). The LC_{50} and EC_{50} are used in the determination of TI because they are automatically calculated by probit analysis programs, and there is the least variation in confidence intervals at the 50% response level. The TI may not correctly identify the teratogenic hazard if

the slopes of the mortality and the malformation curves are different. However, most of the concentration curves obtained during the validation phase of FETAX demonstrated similar slopes for mortality and malformation (Fig. 1, Table 1). There is no reason to assume that the TI for FETAX will be indicative of the TI observed in another species. However, it is likely that these values will be fairly close, given that the predictive accuracy of FETAX is about 90% when compared to chemicals of known mammalian and human developmental toxicity.

A typical FETAX concentration-response curve illustrating how to assess developmental toxicity is shown in Fig. 1A. It is important to remember that most probit analysis programs do not accommodate 0 and 100% response data points. Generally, only partial response concentrations are used to establish the curve, although many other data points are collected (see Fig. 1A). In this figure, each data point represents 50 embryos, and the data closely fit the line. The 96-hour LC_{50} for this experiment was 2.54 mg/ml, whereas the EC_{50} (malformation) was 0.0045 mg/ml. The TI was high (550.6) indicating considerable hazard for developmental malformation. Note the wide concentration range that exists where all exposed embryos would be malformed but would not die. The separation of the two curves is indicative of relative hazard and is represented by the TI.

Treatment with 6-aminonicotinamide causes severe malformations to all organ systems even at concentrations <30% of the 96-hour LC_{50} (Dawson et al., 1989). This is a second criterion for predicting developmental hazard. Some compounds, such as plant glycoalkaloids, have low TI values but cause very severe malformations (Friedman et al., 1990). Saccharin and sodium cyclamate are compounds that have little developmental toxicity because they have low TI ratios and cause only moderate malformations even at very high concentrations (Dawson and Bantle, 1987; Dawson et al., 1989).

Growth inhibition is a third criterion of developmental hazard. The concentration-response curve for growth inhibition is shown in Fig. 1B. Since this particular experiment was designed to delineate the mortality and malformation curves, Fig. 1B shows some discontinuity. Experiments have previously been performed that investigated the effect of 6-aminonicotinamide on growth in the concentration range of 0.01-1.5 mg/ml (Dawson et al. 1989). These early data clearly showed significant growth inhibition (MCIG = 0.01 mg/ml or about 3% of 96-hour LC_{50}) below 30% of the 96-hour LC_{50}. Most chemicals that have high developmental toxicity cause inhibition at <30% of the 96-hour LC_{50} and cause a reduction >20% of control length.

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

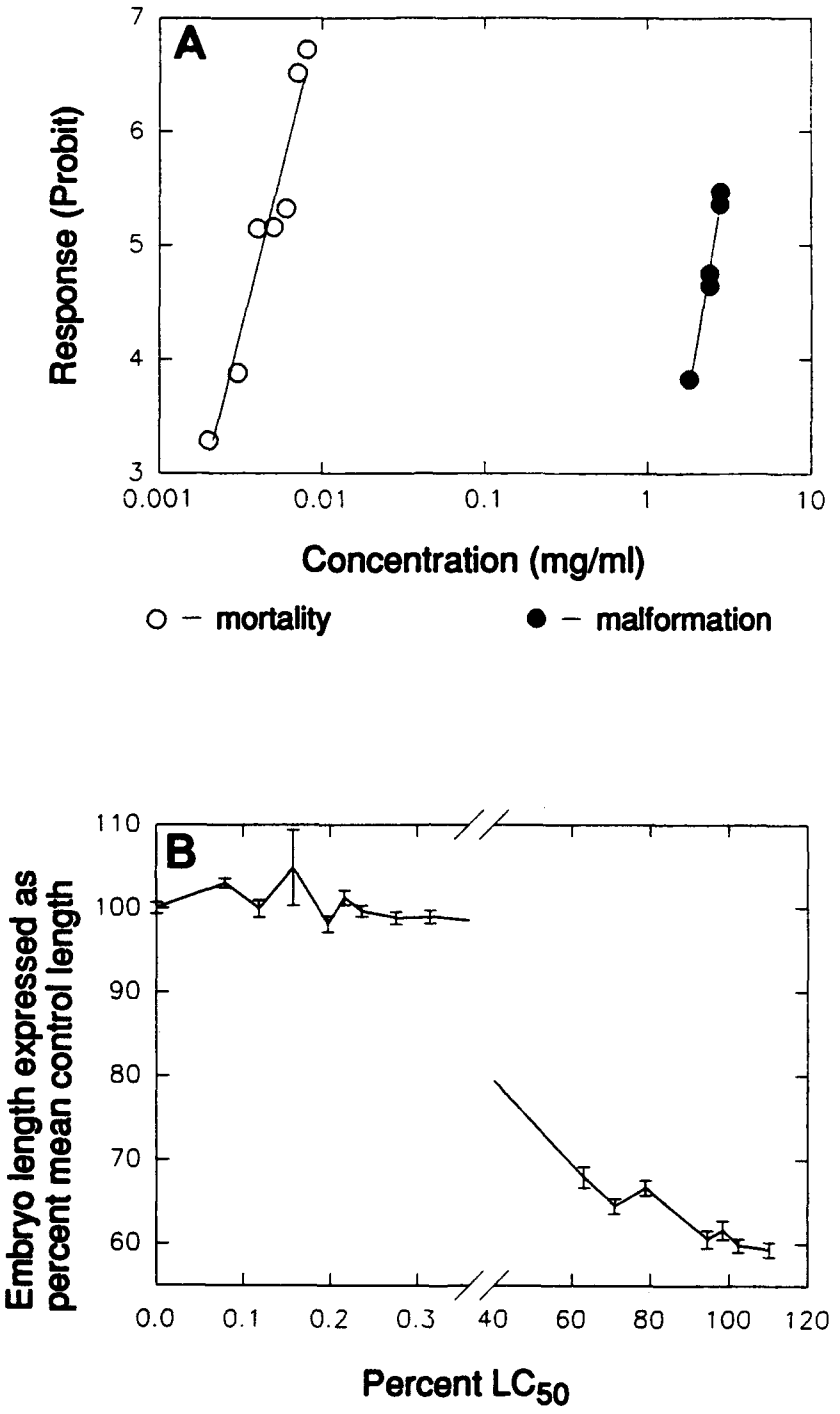

Figure 1. Representative concentration-response curves for mortality, malformation, and growth. (A) Mortality and malformation curves for 6-aminonicotinamide. (B) Growth inhibition. Note that the concentrations selected bracket the mortality and malformation ranges but not the range of growth. This particular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

Table 1. Results of FETAX Validation Studies

Substance	Units	96-hr LC_{50}	96-hr EC_{50}	TI	MCIG	MAS LC_{50}	MAS EC_{50}	TI	MAS MC/G
Acetaminophen	(mg/ml)	0.16	0.14	1.1	0.10				
Acetone	(% v/v)	2.19	1.29	1.7	1.43				
2-Acetylamino-fluorene[a]	(mg/l)	88.50	7.15	12.4		42.50	2.60	16.35	
Acetylhydrazide	(mg/ml)	12.42	0.05	248.4	0.05	11.14 1.80	0.06 0.12	185.6 15.0	0.04 0.02
Actinomycin D [FN][c]	(mg/ml)	0.02	0.22	0.1	0.02				
Amaranth	(mg/ml)	3.39	3.50	1.0	>4.0				
6-Aminonicotinamide	(mg/ml)	3.07	0.01	511.7	0.10				
Ascorbic acid	(mg/ml)	19.70	12.13	1.6	<10.00				
Aspartame	(mg/ml)	13.92	13.14	1.1	7.00				
5-Azacytidine	(mg/ml)	0.52	0.04	11.7	0.09				
Benzo[*a*]pyrene[a]	(mg/l)	>10.0	11.0	0.9		*>10.0*	*1.65*	*6.11*	*none*
Busulfan	(mg/ml)	>0.20	0.16	>1.25					
Caffeine	(mg/l)	0.26	0.13	2.0	0.10				
Chaconine (α)	(mg/l)	1.88	1.73	1.1	<4.00				
Chaconine (β1)	(mg/l)	2.52	1.75	1.4	4.00				
Chaconine (β2)	(mg/l)	>10.0	6.50	>1.5	6.00				
Chaconine (γ)	(mg/l)	>10.0	7.82	>1.28	4.00				
Copper sulfate	(mg/l)	1.19	0.13	9.2	0.05				

Table 1. Results of FETAX Validation Studies (continued)

Substance	Units	96-hr LC_{50}	96-hr EC_{50}	TI	MCIG	MAS LC_{50}	MAS EC_{50}	TI	MAS MC/G
Cotinine	(mg/ml)	4.34	0.72	6.0	0.32				
Coumarin[a] [FP][d]	(mg/ml)	0.13	0.04	3.2					
Cycloheximide	(mg/ml)	0.16	0.12	1.3	0.06				
Cyclophosphamide	(mg/ml)	1.37	0.39	3.5		>10.0	1.65	>6.06	
Cytochalasin D	(mg/l)	461.5	121.0	3.8		805.5	546.0	1.48	
Cytosine arabinoside	(mg/ml)	5.41	0.76	7.1	0.7				
Diazepam	(mg/ml)	0.03	0.02	1.6	0.01				
Dimethylsulfoxide	(% v/v)	1.18	1.31	0.9	1.50				
Diphenhydramine (HCl)	(mg/ml)	0.03	0.00	10.7	0.00				
Diphenylhydantoin[a]	(mg/l)	74.45	32.00	2.3		126.35	69.15	1.83	
Doxlyamine succinate	(mg/ml)	0.22	0.04	4.8	0.04				
Ethanol bromide	(%v/v)	1.44	1.01	1.4	1.00				
Ethidium bromide	(mg/ml)	0.05	0.04	1.4	0.05				
N-Ethyl-*N*-nitrosourea	(mg/ml)	0.26	0.05	5.0	4.87	3.70	7.30	5.07	
5-Flourouracil	(mg/ml)	1.62	0.14	11.8	0.12				
Furazolidone	(mg/ml)	0.01	0.01	2.0	0.01				
m-Hydroxyphenytoin[a]	(mg/l)	>150.0	>150.0	1.0		>150	>150		
p-Hydroxyphenytoin[a]	(mg/l)	>150.0	>150.0	1.0		>150	>150		
Hydroxyurea	(mg/ml)	1.82	0.43	4.2	0.3				

Table 1. Results of FETAX Validation Studies (continued)

Substance	Units	96-hr LC_{50}	96-hr EC_{50}	TI	MCIG	MAS LC_{50}	MAS EC_{50}	TI	MAS MC/G
Isoniazid [FP][d]	(mg/ml)	9.89	0.27	36.6	0.17	8.90 6.34	0.29 0.41	30.17 15.28	0.25 0.13[b]
Isonicotinic acid	(mg/ml)	3.18	1.26	2.5	1.25	3.29 3.29	1.51 153.0	2.18 0.02	1.13 1.27[b]
Methotrexate	(mg/ml)	0.50	0.03	19.4	0.02				
Methylmercury chloride	(mg/l)	0.08	0.02	3.6	0.04				
Naphthalene	(mg/l)	6.54	5.75	1.1					
Nicotine	(mg/l)	136.50	0.41	332.9	0.46	20.35	5.85	3.48	
Nitriloacetic acid	(mg/ml)	0.57	0.52	1.1	0.38				
N-Nitrosodimethyl-amine	(mg/ml)	3.38	2.30	1.5	1.25				
Procarbazine	(mg/ml)	3.17	1.31	2.4	1.00				
Propylene glycol	(% v/v)	2.70	1.74	1.5	1.38				
Pseudoephedrine [FP][d]	(mg/ml)	0.41	0.24	1.7	0.20				
cis-Retinoic acid	(ng/ml)	30.42	2.49	12.2	9.00				
trans-Retinoic acid	(mg/l)	0.38	0.03	11.0	0.07				
Rifampicin[a]	(mg/ml)	>2.00	>2.00	1.0	1.38	0.50	2.75		
Saccharin	(mg/ml)	18.37	19.34	0.9	17.00				
Serotonin	(mg/ml)	3.00	0.37	8.1	0.42				
Sodium acetate	(mg/ml)	4.20	3.30	1.3	2.50				

Table 1. Results of FETAX Validation Studies (continued)

Substance	Units	96-hr LC_{50}	96-hr EC_{50}	TI	MCIG	MAS LC_{50}	MAS EC_{50}	TI	MAS MC/G
Sodium cyclamate	(mg/ml)	16.14	14.84	1.1	12.00				
Sodium salicylate	(mg/ml)	2.32	1.45	1.6	1.25				
Sodium selanate	(mg/ml)	0.02	0.01	3.0	0.01				
Solanine (a)	(mg/l)	10.90	8.80	1.2	12.00				
Solasonine	(mg/l)	5.60	5.11	1.1					
Trichloroethylene	(% v/v)	0.03	0.01	3.9	0.02				
Triethylene glycol	(% v/v)	2.45	2.15	1.1	1.43				
Urethane	(mg/ml)	5.64	1.73	3.3	1.25				
Zinc sulfate [FP][d]	(mg/l)	34.40	2.69	12.8		36.70	2.88	12.72	

Abbreviations: EC_{50}, median effective concentration for malformation; LC_{50}, median lethal concentration; MAS, metabolic activation system; MCIG, minimum concentration that inhibits growth; TI, teratogenic index.

