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OAK RIDGE NATIONAL LABORATORY

MARTIN MARIETTA

Biological (Molecular and Cellular) Markers of Toxicity

Semi-annual Technical Progress Report (No. 5) October 1, 1990, to March 30, 1991

L. R. Shugart

MANAGED BY
MARTIN MARIETTA ENERGY SYSTEMS, INC.
FOR THE UNITED STATES
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ENVIRONMENTAL SCIENCES DIVISION

BIOLOGICAL (MOLECULAR AND CELLULAR) MARKERS OF TOXICITY

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BIOLOGICAL (MOLECULAR AND CELLULAR) MARKERS OF TOXICITY

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Contracting Officer's Technical Representative:

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Fort Detrick, MD 21701-5010

I. PURPOSE AND SCOPE OF RESEARCH EFFORT

The overall objective of this study is to evaluate the use of the small aquarium fish, Japanese Medaka (Oryzias latipes), as a predictor of potential genotoxicity following exposure to carcinogens. This will be accomplished by quantitatively investigating the early molecular events associated with genotoxicity of various tissues of Medaka subsequent to exposure of the organism to several known carcinogens, such as diethylnitrosamine (DEN) and benzo[a]pyrene (BaP).

The Japanese Medaka has recently been used as a bioassay model to screen for carcinogenic compounds in water. The primary endpoint for most small fish carcinogenesis studies is the histopathogenic identification of a neoplastic lesion. Such lesions occur mainly in the liver, although other tissues in which lesions have been observed include nervous tissue, kidney, mesenchymal tissue, skeletal and smooth muscle. The histogenesis of liver neoplasms in fish is similar to that in rodents. Following exposure to chemical carcinogens, preneoplastic lesions appear as eosinophilic foci, basophilic foci, followed by tumor formation (Aquatic Toxicol. 11:113-128;1988). This is identical to the process that appears to occur in rodents and other mammals (Environ. Health Perspec. 75:65070;1987).

Therefore, if we are to understand in any depth the processes by which environmental chemicals exhibit genotoxicity, new and innovative techniques will be needed that elucidate the fundamental cellular nature of the various steps subsequent to exposure to these agents.

Because of the often long latent period between initial contact with certain chemical and physical agents in our environment and subsequent expression of deleterious health or ecological impact, the development of sensitive methods for detecting and estimating early exposure is needed so that necessary interventions can ensue. A promising biological endpoint for detecting early exposure to damaging chemicals is the interaction of these compounds with cellular macromolecules such as Deoxyribonucleic acids (DNA). This biological endpoint assumes significance because it can be one of the critical early events leading eventually to adverse effects (neoplasia) in the exposed organism.

The research will proceed along several interrelated lines of investigation, which have recently been reevaluated and revised:

Task 1. Evaluate current analytical techniques and

methodologies for their effectiveness to detect and quantitate biological responses at the molecular level to the action of genotoxic agents. Particular emphasis will be placed on the adaptation of these methods for the detection of enzyme activities associated with detoxification and damaging events that occur to the DNA in the liver;

Task 2. Develop protocols for the exposure of Medaka under controlled laboratory conditions to DEN, AAF, and BaP and investigate the effects these chemical carcinogens have on the suite of molecular markers selected and for testing potentially new biomarker assays;

Task 3. Evaluate the short-term responses of the molecular markers as suitable biomarkers to estimate exposure and predict cellular effects to genotoxic chemicals.

The work proposed here focuses on the more fundamental aspects of the problems that deal with the detection of exposure to genotoxic agents through the appropriate use of molecular markers and the potential to predict subsequent adverse effects. Many chemical carcinogens and mutagens have the capacity to cause various types of DNA damage as a result of the interaction of highly reactive metabolites with DNA. Such interactions can form stable adducts with DNA, adducts which result in the formation of alkali-labile apurinic sites, and unstable adducts which cause strand breaks (American Sci. 70:386-393;1982). Indirectly, the fidelity of post replicative modification of DNA (such as minor nucleotide composition) can be affected by genotoxic agents. Each type of damage to the integrity of the DNA, if left uncorrected, could trigger a sequence of events that culminates in the appearance of an overt malignancy. Estimates of these various types of damage to DNA, along with an appraisal of the organisms capability to maintain the integrity of it's DNA (repair and chromosomal structure-function), will provide the basis for determining bioavailability of the genotoxic agent and for estimating exposure. In addition the information may be used as a short-term predictor of the potential for long-term deleterious effects.

II. OVERALL PROGRESS

1. Task 1.

The purpose of this task is to evaluate the feasibility of using currently existing analytical techniques to detect molecular markers of genotoxicity, and included: (a) enzymes of detoxication and, (b) changes to the integrity of DNA. This task has essentially been completed and was reported in detail in Semi-Annual Technical Progress Report No.1 (ORNL/M-829, dated

April 1, 1989). With respect to detoxication enzymes in the medaka, methodologies for the measurement of 7-ethoxyresorufin O-deethylase (EROD) and Glutathione S-transferase (GST) were successfully applied. Also, existing methods and techniques for the assessment of DNA integrity (i.e., strand breaks, adduct formation, and minor nucleoside composition) were adapted.

2. Task 2.

The purpose of this task is to evaluate the effects of exposure of the Japanese medaka to known genotoxic agents on the response of a suite of molecular markers. This complex task has been subdivided into several subtasks and the progress on each is reported separately.

a. Protocols for exposure.

A protocol for exposure to DEN was reported in detail in Semi-Annual Technical Progress Report No.1 (ORNL/M-829, dated April 1, 1989). Molecular marker responses observed in Japanese medaka exposed to DEN employing this protocol have been examined (see Task 2.b. and Appendix A of technical progress report no.3).

Protocols for exposure of adult medaka to BaP were developed and reported in Semi-Annual Progress Report No. 4 (ORNL/M-1305). The methodologies and results of DNA strand break, adduct, and chromosomal protein analyses are reported in "Appendix A" (this report). The procedures and results of a medaka embryo assay for developmental toxicity after exposure to BaP are reported in "Appendix B" (this report).

A second exposure of medaka, described in the following paragraph, to DEN has been completed. Biomarker analyses are currently underway and the results will be reported in a later progress report.

> Acclimate 700 adult medaka (approximately 3 months old-60% females and 40% males) at 25°C for two weeks. There are 200 fish per tank and two tanks per treatment group and one control tank. Three exposure groups contain 100 ppm, 50 ppm, and 25 ppm. Exposure lasts for five weeks with 100% renewal of water every third day. Samples are taken at one week intervals until termination of exposure, and at 2, 4, and 8 weeks post exposure. On sampling days, ten (10) fish are taken from each tank, divided into two groups of five fish each. The livers and gills are pooled separately and carcasses kept separate. Samples are placed in 1.5 ml microfuge tubes and frozen in liquid nitrogen for shipment for DNA and protein analysis.

The following protocol (following paragraph) was developed for exposure of medaka to AAF (2-acetylaminofluorene) The

exposure has been completed and the results of biomarker studies will be reported in a later progress report.

Medaka and King Cobra Guppy (Poecilia reticulata),(in two replicate control and AAF exposure tanks for both species; each tank contains 90 fish each; 45 males and 45 females), are exposed to 2 μg/L of 2-AAF for three days (72 h) under conditions of static exposure. Unexposed controls are included for both medaka and guppy. Fish samples treated with AAF are taken at post-exposure times 6, 12, 18, 24, 36, 48, 60, and 72 hours; controls samples though, are taken at 0, 24, 48, and 72 hours. At each sampling time, 6 males and 6 females of both medaka and guppy are taken from exposed and control tanks. The fish are weighed and individually frozen in liquid nitrogen; three specimens are pooled (males and females are separate) to give duplicate samples of each sex. After 6 h in liquid nitrogen, samples are stored at -70°C.

b. Application of new techniques & methodologies.

Flow cytometric analysis for the determination of DNA distribution in hepatocytes (Bull. Environ. Contam. Toxicol. 40:343-349;1988) has been implemented into the suite of molecular markers (see Semi-Annual Technical Progress Report No.2 for details).

Currently under consideration and evaluation are techniques to detect and measure: (a) DNA repair; (b) DNA damage due to free radicals, and cross-linking agents; (c) induction of stress proteins; (d) changes in chromosomal/nuclear proteins and the relationship of these changes to resistance to DNA unwinding in medaka (e) electrophoretic analysis of DNA double and single strand breaks; (f) teratogenesis study on medaka embryos; and (g) dominant lethal analysis. It is anticipated that data from these studies on the exposure of medaka to BaP, DEN, and AAF will be incorporated into future progress reports and publications.