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

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

Use of Cosolvents To Solubilize Hydrophobic Compounds

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

Use of FETAX Onsite

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

Interlaboratory Study

A seven-member interlaboratory study is ongoing to determine the repeatability and reliability of FETAX data. The study is divided into three phases. In the first phase, technicians are trained and then test three compounds previously assayed in FETAX. Each lab was given the concentrations to be tested and the identity of the chemical was known. The second phase was the same as Phase I except that the study was performed using a blind testing format. In Phase III, each lab had to perform range-finding tests and then perform three full or definitive tests on each chemical using a blind testing format. Phase III closely duplicated real-world testing. Between phases, the FETAX protocol was to be reevaluated. Phase I was completed using the test compounds hydroxyurea, isoniazid, and 6-aminonicotinamide. The coefficients of variation (CVs) for the 96-hour LC_{50} and EC_{50} (malformation) were consistently below 75 except for the 6-aminonicotinamide 96-hour EC_{50} (malformation). When the results from a single lab were eliminated, the EC_{50} (malformation) CV fell well below 75. The variation was deemed acceptable and the developmental toxicity of each compound was adequately assessed. The only change in protocol that was necessitated by the Phase I study was the 6-aminonicotinamide positive control. The ASTM task force had suggested that two dishes of 25 embryos each be set up at the

anticipated 96-hour LC_{50} and two dishes at the EC_{50} (malformation). The number dead or malformed had to fall within a 10-90% response range at the end of the test. This occurred in only 32 of 42 measurements. Analysis of the full concentration-response data showed excellent overall results for 6-aminonicotinamide suggesting that the committee set too tight a range for the positive control. Contributing to the problem was the use of different lot numbers for the ASTM guide study and the interlaboratory study. This problem is now being addressed by the ASTM task force. The repeatability and reliability of FETAX data is acceptable as judged by Phase I results. The main source of variation in the study appeared to be the concentration of test chemical and not in judging malformations as expected. By the end of Phase III, the FETAX protocol should be proven.

Past and Future Developments of FETAX for Use as a Screen for Developmental Toxicants That Affect Humans

Research Areas

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

Validation Study Results

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

schemes are based on results from testing in rodents; consequently, validating an *in vitro* teratogenesis screening assay is removed from humans by an additional step. Additional uncertainty is introduced as a result, because rodent systems also have an error rate (Marks, 1991), which makes it very difficult to carry out this type of study. Additional validation studies need to be performed using chemicals for which the human developmental toxicity has already been established. These studies should be carried out in a blind testing format and always with and without a MAS. Clearly, it will not be possible to predict human developmental toxicity in all cases, but FETAX should still be useful in the preliminary screening of compounds for developmental toxicity.

Metabolic Activation System

Xenopus embryos have a limited capability to metabolize xenobiotic compounds. In order to simulate mammalian metabolism and thereby increase the predictive accuracy of FETAX, an *in vitro* MAS using rat liver microsomes was developed (Bantle and Dawson, 1988; Dawson et al., 1988*a*; Fort et al., 1988; Bantle et al., 1989a; Fort et al., 1989; Fort and Bantle 1990*a*; Fort and Bantle 1990*b*; Bantle et al., 1991; Fort et al., 1991). Initial experimentation was performed using cultured hepatocytes and whole rat liver. However, it quickly became clear that S9 supernatant or microsomes were the best choice. Unfortunately, Aroclor 1254-induced S9 supernatant proved toxic to *Xenopus* embryos. Uninduced microsomes were then tried with better results. However, it subsequently proved possible to use Aroclor 1254-induced microsomes to effectively activate proteratogens and inactivate compounds that are normally detoxified by the mammalian liver. Aroclor 1254 is a broad spectrum inducer, which helped improve the predictive accuracy to nearly 90%. Perhaps the best microsomal preparation is a 50:50 mix of Aroclor 1254-induced and isoniazid-induced rat liver microsomes, which covers a very broad spectrum of cytochrome P-450.

Even though the MAS is added daily along with fresh test compound, it must be remembered that the microsomes only work for 5 hours after addition. Any parent test compound remaining is then free to affect the test. This makes it necessary to qualify interpretation of test results. A twofold change in TI and a significant change in the severity of malformations (Fig. 2) are sufficient to lead to a conclusion that a compound is either a proteratogen or it poses little developmental toxicity because of detoxification. When a compound is evaluated for potential human or mammalian developmental toxicity, it is necessary to test with and without microsomes. Preliminary tests with limited concentrations can be performed that will indicate whether there is significant metabolism.

Future research must be performed to replace Aroclor 1254 induction with phenobarbitol and B-napthoflavone induction because of the difficulty of disposing of treated rats and the variability in lots of Aroclor 1254. It is possible that a continuous flow apparatus can deliver toxicant and MAS continuously so that the embryos are exposed to parent compound, intermediate metabolites, and metabolites. This procedure will help in data interpretation. Finally, work must be done on modeling human placentation if FETAX is to attain its maximum predictive accuracy. No matter how well developed and validated FETAX becomes, it will always have a failure rate.

Past and Future Developments of FETAX for Use in Ecotoxicology

Research Areas

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

Surface Water, Groundwater and Sediment Extract Testing

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

FETAX has been tested with metal-contaminated surface waters and sediments, as well as with groundwater contaminated with volatile organics (Dawson et al., 1984; Dawson et al., 1988*b*;

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

Bantle et al., 1989*b*). FETAX also detected chemicals causing high malformation rates in 8 of 12 Yellowstone National Park water samples (Bantle and Peterson, unpublished). Because FETAX is a 4-day test, it is fast enough to be used in TI/Toxicity Reduction Evaluation (TRE) studies. The department of Ecology of the State of Washington is currently evaluating FETAX as a test for soil and sediment toxicity as a part of their biomonitoring program. A field validation program is required in order to prove that data collected with FETAX can be used to explain developmental toxicity in wild amphibian populations. Additional work needs to be performed with sample preparation and evaluation of the role of FETAX in multispecies testing formats.

In Situ Applications

Linder and co-workers have been developing methods for *in situ* applications of FETAX at contaminated waste sites (Linder et al., 1990). They have developed plastic mesh exposure cages to allow *in situ* exposure of developing *Xenopus* embryos. The exposure cage is placed into the test matrix (e.g., sediment and water column) at locations in the field and then secured with stainless steel stakes. This *in situ* exposure method recently has been used at an abandoned mine site in Montana. Care must be taken in performing these experiments to prevent the release of organisms in areas where the frogs can over-winter.

SUMMARY

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

It is likely that multispecies testing is needed to adequately safeguard the environment. Additionally, acute toxicity tests alone may not suffice. It is likely that reproductive and developmental toxicity tests will be needed along with mutagenesis, carcinogenesis, and other testing to adequately evaluate the hazards posed by environmental toxicants. An effort is already being made to replace FETAX with even simpler cellular based tests. FETAX will serve to indicate the predictive value of these new tests. Results of these tests will be compared with

results from FETAX. Because a consistent protocol was followed in FETAX, it will be possible to compare results. Additional efforts must be expended to ensure that developmentally relevant, rapid, and inexpensive tests are available to the scientific community.

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QUALITY ASSURANCE ISSUES IN THE CONDUCT OF SMALL FISH CARCINOGENESIS BIOASSAYS

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ABSTRACT

The Good Laboratory Practices (GLP) regulations came out of audits conducted by the Food and Drug Administration (FDA) on toxicologic studies performed in support of the registration of products of pharmaceutical companies. The audits revealed serious deficiencies in the conduct of product safety tests in laboratory animals. The GLP regulations, in addition to other mandates, require that a facility that conducts nonclinical laboratory tests have a Quality Assurance Unit to monitor the test facility, the conduct of a study, and the reporting of the study results. The GLP regulations apply to tests that use fish as well as to tests that use mammals. This paper is a discussion of GLP regulations and Quality Assurance issues as they might apply to carcinogenesis bioassays with small fish as the test system.

INTRODUCTION

Quality in a toxicologic test should be inherent, especially in tests of compounds to assure safety to human beings. Unfortunately, that has not always been the case. Until the mid-1970s, it was more or less assumed that reports submitted to the FDA in support of the registration of a product accurately represented the study conduct and results. In the 1970s, however, the FDA began to audit studies that had been conducted for major pharmaceutical manufacturers; their inspections revealed unacceptable or questionable laboratory practices and inconsistencies in data that had been submitted to the FDA. As a result of these revelations concerning information submitted to the FDA in support of regulated products, the GLP regulations were promulgated (FDA, 21 CFR Part 58; EPA, 40 CFR Part 792; EPA, 40 CFR Part 160). It is difficult to discuss Quality Assurance in the conduct of a study without including discussion of GLPs, because an organized functional Quality Assurance Unit, considered an essential part of toxicology testing today, was mandated by the GLP regulations legislated in the late 1970s. The activities of the U.S. Army's aquatic toxicology laboratory at Ft. Detrick, Maryland, are not required to be conducted according to GLP regulations, but references are made to GLPs because the GLP regulations are an accepted standard for quality in toxicologic testing.

DISCUSSION

Good Laboratory Practices Regulations

Why were the GLP regulations needed? When the FDA audited the studies that were conducted for various pharmaceutical companies, they identified a variety of problems. There was evidence of poor study design and conduct. The studies were not monitored properly, and recordkeeping and data review were inadequate. Qualifications and training of personnel involved in a study were not properly documented.

Laboratory and data management procedures were not followed. For example, animals were mixed up as to treatment groups, animals were not being dosed as required, animals were listed as dead but later reported as alive, and pathology specimens were lost. In some cases, data on incidence of neoplasia were excluded during evaluation of the test results. There was also failure to verify that final reports were accurate and complete.

As a result of these FDA investigations, criminal charges of fraud were brought and convictions were handed down. Most importantly, perhaps, a great concern was engendered for the validity of toxicological studies in general, those completed and those in progress.

The GLP regulations came from this need by the FDA and by the Environmental Protection Agency (EPA) to assure that the data used in decisionmaking on regulated products were sound. Many of the GLP requirements are "common sense" sorts of requirements. GLPs require that each test have a protocol. The required contents of the protocol are spelled out. GLPs require that a testing laboratory maintain a set of Standard Operating Procedures that are kept current to reflect changes in procedures. There are standards set for laboratory facilities such as requirements for animal rooms, separate storage areas for feeds and bedding supplies, and separate areas for handling test materials. Equipment must be inspected, cleaned, and maintained regularly and calibrated, if appropriate, before use. Test and control materials have to be characterized as to identity, strength, quality, and purity. GLPs define what records are to be kept and how the records are to be maintained. They also detail what is to be included in the final report of a study.

These requirements reflect the demand for the basic experimental structure necessary for conducting any high-quality, scientifically sound study. The GLP regulations have gone beyond these basics, however, by requiring certain additional securities for every study, including a Study Director, who assumes overall responsibility, and a Quality Assurance Unit. The Study Director and the Quality Assurance Unit, in cooperation with the management of the testing facility, are responsible for assuring that a test is properly conducted. The testing facility management

designates the Study Director and assures that there is a functional Quality Assurance Unit (Baldwin and Hoover, 1986).

The Study Director has the overall responsibility to interpret, analyze, document, and report the results of the study, and represents the single point of study control. The Study Director is responsible for and must sign the protocol for a study and must provide a copy of the protocol to the Quality Assurance Unit. The protocol for a study is directive; it is not the documentation. Adherence to the protocol directive is confirmed through the documentation of the conduct of the study.

Quality Assurance

Quality Assurance in a testing laboratory is an auditing examination process that oversees all aspects of a study from its conception in the protocol to the review of the final report (Berny et al., 1989; Morris, 1989; Schroeder, 1989; Simon, 1986). The Quality Assurance Unit accomplishes its task by monitoring the study conduct, the test facility, and the final report of the study. These monitoring activities insure compliance with GLPs. The Quality Assurance Unit is an independent unit that reports to management. The unit's personnel may not participate in both the Quality Assurance monitoring and the actual conduct of a particular study.

The Quality Assurance Unit depends upon documentation through both physical observations of procedures and review of data generated during the course of a study. The documentation procedures are designed with the objectives listed here:

1. To determine if quality control operations are being carried out. For example, quality control might dictate that a pH meter be calibrated against standard buffer solutions each day it is used. The Quality Assurance Unit is able to monitor this quality control task because the results of this calibration must be written down in a notebook each day the pH meter is used.
2. To assure accountability of data. For example, if certain water samples are to be taken, safeguards should be in place to minimize the possibility of a mix-up of samples taken from various aquaria.
3. To assure traceability of reported data. For example, water quality data recorded for a particular day during a test should be traceable to the individual who performed the procedure, the instruments that were used, and the status of the quality control system for that instrument at the time the water quality parameter was measured.

4. To demonstrate that precautions are being taken against the chance that data might be falsified. For example, certain requirements must be met if data are corrected. The original data cannot be obscured by erasure or white-out. A line is drawn through the entry that is in error, the correction is made, a reason for the change is stated, and the correction is initialed and dated.

Appropriate documentation that facilitates the Quality Assurance function is implemented to a great degree by a facility's Standard Operating Procedures. Standard Operating Procedures are mandated by the GLP regulations.

Facility and Study Audit, Research Methods Branch of the U.S. Army Biomedical Research and Development Laboratory

In 1991, the Research Methods Branch requested an audit of its aquatic animal laboratory facility, including an audit of one of the medaka carcinogenesis bioassays (Loeb et al., 1989). Interesting differences between fish and mammals had to be considered as the study data were evaluated. With mammals, it is required that individual animals be uniquely identified. This is not possible with the small aquarium species used in the U.S. Army's tests, so the requirement for fish is satisfied by adequate identification of the aquarium containing the fish by test number, group number, and the concentration of test material. This method of identifying fish by groups is spelled out in the EPA Toxic Substances Control Act Aquatic Guidelines for Fish Acute Toxicity Tests (EPA, 40 CFR Parts 796, 797, and 798).

With mammals, it is easy to keep track of the total number of animals on test at one time because the animals are housed individually or in small groups and are easily counted. With fish, it is virtually impossible to count all the fish at any one moment because there may be 50 or more fish in constant motion in one aquarium. Also, it is conceivable that a fish could die unnoticed late in the day and disintegrate or be cannibalized before the fish are observed again the following day. This phenomenon must be considered when there is a discrepancy between the total number of fish in a group at the beginning of a study and the total number at the end. Except in certain circumstances, GLPs require that sexes be housed separately during a test. But this is not possible with certain species of small aquarium fish, because males and females cannot be distinguished at the time the fish are started on test.

Environmental conditions that must be monitored in mammalian animal rooms include air temperature, humidity, and photoperiod. With fish, environmental conditions include water temperature, photoperiod, light intensity, and a battery of physical and chemical qualities of the

water including such parameters as dissolved oxygen, un-ionized ammonia, conductivity, and hardness. This means that documentation of proper environmental conditions for fish requires more paperwork than for mammalian testing situations.

During tests with mammals, individual animals are weighed periodically throughout the study. Fish, especially small aquarium fish, are not usually weighed during testing because this procedure would be stressful to the fish. In fish nutrition studies, when body weight data are critical to the interpretation of the study, fish are often weighed periodically as a group, and an average weight is determined. In a toxicologic test, the fish are usually weighed individually only at the end of the test, after euthanasia.

After the audit of the U.S. Army facility and study, a report was issued. The report noted some deficiencies in the conduct of the study against the background of GLP regulations. One outcome of the report was that Standard Operating Procedures for the Research Methods Branch were to be reorganized and rewritten. The final document will include 250-300 individual Standard Operating Procedures for the Research Methods Branch.

The Standard Operating Procedures or "Standing" Operating Procedures, as they are called by the U.S. Army, will include a section on Quality Assurance and associated areas such as protocol preparation, recordkeeping, reporting requirements, initiating and revising Standing Operating Procedures, and archiving raw data, specimens, and reports.

One function of the Quality Assurance Unit that is defined in the new Standing Operating Procedures is that of examining critical phases of each toxicologic test while the test is in progress for compliance with the written protocol and with the Standing Operating Procedures that are operative for a particular study phase.

Critical Phases of Small Fish Bioassays

The potential critical phases of small fish bioassays that are to be observed by Quality Assurance personnel are outlined in the Standing Operating Procedures, and those pertinent to a given test are defined in the study protocol. For a test with aquatic animals, fish in this case, some critical phases that might be considered for Quality Assurance inspection purposes are as follows:

1. Test system identification and housing. In fish studies, the test system is identified as a group of fish in a particular aquarium. Quality Assurance personnel would verify that the identification of the aquarium is adequate and that the fish are housed in the size and type of aquarium specified by protocol.

2. Test substance preparation. Quality Assurance personnel would observe preparation of the test substance. If the test material is made up in a particular concentration, such as would be done in the static exposure of medaka to diethylnitrosamine, the Quality Assurance personnel would observe the preparation of the required concentration. In cases when fish are exposed to varying concentrations being delivered by a proportional diluter, the Quality Assurance reviewer's observation might include the calibration of the diluter as to cycle time and volume distribution, and the collection of samples to verify that the system is set up to deliver protocol-required concentrations of test substance to particular aquaria. Verification is finally accomplished after review of the chemical analysis of the samples that are taken.
3. Addition of test substance and the test system to test aquaria. Quality Assurance personnel would observe the addition of test substance to the aquaria and also the process of placing fish in the aquaria including randomization procedures.
4. Water quality analysis. Quality Assurance personnel would observe how water samples are collected for water quality analysis as well as for chemical analysis. The chemical analyses could be to check the dilution water for contaminants or to verify the concentration of test material in the dilution water.
5. Fish euthanasia. Quality Assurance personnel would observe the euthanasia procedure and how the fish are weighed and measured.

Records of these critical-phase inspections are maintained. If there are problems, these are brought to the attention of management and the Study Director. Actions recommended for solution of problems and actions taken to solve problems are documented in the Quality Assurance inspection report. A date may be scheduled for reinspection of some study phase if it is warranted.

CONCLUSION

The FDA and the EPA periodically inspect laboratories that conduct nonclinical laboratory tests. Laboratory operations and study documentation have improved significantly since the implementation of the GLP regulations (Lepore, 1986), but departures from GLP regulations still occur and are cited during inspections. Some significant departures include discrepancies between raw data and the final report, failure to correct raw data properly, failure to follow the protocol, and failure to update the Standard Operating Procedures.

A conscientious Quality Assurance Unit can be highly beneficial during the conduct of a toxicologic test. One benefit is that the unit puts a testing facility into compliance with GLPs. This is secondary, however, to the help the Quality Assurance Unit provides in anticipating and preventing problems, enabling improved facility operation and quality of studies, and creating heightened awareness in all levels of laboratory personnel.

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MODELING AND STATISTICAL ANALYSIS OF MEDAKA BIOASSAY DATA

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ABSTRACT

A histopathologic examination of tissues from *Oryzias latipes* (Japanese medaka fish) was performed to evaluate the carcinogenic potential of trichloroethylene (TCE) in groundwater. The data were reported by Experimental Pathology Laboratories, Inc., in a report dated January 19, 1990, submitted to the Army Biomedical Research and Development Laboratory, Ft. Detrick, Maryland. This paper provides a brief statistical analysis of some aspects of those data. The analysis does not reveal a strong positive relationship between TCE concentration over the range considered and probability (risk or hazard) of incurring at least one end-point manifestation (here, cystic degeneration or liver neoplasm) in a fish. Uncertainties in the point estimates are assessed by bootstrapping. Both nonparametric (weak statistical assumptions) and parametric (stronger statistical assumptions) analyses give similar inconclusive dose-response indications. A brief discussion is included of a biologically based mathematical model that is likely to form an appropriate basis for more sophisticated data analysis. One contribution of this paper is to discuss and illustrate techniques for quantitative analysis of other similar data. The methods can also be used to assist in choosing an experimental design.

INTRODUCTION

The Japanese medaka fish (*Oryzias latipes*) has come to be of great interest as an indicator of groundwater toxicity; see Van Beneden et al. (1990), Gardner et al. (1990). The Research Model Branch, Health Effects Research Division of the Army Biomedical Research and Development Laboratory, Ft. Detrick, Maryland, has conducted extensive experimentation with medaka on its response to various known or suspected toxic agents or carcinogens. This paper provides a statistical analysis of data from such an experiment. Analysis provides a quantitative and focused perspective on the message of the data that usefully supplements the more usual simple qualitative observations.

Design of Experiment

The experiment was planned and conducted as follows, and the data were analyzed. Eight groups of medaka were treated as shown in Table 1. Those groups treated with diethylnitrosamine (DEN) received pretreatment with 10 mg/l for 48 hours at 17 days after hatch. The trichloroethylene (TCE)-treated groups received various concentrations (100%, 50%, 25%, and 0%) on a biologically based scale: 100% refers to undiluted groundwater containing TCE, and 50% and 25% refer to corresponding dilutions with pure water.

Table 1. Treatment Combinations and Cystic Degeneration Responses

Group	Treatment			Number of fish with symptom/ Number of fish killed at sacrifice time of	
	DEN	no DEN	%TCE	3 months	6 months
1		X	0	6/25	4/15
3		X	25	4/25	5/13
5		X	50	2/25	4/14
7		X	100	3/25	3/14
2	X		0	11/25	6/12
4	X		25	4/25	8/13
6	X		50	6/25	5/12
8	X		100	7/25	3/8

The individual fish were assigned to tanks of water, presumably maintained at standard temperature, also presumably in a random manner. There do not appear to have been replicate tanks. After 3 months an interim sacrifice was made of 25 fish in each group; the number of fish showing cystic degeneration (CD) after 3 months and 6 months appear to the left of the slash in the table. Thus Group 1 contained 6 fish out of 25 with CD after 3 months, and 4 out of 15 after 6 months. In the latter case 15 fish were exposed to the original concentration for the entire 6 months; this is referred to as the *chronic group*. Another group, the so-called *recovery group*, was placed in pure water for the second 3-month period. This group's response is not analyzed in this paper. Table 2 reports the incidence of liver neoplasms for the same fish in groups 2, 4, 6, and 8 that were pretreated with DEN.

Table 2. Incidence of Liver Neoplasms in Medaka for Groups Pretreated with DEN

Group	Treatment	Number of fish with symptom/ Number of fish killed at sacrifice time of	
	%TCE	3 months	6 months
2	0	0/25	1/12
4	25	2/25	3/13
6	50	0/25	1/12
8	100	2/25	4/8

Model-Based Analysis

The experimental outcomes are viewed from the following perspective. Each individual fish subjected to a particular DEN-TCE treatment (e.g., DEN = 0, TCE = 50%) is initially thought of as a member of a population of similar fish. For various reasons, including that of genetic diversity, the individual fish will exhibit particular symptoms, i.e., reach specified biological end points such as cystic degeneration or neoplasms within specified organs, at widely different times. In addition, some fish may die before any such symptoms manifest themselves. Consequently, it is reasonable and natural to think of the occurrence of a particular end point as a probabilistic (or random, or stochastic) phenomenon, much like a coin flip or dice throw, or in the same way that actuarial scientists regard human life durations when proposing life insurance contracts. That is, let T, the time to occurrence of a particular end point, such as cystic degeneration, be a *random variable*, a quantity whose value (for a particular fish) is determined by sampling from a population with a fixed distribution function that in turn depends upon the treatment of interest (DEN and TCE), but also upon water temperature and presence of other elements in the fish tank, and also individual fish traits. This distribution function is

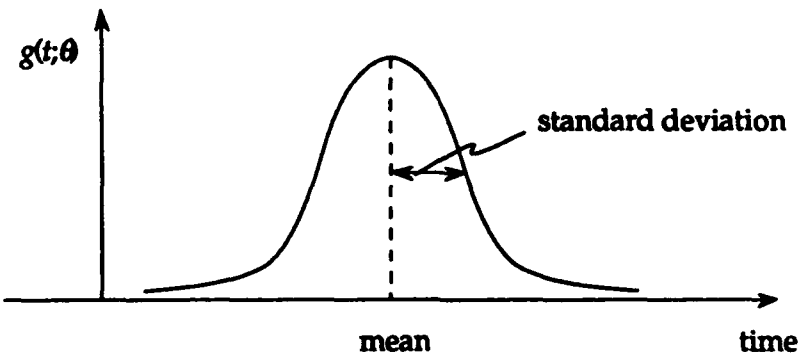
$$\begin{array}{l}\text{Population fraction of fish with symptom} \\ \text{Times, } T\text{, less than } t \text{ } (t = 3 \text{ months, } 6 \text{ months})\end{array} = G(t,\theta)$$

The population parameter values that identify the particular distribution are called $\theta = (\theta_1, \theta_2, \ldots, \theta_p)$. For instance, θ_1 might be the population mean, and θ_2 the population standard deviation. Occasionally the specific distribution used to model the variability in a population of times is

suitably assumed to be *normal*, i.e., its density function

$$g(t;\theta) = dG/dt = e^{-\frac{1}{2}(t-\theta_1)^2/\theta_2^2} \Big/ \sqrt{2\pi}\theta_2,$$

is plotted as the familiar bell-shaped curve:



However, frequently that data variability is better described as *lognormal*: Logarithm of $T = X$ has a bell-shaped density, for the raw T-values tend to straggle off to the right, i.e., the density of T is (possibly) "positively skewed." A simpler form that may be appropriate is the *exponential*:

$$G(t;\lambda) = 1 - e^{-\lambda t}.$$

In this model, $\lambda = 1/$(mean time to exhibit symptoms, e.g., CD). We will use this exponential model below in an illustrative analysis. Still another form that may be appropriate for describing the time to the onset of a cancerous growth is the Weibull, which describes an increasing, or decreasing, time of exposure effect, depending upon data requirements. We describe below a distribution that arises from plausible biological assumptions, particularly when cancer is considered—the Moolgavkar family of clonal expansion models (Moolgavkar, et al. 1979). Use of the latter "biologically based" family requires that at least four parameter values be estimated from data. In light of the current experimental design, such models are somewhat difficult to identify.

The exponential distribution will be used in this report to illustrate a parametric analysis (one using a specific assumed form for $G(t;\theta)$).

Use of the Conceptual Model

Since TCE is a toxic substance it might be anticipated that (a) the mean or average fraction of fish exposed to x% of TCE that exhibit symptoms after t = 3 months (the first 3 months) would increase with x (the TCE concentration); likewise for 6 months of (chronic) exposure; and (b) the mean fraction of the fish that survive the first 3 months that exhibit symptoms in the second 3 months (between 3 months and 6 months of exposure) *might* increase over the mean fraction in the first 3 months. The latter occurrence would imply an *increasing hazard* property attributable to dosage with TCE. The increasing hazard property is consistent with the idea that prolonged exposure (to TCE here) increases the chances that particular end points will occur as time goes on. However, evidence for such behavior from the current data is not strong in light of the uncertainties associated with sampling errors as assessed by bootstrapping.