3. Task 3.

The purpose of this task is to evaluate the short-term responses of the suite of molecular markers as suitable biomarkers to estimate exposure and predict cellular effects to genotoxic chemicals. It is anticipated that this study will provide data to demonstrate the usefulness of the Medaka as a bioassay model to evaluate the potential of environmental genotoxicity. The responses to be studied are important because they have been selected on the basis of existing experimental observations (i.e., they are key ingredients that provide evidence that exposure to genotoxic agents is resulting in toxicological interactions) and they can be used to make positive predictions.

III. PROBLEM AREAS

Because of the small size of the Medaka, and therefore the amount of tissue available for analyses, most of our procedures require adult fish for study. We currently are well stocked with adult medaka (2000) which meets our current requirements. Continued exposures of medaka are planned to study developmental teratogenesis and dominant lethality in medaka exposed to xenobiotics. Improvements in isolation of sufficient amounts of highly purified DNA have been successful for small tissue samples such as blood and liver. Preparation of small tissue samples for other types of analyses are being refined and should permit bioassay studies on whole fish, gills and microsomes. Improvements in DNA yield now permit multiple analysis (strand breaks, DNA adducts, ^{5}m-CYD etc...) from one sample. Liver and gill tissue can be easily obtained for flow cytometric analysis which can measure aneuploidy, polyploidy and other changes in nuclear DNA content. Additional small scale molecular and cellular assays are being developed to compensate for the small size of the medaka and are being studied.

IV. WORK TO BE PERFORMED DURING THE NEXT SIX MONTHS

At present, approximately 2000 adult medaka are available for experimentation with sufficient fry being maintained to supplement those used for this purpose.

Studies will continue to focus on alterations to DNA integrity and changes in detoxication systems of medaka exposed to BaP, DEN, and AAF however, it is anticipated that exposure protocols will be modified to include mixtures of potential genotoxic agents.

The study to determine if chromosomal proteins change as a result of xenobiotic exposure is being continued to identify and characterize proteins which signal changes in gene expression or DNA repair activity and further characterize the proteins responsible for the resistance of medaka DNA to alkaline unwinding and salt extraction. This resistance is induced in BaP exposed fish. Additional studies to measure developmental abnormalities caused by BaP and contaminant mixtures from field sites are being continued. The development of dominant lethal assay protocols using microinjection are planned; this assay is a measure of inherited genotoxic effects.

As mentioned in Task 2.b. above, the evaluation of new techniques to detect and measure DNA repair and DNA damage due to free radicals, cross-linking agents, and other DNA interacting compounds is in progress.

As data generated from the BaP exposure experiment becomes available, it will be compared to similar data generated from the DEN and AAF exposure experiments in an effort to evaluate the usefulness and effectiveness of the medaka bioassay model to predict exposure to genotoxic chemicals.

V. ADMINISTRATION COMMENTS

This report was prepared according to the instructions found in the U.S. Army Medical Bioengineering Research & Development Laboratory document entitled "Contractor Reporting Requirements for the Health Effects Research Division" dated march 1984.

VI. GANTT CHART

Attached.

VII. COST SUMMARY REPORT

1. PROJECT COST SUMMARY

Attached.

2. COST SUMMARY GRAPH

Attached.

VIII. APPENDIX "A"

Analysis of DNA Adducts, Strand Breaks, and Chromosomal Proteins in the Japanese Medaka.

Attached.