NONPARAMETRIC ANALYSIS

Suppose the above general sampling mode prevails. Then an estimate of the probability that a fish exhibits a particular symptom, e.g., CD, within time t (= 3 months) is

$$\text{Estimate of probability that } T \le t \text{ (=3 months)} \equiv \hat{G}(3;\theta) = \frac{\text{number of fish sacrificed at } t \text{ (= 3) that exhibit symptom (e.g., CD)}}{\text{number of fish exposed (for } t = 3)}$$

This estimate is easily calculated for all treatments; however, sometimes it is zero. This suggests that the number of fish exposed is too small to be detectably influenced by the dosage; in general we might expect *some* response. Since $G(3;\theta)$ is the so-called hazard associated with the appearance of the particular symptom during the first 3 months of exposure, $\hat{G}(3;\theta)$ is an estimate thereof on the basis of only 25 exposed fish; for a different 25 fish treated equivalently we generally anticipate a different numerical value of $\hat{G}$. By *resampling* (bootstrapping) it is possible to appraise the sample variation in the estimate $\hat{G}$: sample from a binomial distribution with $\hat{G}$ being the probability of "success" = symptom occurrence with t = 3, and N (= 25), the number exposed, to obtain a pseudosample or bootstrapped sample number of fish exhibiting the symptom, and from this, divided by N, a possible sample value of hazard, i.e., $\hat{G}_1$. Repeat to get

$\hat{G}_2$, again to obtain $\hat{G}_3, \ldots, \hat{G}_B$, where B is "large." The sampling has been repeated $B = 500$ times. Then compute

$$\text{Variance } \hat{G} = \frac{1}{B}\sum_{b=1}^{B}\left(\hat{G}_b - \overline{G}_B\right)^2$$

and the standard error of the original estimate, $\hat{G}$, is SE[$\hat{G}$] = (variance $\hat{G}$)$^{\frac{1}{2}}$ where $\overline{G}_B$ is the sample mean of the bootstrap estimates; $\overline{G}_B = \sum_{b=1}^{B}\hat{G}_b / B$. For data from the first 3-month period, the above standard error can actually be calculated directly (no resampling necessary): SE ($\hat{G}$) = [$\hat{G}$ (1-$\hat{G}$)/N]$^{\frac{1}{2}}$ but this formula approach is not so easy for the second 3-month period. Roughly speaking, the true value of $G(3)$ lies within $\hat{G}$ - 2SE[$\hat{G}$] and $\hat{G}$ + 2SE[$\hat{G}$]. So an estimate, and an error estimate, for initial 3 months' hazard is obtained. See Table 3 for quoted estimates and standard errors (in parentheses).

To compare to the second 3 months' hazard compute

Estimate of probability that $T \le t = 6$ months, Given that $T > t = 3$ months $= \dfrac{\hat{G}(6,\theta)-\hat{G}(3,\theta)}{1-\hat{G}(3,\theta)} \equiv \hat{G}(6,3;\theta)$

$=$ Estimate of second 3 months' hazard.

Notice that the estimates $\hat{G}(6;\theta)$ and $\hat{G}(3;\theta)$ must be obtained from different sets of counts, just as was done earlier, and consequently that there is no guarantee that $\hat{G}(6;\theta)$ is greater than $\hat{G}(3;\theta)$. Although no case of such reversal occurs in the present data, a few reversals have occurred when resampling or bootstrapping is done; in such cases the hazard value is set equal to zero. Standard errors of the second 3 months' hazards are calculated by bootstrapping $\hat{G}(6,2;\theta)$ by resampling for each component, $\hat{G}(6;\theta)$ and $\hat{G}(3;\theta)$, and combining as in the formula above.

Tables 3 and 4 summarize the results of the point estimates and their standard errors. Table 3 refers to CD, and Table 4 addresses neoplasms. Figures 1 through 5 graphically display the actual hazard sampling variations as assessed by bootstrapping. Figures 1-3 present boxplots of the bootstrap sample hazards $\hat{G}_b(3;\theta)$ and $\hat{G}_b(6,3;\theta)$.

The following description of the boxplot is taken from the documentation of GRAFSTAT, a developmental product of IBM that the Naval Postgraduate School is using under a test agreement with IBM.

The box portion of the plot extends from the lower quartile of the sample to the upper quartile. (The lower quartile is the point for which one quarter of the sample lies below and three quarters above. The upper quartile is analogous.) The line across the center of the box marks the median. The circle in the box represents the mean.

The distance from the lower to the upper quartile is called the **interquartile distance**, and it will be represented by Q. The points at the ends of the two lines (called **whiskers**) are the smallest and largest points, respectively, within 1.5 Q of the quantiles. The points beyond the whiskers are outlying values.

Figures 1 and 2 present the boxplots for the CD hazards. Figure 3 presents boxplots for the neoplasm. The boxplots are grouped by level of TCE, which is indicated at the bottom of the figure. The left boxplot in each grouping is for the 3-month hazard. The right boxplot in each group is for the 6-month hazard. Comparison of the boxplots for the 3- and 6-month hazards in Figures 1 and 2 suggests that the 3- and 6-month hazards are roughly the same. Comparing Figures 1 and 2 suggests that the pretreatment with DEN tends to increase the hazard. Comparison of the 3- and 6-month boxplots in Figure 3 suggests the respective hazards are the same except for the 6-month hazard at the 100% TCE level, which appears to be somewhat higher than the others for neoplasms.

Figure 4 presents the histograms of the CD hazard bootstrap samples. Once again the major effect seen is the increase in hazard for the fish pretreated with DEN.

Figure 5 presents the histograms of the neoplasm hazard bootstrap samples. Once again the only histogram that appears different is the histogram for the 6-month hazard at 100% TCE.

Conclusions

The general conclusion from the above analysis is that there is only a weak effect from TCE treatment change, regardless of whether DEN is used. The effect of DEN is noticeable: the second 3 months' hazard is always somewhat larger when DEN is used than is the case with no DEN. This is anticipated, but the quantitative degree of enhancement may be of interest.

Table 3. Nonparametric Hazard for Cystic Degeneration
(Bootstrap Standard Error)

Group	Treatment			Estimated hazard (standard error) $[(\hat{p}\,\hat{q}/25)^{\frac{1}{2}}]$ for sacrifice time	
	DEN	no DEN	%TCE	3 months	6 months
1		X	0	0.24 (0.09) [0.09]	0.04 (0.11)
3		X	25	0.16 (0.07) [0.07]	0.27 (0.16)
5		X	50	0.08 (0.05) [0.05]	0.22 (0.14)
7		X	100	0.12 (0.07) [0.06]	0.11 (0.11)
2	X		0	0.44 (0.10) [0.10]	0.11 (0.20)
4	X		25	0.16 (0.07) [0.07]	0.54 (0.17)
6	X		50	0.24 (0.09) [0.09]	0.23 (0.18)
8	X		100	0.28 (0.09) [0.09]	0.13 (0.19)

Table 4. Nonparametric Hazard for Neoplasms
(Bootstrap Standard Error)

Group	Treatment	Estimated hazard (standard error) $[(\hat{p}\,\hat{q}/25)^{\frac{1}{2}}]$ for sacrifice time	
	%TCE	3 months	6 months
2	0	0 (0) [0]	0.08 (0.07)
4	25	0.08 (0.05) [0.05]	0.16 (0.12)
6	50	0 (0) [0]	0.08 (0.07)
8	100	0.08 (0.06) [0.05]	0.46 (0.16)

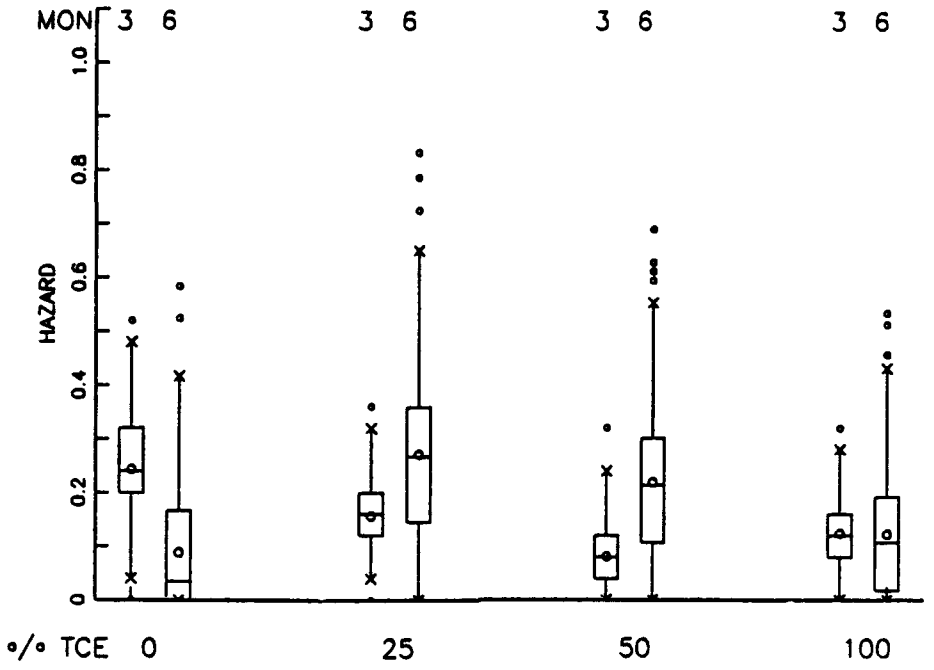


Figure 1. Nonparametric analysis of hazard for cystic degeneration (CD), as assessed by bootstrapping, for fish exhibiting the symptoms of treatment with trichloroethylene (TCE, at percentages shown on *x*-axis) and killed at 3 or 6 months. These fish, from groups 1, 3, 5, and 7, were not pretreated with diethylnitrosamine (DEN). Boxplots are explained in the text.

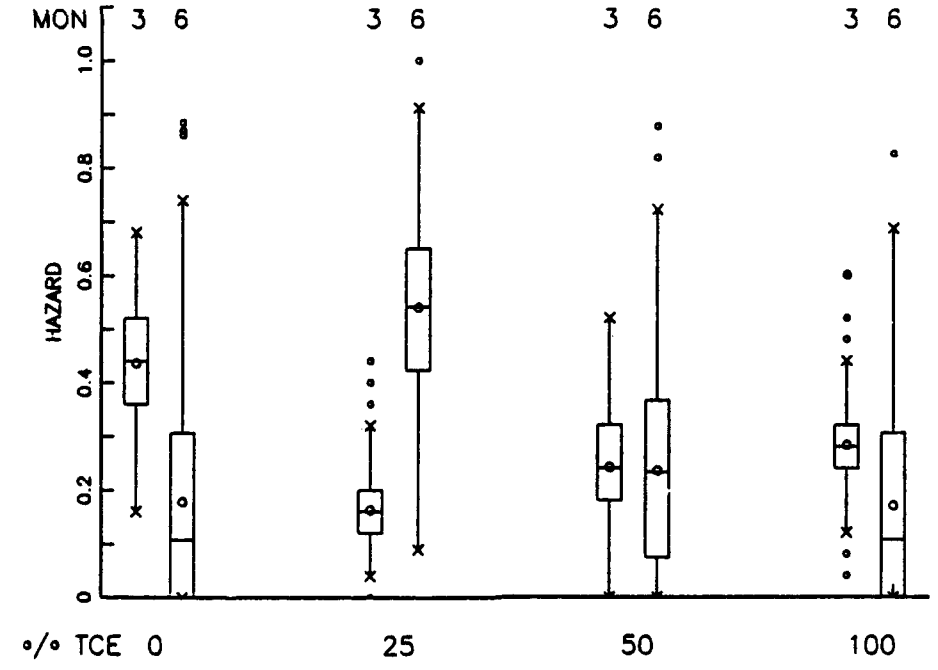


Figure 2. Nonparametric analysis of hazard for CD, as assessed by bootstrapping, for fish exhibiting the symptoms of treatment with TCE (at percentages shown) and killed at 3 or 6 months. These fish, from groups 2, 4, 6, and 8, were pretreated with DEN.

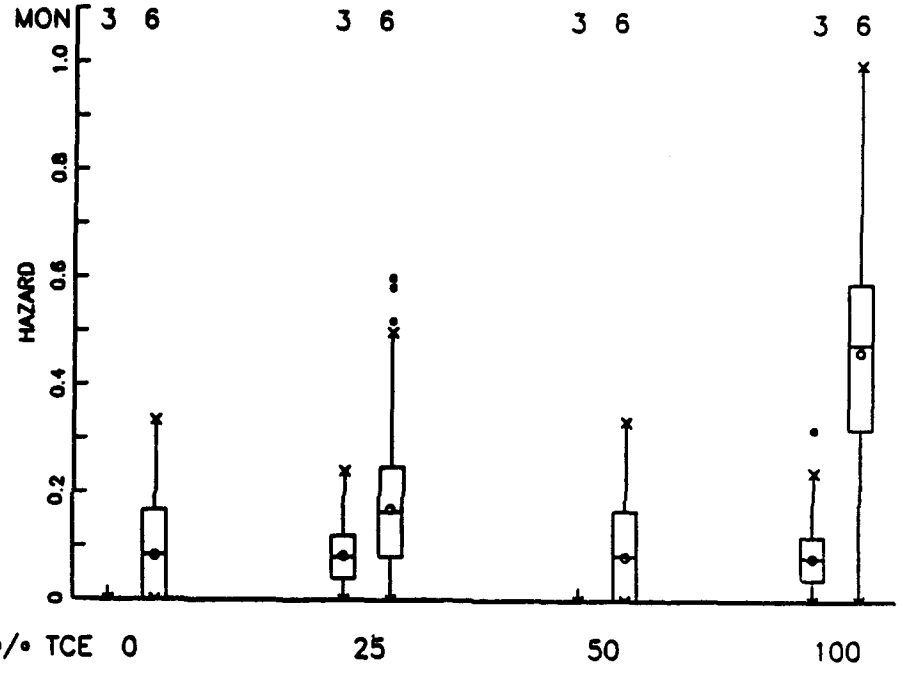


Figure 3. Nonparametric analysis of hazard for neoplasms, as assessed by bootstrapping, for fish exhibiting the symptoms of treatment with TCE (at percentages shown) and killed at 3 or 6 months. These fish, from groups 2, 4, 6, and 8, were pretreated with DEN.

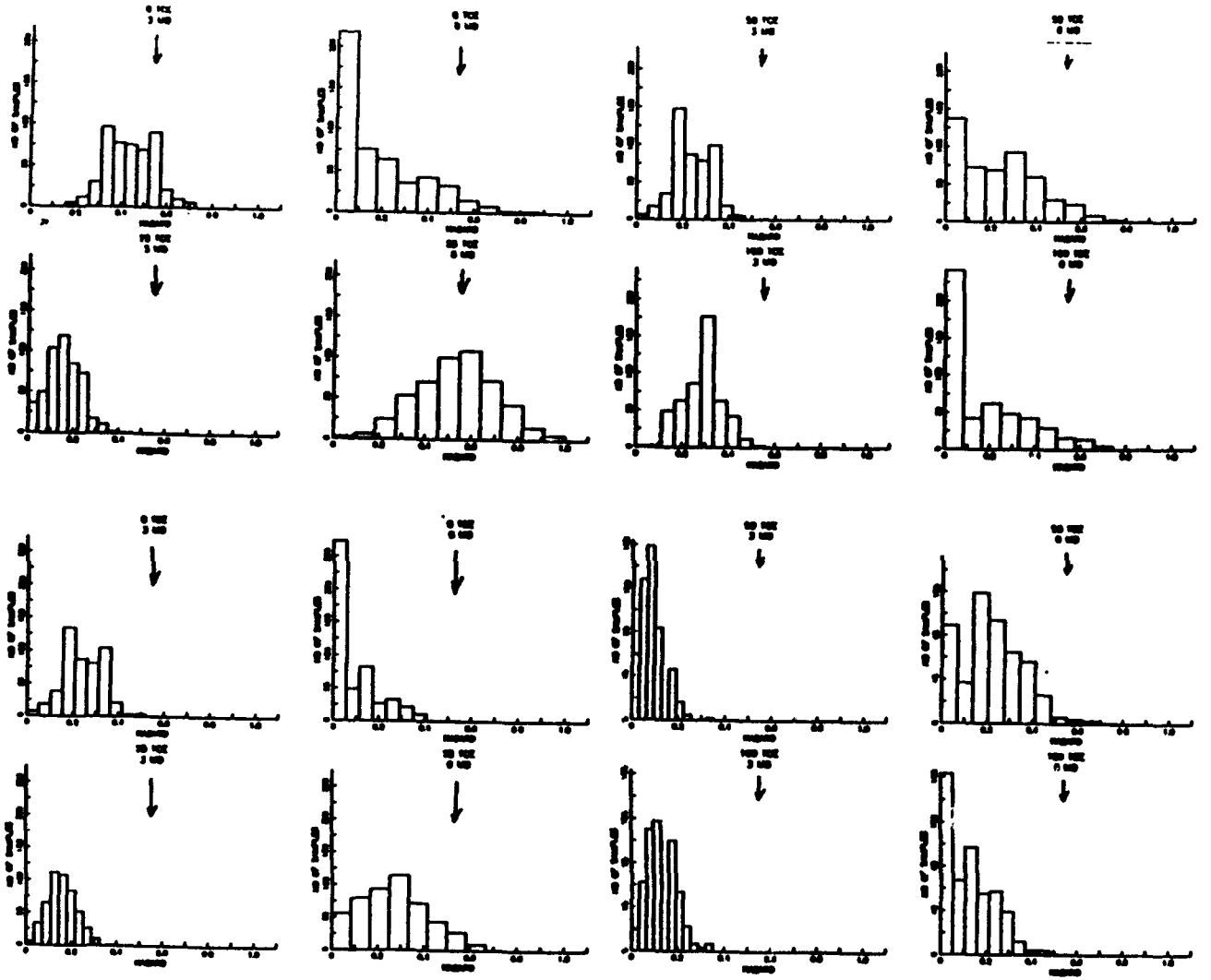


Figure 4. Sample distribution for hazard of CD, hazard assessed by bootstrapping, for fish exhibiting the symptoms of treatment with TCE (at percentages shown) and killed at 3 or 6 months. Histograms for fish pretreated with DEN are in the top half of the figure; those for fish not pretreated with DEN are shown in the lower half of the figure.

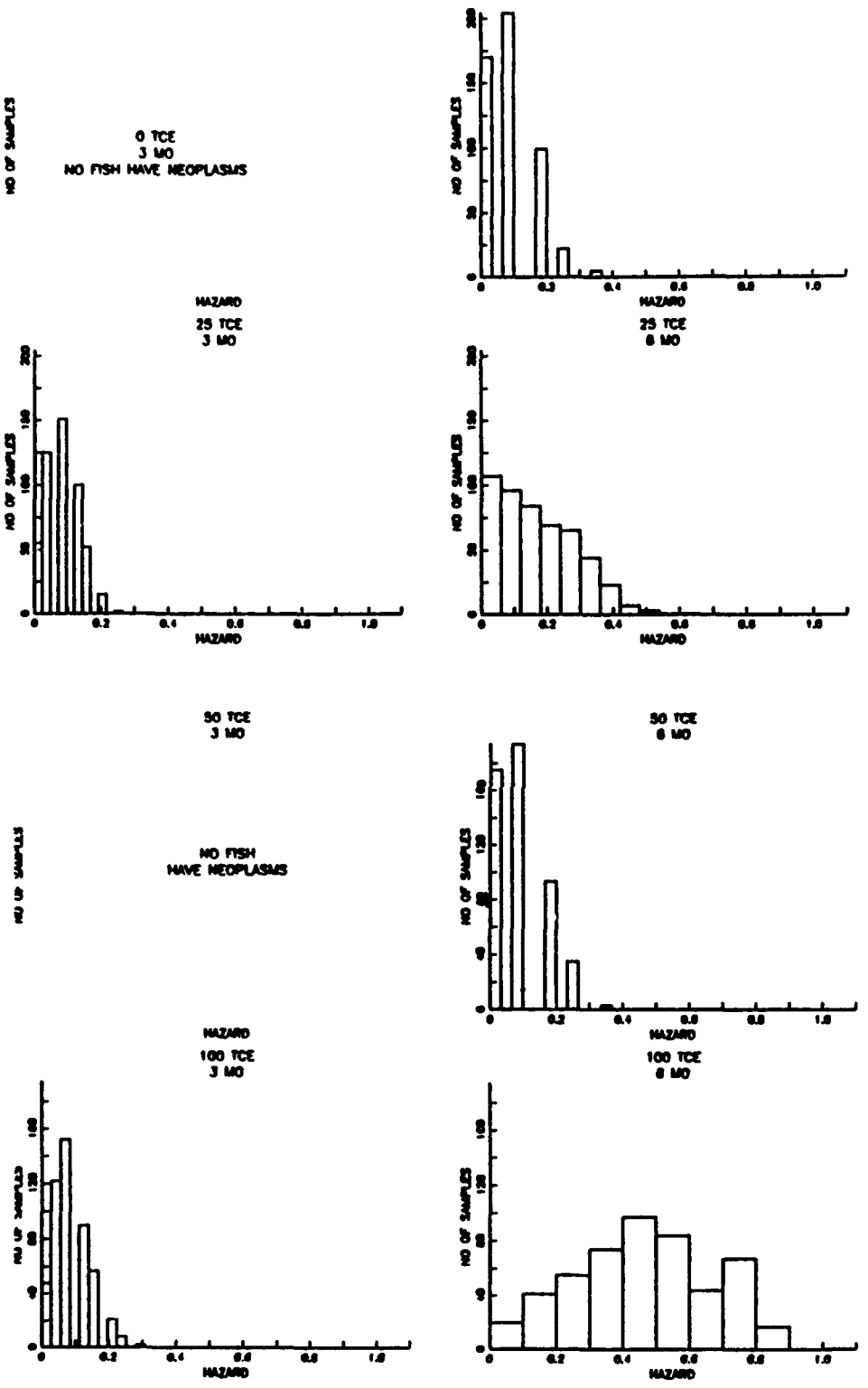


Figure 5. Sample distribution for hazard of neoplasms, hazard assessed by bootstrapping, for fish exhibiting the symptoms of treatment with TCE (at percentages shown) and killed at 3 or 6 months. Fish were pretreated with DEN.

PARAMETRIC ANALYSIS

In the present context, a parametric analysis of data means that a particular mathematical form is adopted for the distribution of T, the time to symptom occurrence. It is desirable that such a form have a plausible biological origin, i.e., that it can be derived from suitable biological considerations, and that it adequately represent the data. The models of Moolgavkar et al. (1973, 1979, 1983) seem to satisfy the former requirement, but involve at least four parameters, which is too many to attempt to fit using data from the present design. Instead, the simple exponential distribution,

$$G(t;\lambda) = 1 - e^{-\lambda t}$$

is adopted for illustration. Note that the single parameter, λ, is actually interpretable as *the inverse of the mean of T* (time to symptom occurrence) *in the population.* If this model agrees reasonably well with the data then 1/estimated $\lambda = 1/\lambda$ is easily understood and interpreted. The exponential model also implies that the theoretical first and second 3-month hazards are the same. Notice that since no actual times to symptom appearance are ever observed, such a quantity is not available from nonparametric methodology. The parameter λ (actually it is best to estimate $\gamma = \log \lambda$) must be estimated from the counts at 3 months and 6 months. The method used here is that of *maximum likelihood*; details are provided in an appendix. Tables 5 and 6 exhibit the results of the analysis.

These results seem surprising, since mean time to exhibit the CD symptom appears to *increase* with TCE dosage; as anticipated the effect of DEN is to reduce the time to symptom appearance; these results are in rough qualitative agreement with the nonparametric results. See also Figures 6 and 7, which indicate the uncertainty associated with the above numerical values. These results were obtained by bootstrapping.

Figure 6 displays boxplots of the values of $-\gamma$, the log mean time to CD for the bootstrap samples. Once again the boxplots are grouped in pairs by level of TCE, which is indicated on the bottom of the figure. The leftmost boxplot in a group is for the fish not pretreated with DEN, the rightmost is for those pretreated with DEN. Once again the major effect is a decrease in mean time to occurrence of CD with pretreatment with DEN. The variability of the estimate makes other conclusions suspect. Figure 7 displays the histograms of the bootstrap estimate values of the $-\gamma$, the log mean time to CD.