IX. APPENDIX "B"

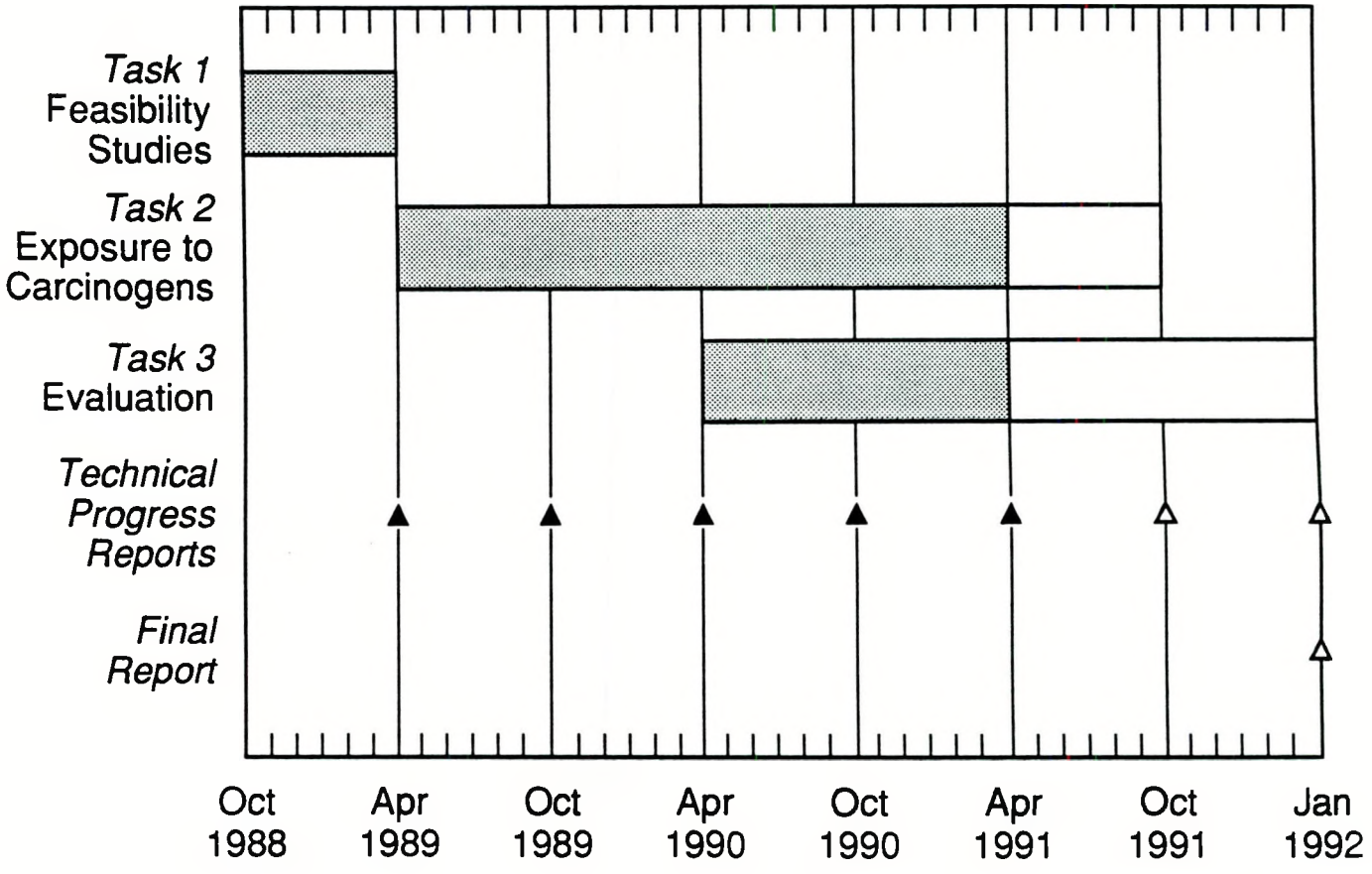
Japanese Medaka Embryo Assay for Developmental Toxicity: Abnormalities Observed After Exposure to Benzo[a]Pyrene.

Attached

Lee R. Shugart, April 1, 1991

ORNL-DWG 91M-7473

Project Title: Biological (Molecular and Cellular) Markers of Toxicity
Contract No.: U.S. Army 88PP8861
IAG 1016-B047-A1
Reporting Period: October 1, 1990 to April 1, 1991
Performing Organization: Oak Ridge National Laboratory
Principal Investigator: L. R. Shugart
Date: April 1, 1990



PROJECT COST SUMMARY

Project Title No.: Biological (Molecular and Cellular) Markers of Toxicity/U.S. Army 88PP8861
Organization/PI: Oak Ridge National Laboratory/Lee R. Shugart
Reporting Period: October 1, 1990 to January 15, 1992

Total Allocation: $ 44,590 Carry-over from 2nd year
$130,000 Allocated 3rd year
$174,590