Figures 8 and 9 present boxplots comparing the bootstrap estimates of the probability that CD occurs before 3 months obtained from the parametric exponential model and the nonparametric analysis. The estimate for the probability using the exponential model is

Table 5. Maximum Likelihood Estimates of Mean Time to Exhibit CD
(Bootstrap Standard Error)

Group	DEN	no DEN	%TCE	Log mean time to CD (Standard error)	$P\left\{\begin{array}{c}\text{CD occurs}\\ \text{before 3 months}\end{array}\right\}$ (Standard error)
1		X	0	2.66 (0.32)	0.19 (0.05)
3		X	25	2.68 (0.34)	0.19 (0.05)
5		X	50	3.17 (0.45)	0.12 (0.04)
7		X	100	3.19 (0.60)	0.12 (0.04)
2	X		0	1.85 (0.26)	0.38 (0.07)
4	X		25	2.3 (0.31)	0.26 (0.07)
6	X		50	2.40 (0.34)	0.24 (0.07)
8	X		100	2.33 (0.33)	0.25 (0.07)

Table 6. Maximum Likelihood Estimates of Log Mean Time to Exhibit Neoplasms
(Bootstrap Standard Error)
Exponential Model, Pretreatment with DEN

Group	%TCE	Log mean time to neoplasms	$P\left\{\begin{array}{c}\text{Neoplasms occur}\\ \text{before 3 months}\end{array}\right\}$
2	0	4.97 (4.47)	0.02 (0.02)
4	25	3.34 (0.99)	0.10 (0.04)
6	50	4.97 (4.60)	0.02 (0.02)
8	100	2.88 (0.39)	0.15 (0.05)

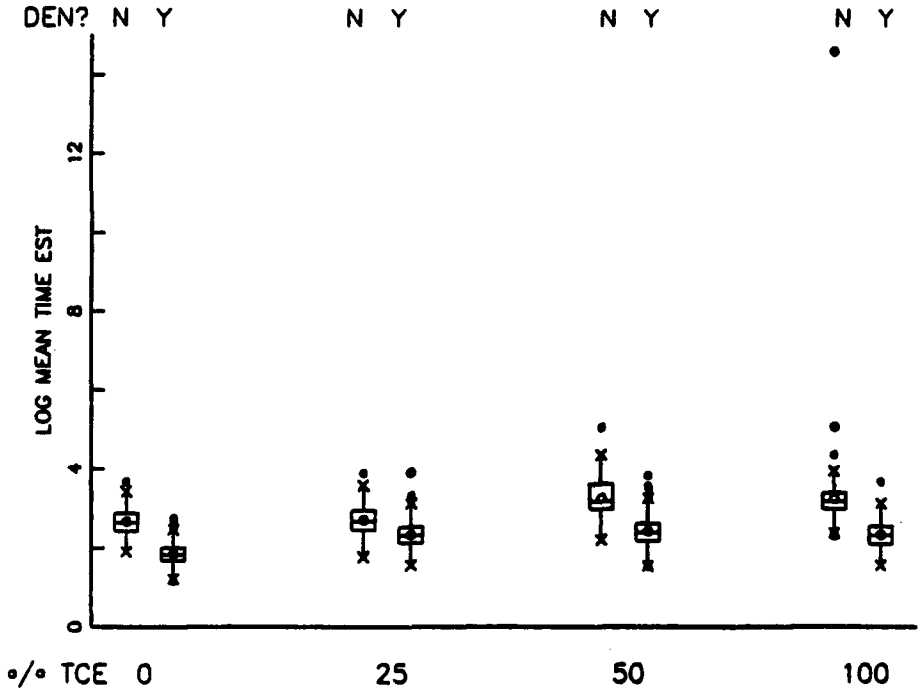


Figure 6. Parametric hazard, estimated log mean time to CD, 500 bootstrap replications per group, for fish exhibiting the symptoms of treatment with TCE (at percentages shown) and with or without pretreatment with DEN.

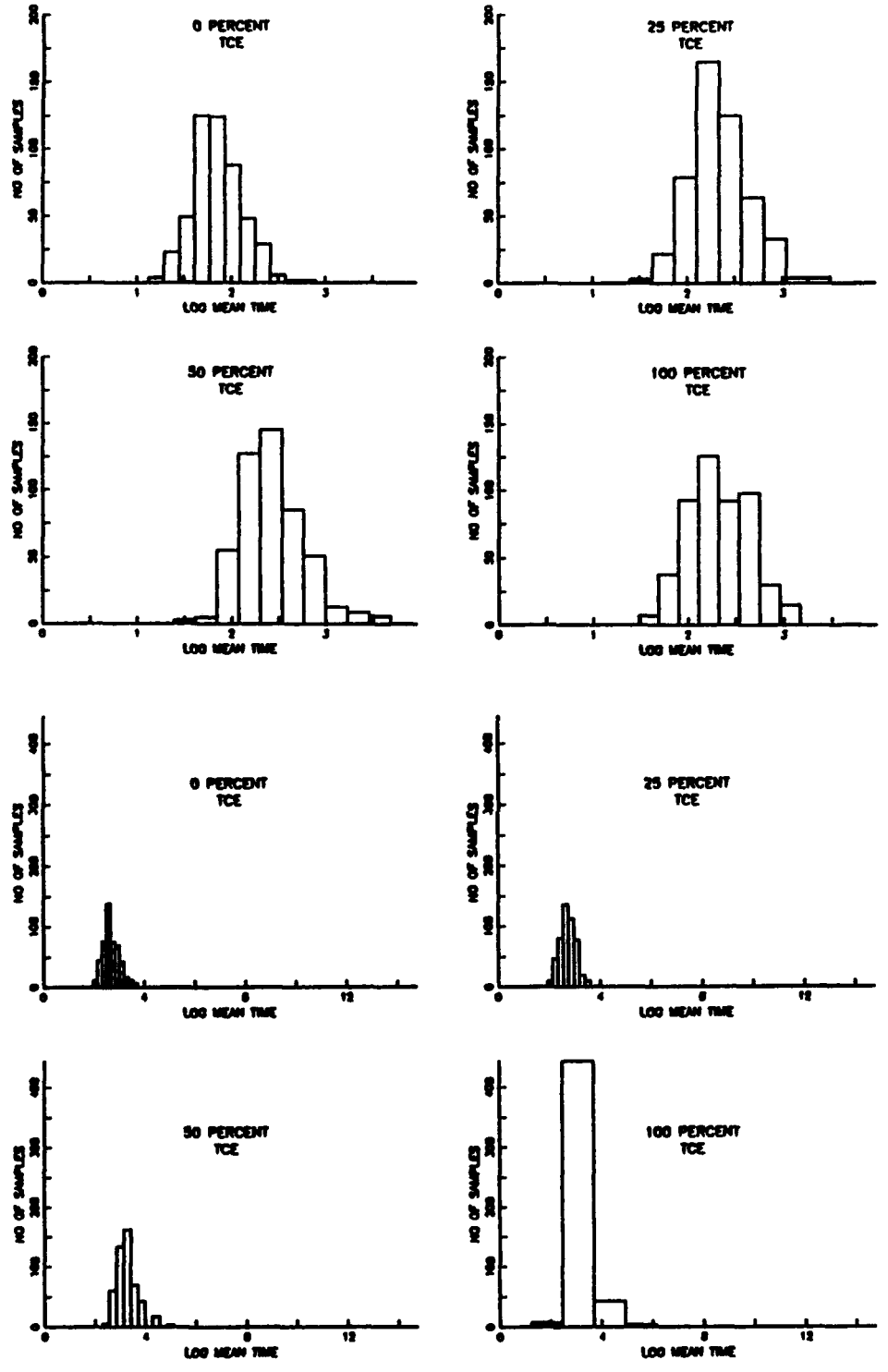


Figure 7. Sample distribution for estimated log mean time to CD, hazard assessed by bootstrapping. Histograms for fish pretreated with DEN are in the top half of the figure; those for fish not pretreated with DEN are shown in the lower half of the figure.

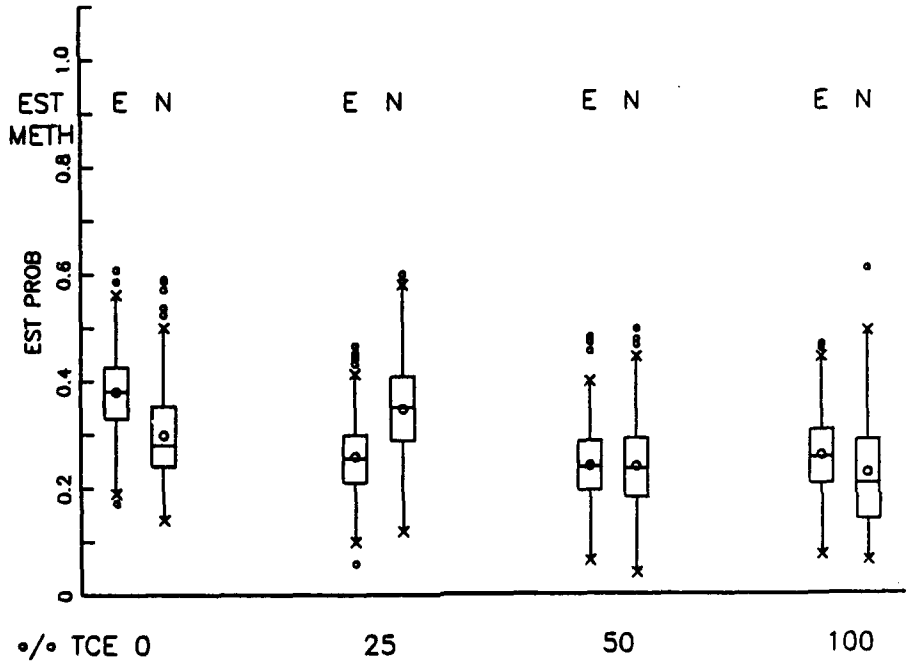


Figure 8. Comparison of the estimates of the probability that CD occurs before 3 months from the parametric exponential model (E) and the nonparametric (N) analysis. Fish were pretreated with DEN and treated with TCE at the percentages shown.

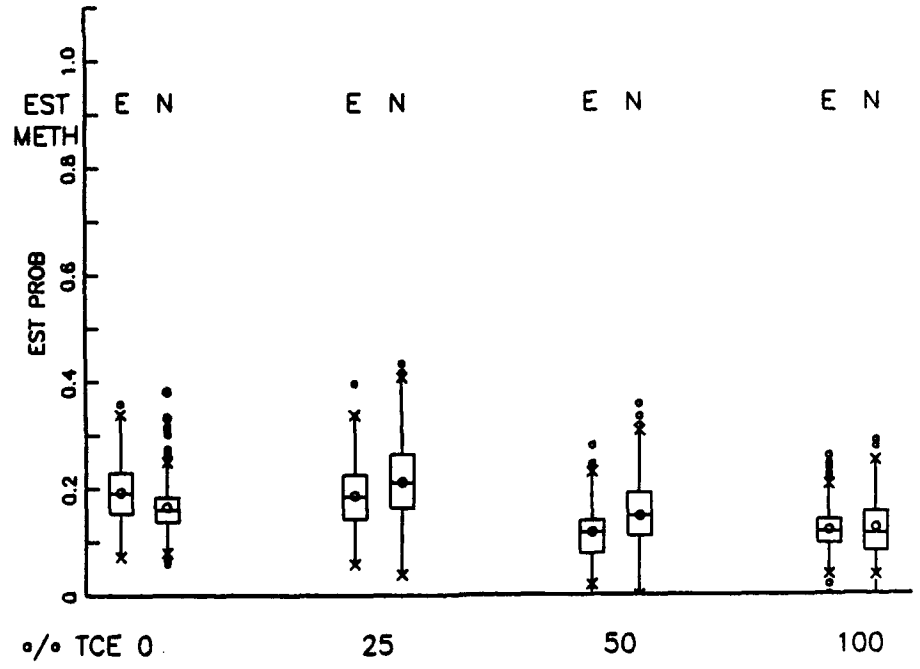


Figure 9. Comparison of the estimates of the probability that CD occurs before 3 months from the parametric exponential model (E) and the nonparametric (N) analysis. Fish were not pretreated with DEN and were treated with TCE at the percentages shown.

$$\hat{p}_e = 1 - \exp\left\{-e^{\hat{\gamma}} 3\right\}.$$

The estimate of the probability using the nonparametric hazard is the average of the first and second 3-month hazard. The boxplots are grouped by level of TCE. The left (respectively right) one in each group is the bootstrap estimate for the parametric exponential model (respectively the nonparametric hazard). The figures suggest that the two procedures yield roughly the same estimate.

Figure 10 presents similar boxplots for the bootstrap estimates of the probability that neoplasms occur before 3 months. Note that the exponential model estimates suggest that there is no effect at 100% TCE. Note that only the chronic data are being examined.

Figure 11 presents histograms for a *simulation* experiment to illustrate the effect of using more fish in the experiments. Our experiment is extreme in that 200 fish are used in each group; 100 are sacrificed at 3 months and 100 sacrificed at 6 months. The nonparametric estimates of $G(3;\theta)$ and $G(6;\theta)$ for each group of the CD data are used as the true probabilities of CD occurring at 3 and 6 months, respectively. For each simulation replication two random numbers are drawn: one from a binomial distribution with 100 trials and probability the estimate of $G(3;\theta)$ and the other from a binomial distribution with 100 trials and probability the estimate of $G(6;\theta)$. For each group 500 simulation replications are done and two 3-month hazards are computed for each replication as before. A comparison of the histograms in Figures 4 and 11 shows the amount of decrease in the variability of the estimates that can be achieved by increasing the number of fish used in the experiment.

BIOLOGICALLY BASED MODEL DESCRIPTION

It is widely believed that precancerous conditions in an organ (the liver) occur as a result of cell clonal expansion, followed by a promotion (to tumor) event. Specific models for this have been proposed and developed Moolgavkar and co-workers. More recent work is by C.J. Portier and co-workers.

The basic mechanism is treated as random or probabilistic: an **initiating event**, e.g., caused by contact with toxin, affects a cell within an organ in accordance with a simple Poisson process with rate parameter λ. That is, the chance of an uninitiated cell being initiated in time interval $(t, t+h)$ is approximately λh. If a cell is initiated during exposure time, it clones itself into other cells at rate β; the original cell and its clones die randomly at rate δ. All cells in the organ perform thus independently, according to the model. Depending upon the values of β and δ (birth and death rates, respectively) a colony of initiated cells (precancerous, presumably) tends either to

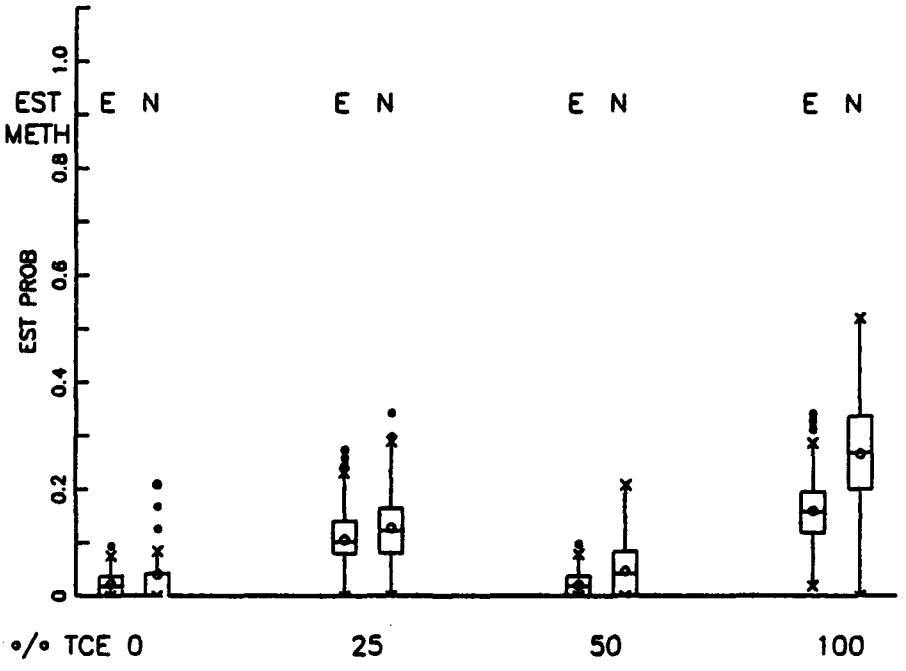


Figure 10. Comparison of the estimates of the probability that neoplasms occur before 3 months from the parametric exponential model (E) and the nonparametric (N) analysis. Fish were pretreated with DEN and were treated with TCE at the percentages shown.

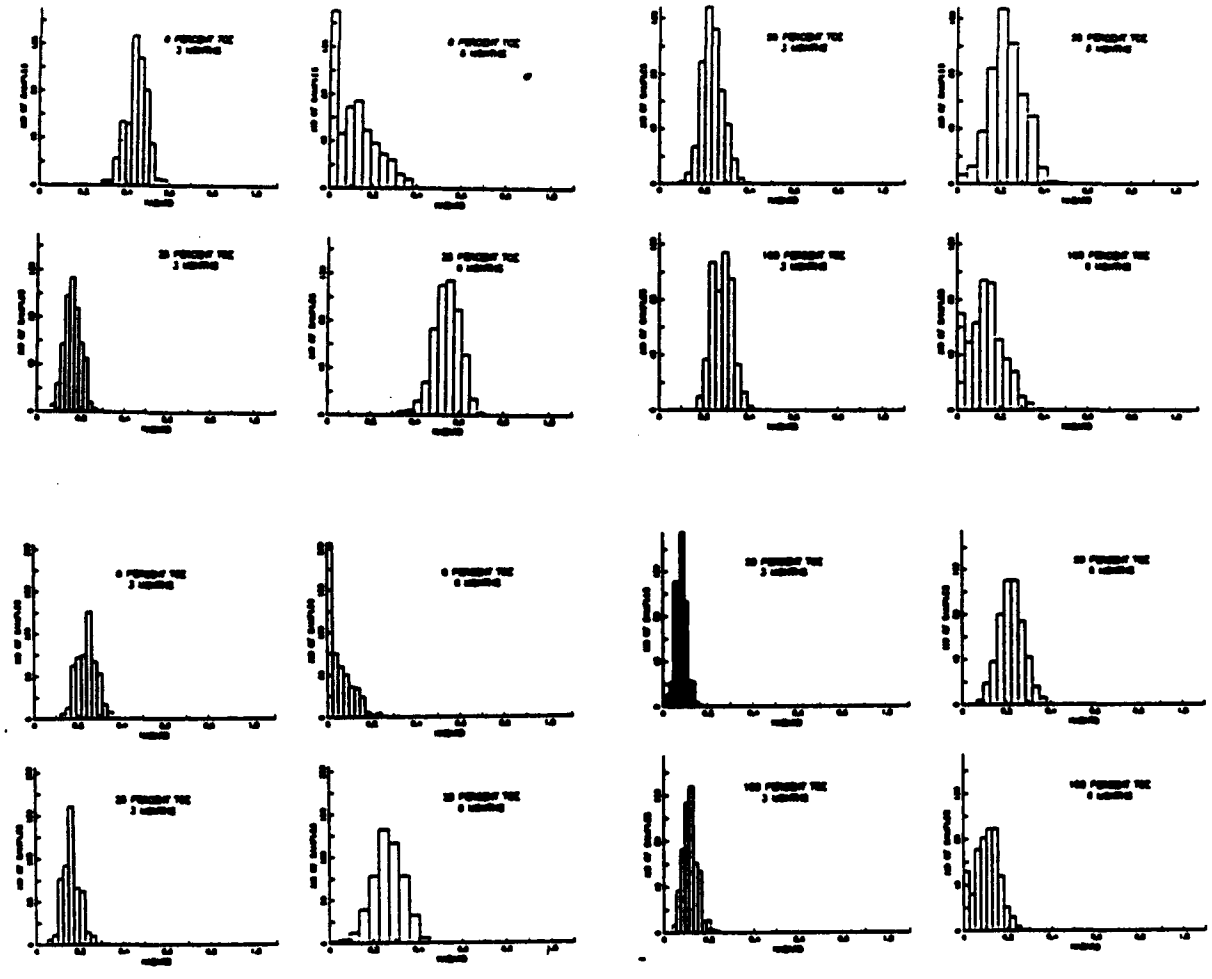


Figure 11. Sample distribution for hazard for a *simulation* experiment in which number of fish and number of trials are multiplied (see text). Histograms for fish pretreated with DEN are in the top half of the figure; those for fish not pretreated with DEN are shown in the lower half of the figure.

grow exponentially or to die off to zero (also exponentially fast). The fates of colonies characterized by the same values of birth rate and death rate may actually be entirely different, as befits experience with variability characteristic of the real biological world. This behavior is *roughly* analogous to that of the flipping of the same coin: on one occasion 10 flips may well result in an excess of 5 heads (7 heads and 3 tails), analogous to more births (heads) than deaths (tails); on another sequence of 10 flips with the same coin the result may be exactly reversed (7 tails, 3 heads). Processes analogous to coin flipping or dice rolling can describe much, but possibly not all, interesting biological variability pertinent to risk analysis. Other options are suggested below.

The values of β and δ describe clone colony properties in a precise probabilistic manner *if* the model is correct. It is certainly only approximate, but may still provide a useful tool for quantifying risk of tumor formation. The second step in the malignant cell development process is postulated to be *promotion*. A model for this is that a rate μ, i.e., with probability μh in time $(t, t+h)$, a promotion event occurs that affects one of the clone colony members in proportion to the current size of the colony; such events are assumed to occur in accordance with a Poisson process with rate proportional to instantaneous clone population size. At the instant that the *first* such promotion event occurs, the clone colony (if one exists, i.e., has been initiated) will be said to have developed a tumor, at least in informal layman's terms. Note that all original cells in an organ are assumed to be independently exposed to initiation and, thereafter, to promotion. Therefore, all organ cells and subsequent clones, if any, must survive from initiation to the end of the observation period without being promoted in order for the organ to survive throughout.

The probabilistic mechanism described has been used to obtain a formula for the survival probability for an organ for any observation time t. See Appendix 2 for the formula and its derivation. Similar formulas have been derived also by Moolgavkar and others. Our formula provides the basis for statistically estimating from pathology data (combinations of) the parameters: λ, the initiation rate; μ, the promotion rate; and β and δ, the clonal birth and death rates. Such estimates can, in turn, be used to estimate the probability of cell, and organ, survival for any time period. Appendix 1 contains a discussion of maximum likelihood estimation from data so as to specify parameters of a preliminary model. Further work is required to obtain additional statistical models and procedures to analyze other experimental data.

Extensions to the Model: Extravariation of Parameters

The above model, and the consequences thereof in the form of a survival probability function, are appealing since they have a plausible biological basis. Organ-to-organ outcomes

(tumor occurrence or not) vary randomly, but according to precisely the same mechanism in each organ; i.e., the same values of λ, μ, β, and δ are assumed to hold for each organ. Note that this ignores likely variability between organs in different subjects (e.g., fish). Different, but superficially identical, biological entities, be they fish, rats, or humans, can be expected to have some differences; these can be said to be the result of genetic diversity. Specifically these differences may cause the effective parameters λ, μ, β, and δ to differ substantially across animals and can lead to more variability in the time to tumor occurrence than predicted by the simple model. If the above are estimated from data without recognition of the possibility of this extravariation, biased results will be obtained. See Harris (1990) for biological explanations of interorgan (subject) variability.

There are several possible simple and preliminary ways of dealing with the above problem. One is by attempting to "explain" parameter variation by representing it as a function of some causal variable, such as the age, sex, weight, etc., of the host subject. The technique is a variation of ordinary regression analysis; methods of McCullagh and Nelder (1983) suggest themselves. A description of a preliminary computational procedure to estimate model parameters is described in Appendix A. This procedure is used to estimate model parameters for a particular data set. A second approach is to assume that the variability between individual host organs can be represented by treating some or all of the parameters as random variables with their own distributions. A typical survival function is then obtained by *mixing*: the parametric survival function of Appendix B is "simply" randomized according to the (joint) distribution of the parameters. In principle it is desirable to recognize both sources of variability between individuals, adjusting for known sources of variation by a regression technique where possible, but recognizing the "unexplainable" variation by use of a mixing technique. The latter has been carried out to a limited extent (see Gaver and Jacobs, 1992).

CONCLUSIONS AND SUMMARY

This report covers an initial short piece of research conducted under the sponsorship of the U.S. Army Biomedical Research and Development Laboratory. Its main contribution is to propose and illustrate quantitative assessments of treatment (here, groundwater concentration) effects upon medaka. Those quantifications include the estimation of statistical sampling errors by the resampling or bootstrapping technique.

The somewhat inconclusive dose-response relationships revealed seem to imply the need for more sensitive experiments. Possibly such sensitivity can be achieved by working with more genetically homogeneous animals (medaka). Possibly, larger numbers of animal subjects will be

helpful as well. Control and measurement of experimental conditions (e.g., tank temperature) and adjustments for their variation can play a useful part in the investigation.

It is hoped that the mathematical and statistical approaches illustrated here will help to promote an interest in the further use of such ideas among biologists and toxicologists.

APPENDIX 1: MODEL-FITTING METHODS FOR QUANTIFYING BIOASSAYS

Preliminary Statistical Models and Methods for Analyzing Bioassay Data

Suppose N organisms (e.g., fish) are used in an experiment. Groups of these organisms may be exposed to different treatments. Let T_i be the random time until organism i develops a particular symptom, e.g., cystic degeneration. Let $X_i = (X_{i1}, X_{i2}, \ldots, X_{ip})$ be covariates which (possibly) influence T_i; the X_i could be levels of substances having possible toxic effects to which the organisms are exposed. Let $G(t; x_i) = P\left\{T_i \le t \mid X_i = x_i\right\}$. We will assume that the organisms develop symptoms independently of each other. In this initial model, the symptom is either present or not.

Suppose that n_k organisms are sacrificed at time t_k with $t_1 < t_2 < \ldots < t_K$. We will label the organisms so that organisms 1 through n_1 are sacrificed at time t_1; organisms $n_1 + 1, \ldots, n_1 + n_2$ are sacrificed at time t_2; etc. Let $s_i = 1$ if organism i exhibits the symptom when it is examined. Under the assumption of independence, the likelihood function is

$$L = \prod_{k=1}^{K} \prod_{i=1}^{n_k} G\left(t_k; x_{n_{k-1}+i}\right)^{s_{n_{k-1}+i}} \left[\bar{G}\left(t_k; x_{n_{k-1}+i}\right)^{\left(1-s_{n_{k-1}+i}\right)}\right] \qquad \text{(A.1)}$$

where $n_0 = 0$ and $\bar{G}(t;x) = 1 - G(t;x)$. The likelihood functions form the basis for estimation of parameters in the distributions that model survival times, i.e., G.

Example (simple binomial model)

If there are no covariates, then (A.1) becomes

$$L = \prod_{k=1}^{K} \binom{n_k}{f_k} G(t_k)^{f_k} \bar{G}(t_k)^{n_k - f_k} \qquad \text{(A.2)}$$

where f_k is the number of the n_k organisms exhibiting the symptom.

A procedure to estimate the parameters of the distribution G for the simple binomial model is as follows.

Maximum Likelihood Estimation in the Simple Binomial Model

Likelihood and Parameter Estimation Formulas

Assume the distribution of the time to appearance of a symptom, G, is a function of the parameters θ_j, $j = 1, \ldots, J$. In this section we discuss maximum likelihood estimation of θ_j for

the simple binomial model. Presumably the n_k subjects examined at time t_k, $k = 1, 2, \ldots, K$ have all been subjected to a common dosage of a potential toxin. The purpose of the present analysis is to predict survival probabilities as they depend on such dosage. The log-likelihood function for the simple binomial model is

$$l = \sum_{k=1}^{K} \ln\binom{n_k}{f_k} + f_k \ln G(t_k;\theta) + (n_k - f_k)\ln \bar{G}(t_k;\theta) \tag{A.3}$$

where $\theta = (\theta_1, \ldots, \theta_J)$. Differentiating, we obtain

$$\frac{\partial}{\partial \theta_j} l = \sum_{k=1}^{K} \frac{f_k}{G(t_k;\theta)} \frac{\partial}{\partial \theta_j} G(t_k;\theta) + \frac{(n_k - f_k)}{\bar{G}(t_k;\theta)} \left[-\frac{\partial}{\partial \theta_j} G(t_k;\theta) \right]$$

$$= \sum_{k=1}^{K} \frac{f_k[1 - G(t_k;\theta)] - (n_k - f_k)G(t_k;\theta)}{G(t_k;\theta)\bar{G}(t_k;\theta)} \left[\frac{\partial}{\partial \theta_j} G(t_k;\theta) \right]$$

$$= \sum_{k=1}^{K} \left[\frac{f_k - n_k G(t_k;\theta)}{G(t_k;\theta)\bar{G}(t_k;\theta)} \right] \frac{\partial}{\partial \theta_j} G(t_k;\theta). \tag{A.4}$$

Since $E[f_k] = n_k G(t_k;\theta)$

$$E\left[\frac{\partial^2}{\partial \theta_j \partial \theta_m} l \right] = -\sum_{k=1}^{K} n_k \frac{\frac{\partial}{\partial \theta_j} G(t_k;\theta) \frac{\partial}{\partial \theta_m} G(t_k;\theta)}{G(t_k;\theta)\bar{G}(t_k;\theta)}. \tag{A.5}$$

Thus a Newton procedure for finding the maximum likelihood estimates of $\{\theta_j;\ j = 1, \ldots, J\}$ would iteratively solve the system of linear equations

$$0 = \frac{\partial}{\partial \theta_j} l(\theta^0) + \sum_{m=1}^{P} E\left[\frac{\partial^2}{\partial \theta_j \partial \theta_m} l \right] (\theta_m - \theta_m^0) \tag{A.6}$$

where $\theta^0 = (\theta_1^0, \ldots, \theta_J^0)$. Such iterative procedures can be programmed for a digital computer, and the resulting parameter values can be used to compute predictions for survival probabilities, or risk, as the latter depend upon the parameters of such models as described in Appendix 2.

APPENDIX 2: TWO-STAGE CLONAL-EXPANSION MODEL

We present here a birth-death model for the distribution of time until a normal cell becomes promoted to a tumor. We first develop an expression for the distribtion of random time, S, until an initiated cell or one of its descendants becomes malignant. Assume that there is one initiated cell at time 0. Such cells divide at an exponential rate β, and die at an exponential rate δ. Any initiated cell turns malignant at an exponential rate μ; i.e., μ is the **promotion rate.**

Time to Promotion of an Initiated Cell

Let S be the random time at which some initiated cell or its descendant turns malignant; note that S may actually be infinite if the population of initiated cell and its descendants dies out. Put

$$z(t) = P\{S > t\}.$$

The following probability argument provides an equation for $z(t)$: the event that $S > t+\Delta$ ($\Delta > 0$) occurs if (i) neither birth (cloning), death, nor promotion occurs in $(0,\Delta)$ *and* promotion does not occur in $(\Delta, t+\Delta)$; the probability of this is $[1 - (\beta+\delta+\mu)\Delta + o(\Delta)]z(t)$; *or* (ii) birth/cloning occurs in $(0, \Delta)$ and no promotion occurs in $(\Delta, t+\Delta)$; the probability of this event is $[\beta\Delta + o(\Delta)]z^2(t)$, where the square recognizes that at time Δ there are now two independent clonal families to be considered; *or* (iii) the original initiated cell dies in $(0, \Delta)$, the probability of which is $\delta\Delta + o(\Delta)$. Sum these three terms to obtain the probability that $S > t+\Delta$:

$$z(t + \Delta) = (1-(\beta + \delta + \mu)\Delta)z(t) + \beta\Delta z^2(t) + \delta\Delta$$

Now subtract $z(t)$ from each side, divide by Δ, and let $\Delta \to 0$. The result is the differential equation

$$\frac{dz(t)}{dt} = -(\beta + \delta + \mu)z(t) + \beta z^2(t) + \delta. \tag{B.1}$$

Hence $z(t)$ satisfies a Riccati equation with initial condition

$$z(0) = 1. \tag{B.2}$$

The solution to (B.1) with initial condition (B.2)

$$z(t) = \frac{\rho_1(1 - \rho_2) - \rho_2(1 - \rho_1)e^{\beta(\rho_1 - \rho_2)t}}{1 - \rho_2 - (1 - \rho_1)e^{\beta(\rho_1 - \rho_2)t}}, \tag{B.3}$$

where $\rho_{1,2}$ are the solutions to the quadratic equation

$$x^2 - \left(1 + \frac{\delta}{\beta} + \frac{\mu}{\beta}\right)x + \frac{\delta}{\beta} = 0; \tag{B.4}$$

$$\rho_{1,2} = \frac{1}{2}\left[\left(1+\frac{\delta}{\beta}+\frac{\mu}{\beta}\right) \pm \left[\left(1+\frac{\delta}{\beta}+\frac{\mu}{\beta}\right)^2 - 4\frac{\delta}{\beta}\right]^{\frac{1}{2}}\right]. \tag{B.5}$$

Since $\left[\left(1+\frac{\delta}{\beta}+\frac{\mu}{\beta}\right)^2-4\frac{\delta}{\beta}\right]^{1/2} \le \left(1+\frac{\delta}{\beta}+\frac{\mu}{\beta}\right)$, both ρ_1 and ρ_2 are positive. Furthermore, $\rho_2 \le 1$ and

$$\rho_1-\rho_2=\left[\left(1+\frac{\delta}{\beta}+\frac{\mu}{\beta}\right)^2-4\frac{\delta}{\beta}\right]^{\frac{1}{2}}>0.$$

Hence,

$$\lim_{t\to\infty} z(t)=\rho_2. \tag{B.6}$$

If the death rate $\delta = 0$, then $\rho_2 = 0$ and $\lim_{t\to\infty} P\{T > t\} = 0$; if $\delta = 0$, then there is no death of initiated cells and thus an initiated cell will transition to a malignant cell in a finite time with probability 1. If $\delta > 0$, then the initiating cells can die, thus preventing a transition to malignancy and hence $\lim_{t\to\infty} P\{T > t\} = \rho_2 > 0$.

Model for the Time until a Normal Cell Becomes Malignant (is promoted to tumor)

Assume that each normal cell is initiated at an exponential rate λ_0. Let N be the total number of normal cells in an organ. Let T denote the first time a normal cell transitions to a malignant cell.

$$P\{T\ge t\}=\left[e^{-\lambda_0 t}+\int_0^t \lambda_0 e^{-\lambda_0 s} z(t-s)ds\right]^N \tag{B.7}$$

where z is a given in (B.3). Assume λ_0 is small and put $\lambda = \lambda_0 N$, a constant. Then

$$P\{T>t\}\approx \exp\left\{N\ln\left[1-\frac{\lambda}{N}t+\frac{\lambda}{N}\int_0^t z(s)ds\right]\right\} \tag{B.8}$$

(B.9)

$$\approx \exp\left\{-\lambda t+\lambda\int_0^t z(s)ds\right\} \tag{B.10}$$

$$=\exp\left\{\lambda(\rho_1-1)t-\lambda\frac{1}{\beta}\ln\left[\frac{1-\rho_2+(\rho_1-1)e^{\beta(\rho_1-\rho_2)t}}{\rho_1-\rho_2}\right]\right\}.$$

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APPENDIX A

AGENDA FOR 1990 RESEARCH METHODS BRANCH THIRD ANNUAL CARCINOGENICITY WORKSHOP

RESEARCH METHODS BRANCH THIRD ANNUAL CARCINOGENICITY WORKSHOP

U.S. Army Biomedical Research and Development Laboratory
Fort Detrick
Frederick, Maryland

14-15 August 1990

AGENDA

14 August

TIME	TOPIC	PRESENTER
0830-0845	Welcome	COL Steve Hembree Commander, U.S. Army Biomedical Research and Development Laboratory
0845-0900	Opening Remarks	Mr. Hank Gardner Research Methods Branch
0900-0945	Guest Speaker	Dr. John Harshbarger Smithsonian Institution
0945-1000	Break	
1000-1030	Molecular Characterization of Carcinogenesis in Medaka	Dr. Rebecca Van Beneden Duke University Marine Laboratory
1030-1100	Immunotoxicology in the Medaka	Dr. Bob Anderson University of Maryland
1100-1130	Assessment of DNA Modifications in Medaka	Dr. Russom Haimanot Pacific NW Research Foundation
1130-1200	Single Cell Gel Assay Techniques	Dr. Ray Tice Integrated Laboratory Systems
1145-1300	Lunch	
1300-1330	Medaka Hepatocytes in Cell Culture	Dr. Robert Finch Research Methods Branch
1330-1400	Epigenetic Carcinogen Studies in Fish	Dr. Ed Calabrese University of Massachusetts
1400-1430	Biological (Molecular and Cellular) Markers of Toxicity	Dr. Lee Shugart Oak Ridge National Laboratory
1430-1445	Break	
1445-1515	Cancer Studies in Fish	Dr. Jerry Hendricks Oregon State University

14 August (continued)

TIME	TOPIC	PRESENTER
1515-1545	Dietary Refinements in a Sensitive Fish Liver Tumor Model	Dr. Dave Hinton University of California-Davis
1545-1615	Discussion	
15 August		
0820-0830	Introduction	Mr. Hank Gardner
0830-0900	Tumor Immunodiagnosis	Dr. Tracie Bunton Johns Hopkins University
0900-0920	Delivered Dose Studies in Fish	Dr. Patricia Schmieder U.S. Environmental Protection Agency
0920-1000	Probing the Carcinogenic Sensitivity of Medaka	Mr. Joseph Tietge AScI Corporation/EPA
1000-1015	Break	
1015-1045	Development of Carcinogenesis Bioassay Models: Response of Small Fish Species to Various Classes of Carcinogens	Dr. Bill Hawkins Gulf Coast Research Laboratory
1045-1115	Determining Dose Response at Low Concentration Using Medaka	Dr. Bill Walker Gulf Coast Research Laboratory
1115-1145	FETAX Developmental Toxicity Screening Test	Dr. Jack Bantle Oklahoma State University
1145-1300	Lunch	
1300-1330	Pathology Assessment Issues	Dr. Marilyn Wolfe Experimental Pathology Laboratories
1330-1345	Disease Surveillance and Strain Characterization	Dr. Roger Herman U.S. Fish & Wildlife Service
1345-1430	Applications of New Models	Mr. Hank Gardner Research Methods Branch
1430-1445	Break	
1445-1530	Applications of New Models Continued	Mr. Hank Gardner Research Methods Branch
1530-1630	Discussion	

APPENDIX B

LIST OF PRESENTERS FOR 1990 RESEARCH METHODS BRANCH CARCINOGENICITY WORKSHOP

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APPENDIX C

AGENDA FOR 1992 RESEARCH METHODS BRANCH RESEARCH REVIEW

RESEARCH METHODS BRANCH RESEARCH REVIEW

U.S. Army Biomedical Research and Development Laboratory
Fort Detrick
Frederick, Maryland

21-22 April 1992

AGENDA

21 April

TIME	TOPIC	PRESENTER
0830	Welcome	Mr. Hank Gardner Research Methods Branch
0845	Development of an Aquatic Bioassay Using the Medaka (*Oryzias latipes*) to Assess Human Health Risk: Tumor Immunodiagnosis	Dr. Tracie Bunton Johns Hopkins University
0915	Molecular Analysis of Medaka Tumors: New Models for Carcinogenicity Testing	Dr. Rebecca Van Beneden Duke University Marine Laboratory
0945	Break	
1030	Assessment of DNA Modifications in Medaka	Dr. Donald Malins Pacific Northwest Research Foundation
1100	Biological (Molecular and Cellular) Markers of Toxicity	Dr. Stephen D'Surney Oak Ridge National Laboratory
1130	Fish as a Predictive Model for Epigenetic Carcinogens	Dr. Ed Calabrese University of Massachusetts
1200	Discussion	
1215	Lunch	
1345	Dietary Refinements in a Sensitive Fish Liver Tumor	Dr. Dave Hinton University of California
1415	Development of Carcinogenesis Bioassay Models: Response of Small Fish to Various Classes of Carcinogens	Dr. Bill Hawkins Gulf Coast Research Laboratory
1445	Low-Dose Carcinogenicity	Dr. Bill Walker Gulf Coast Research Laboratory
1515	Break	

21 April (continued)

TIME	TOPIC	PRESENTER
1545	Development of the Zebra Danio Model: Carcinogenesis and Gene Transfer Studies	Dr. Jerry Hendricks Oregon State University
1615	Biochemical Testing of Potentially Hazardous Chemicals for Toxicity Using Mammalian Liver Cell Cultures	Dr. Curtis Parker Morehouse School of Medicine
1645	Discussion	

22 April

0830	Immunotoxicological Methods Development in Medaka	Dr. Lorraine Twerdok *GEO-CENTERS, INC.*/USABRDL
0900	Immunotoxicology Assessment Using Japanese Medaka	Dr. Bob Anderson University of Maryland
0930	Further Development and Validation of the Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX)	Dr. Jack Bantle Oklahoma State University
1000	Break	
1045	Quality Assurance Issues in the Conduct of Small Fish Carcinogenesis Bioassays	Dr. Marilyn Wolfe Experimental Pathology Laboratories
1115	Modeling and Statistical Analysis of Bioassay Data	Dr. Donald Gaver Naval Postgraduate School
1145	Discussion	
1200	Close	
1215	Optional Tour of USABRDL Facilities	Mr. Hank Gardner Research Methods Branch

APPENDIX D

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APPENDIX E

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