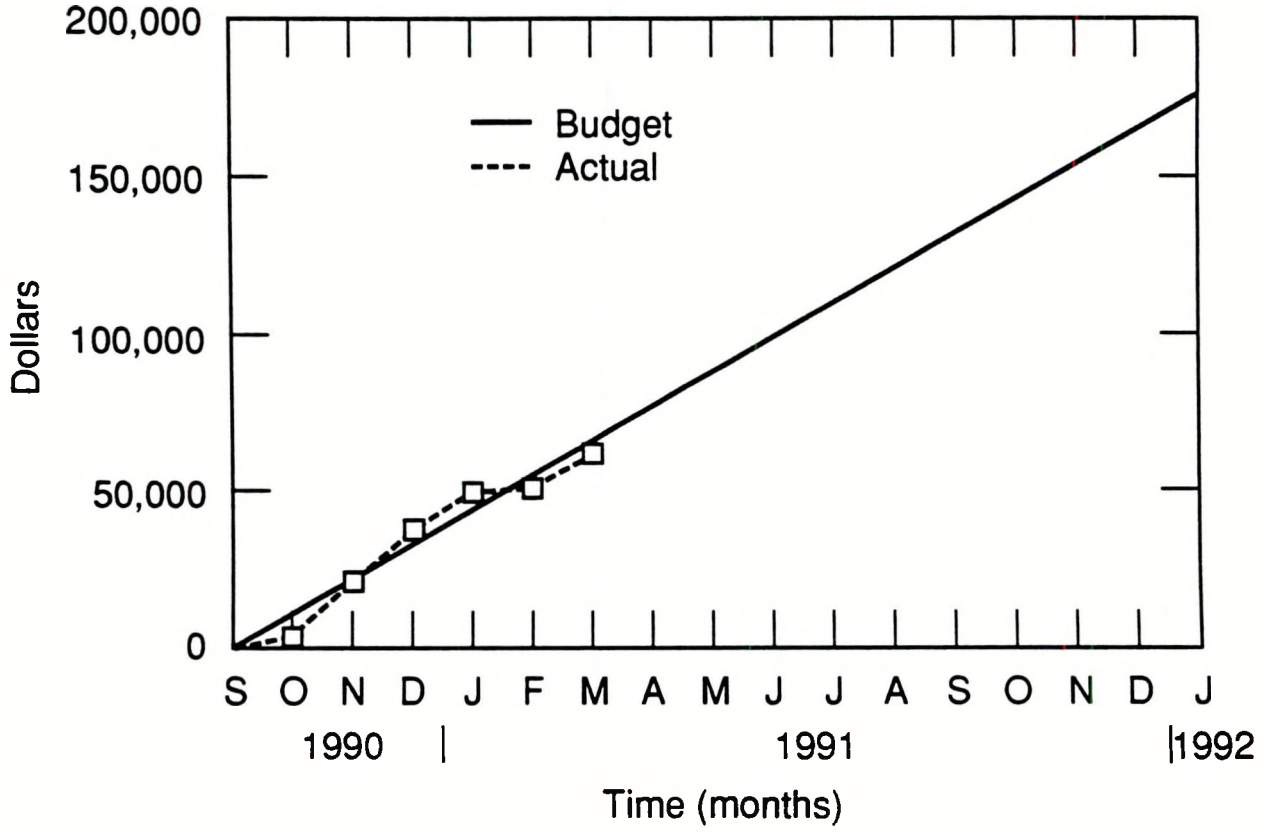
	Monthly Expenditures			Cumulative Expenditures			Available
	Budget	Actual	Variance	Budget	Actual	Variance	Balance
							174,590
Oct	10,911	4,469	-6,442	10,911	4,469	- 6,442	170,121
Nov	10,911	17,453	+6,542	21,822	21,922	+ 100	152,668
Dec	10,911	16,443	+5,532	32,733	38,365	+ 5,632	136,225
Jan	10,911	13,177	+2,266	43,644	51,542	+ 7898	123,048
Feb	10,911	366	-10,545	54,555	51,908	- 2,647	122,682
Mar	10,911	11,682	+ 771	65,466	63,590	- 1,876	111,000
Apr	10,911						
May	10,911						
Jun	10,911						
Jul	10,911						
Aug	10,911						
Sep	10,911						
Oct	10,911						
Nov	10,911						
Dec	10,911						
Jan	10,925						

Note: Cost data as of end of each month.
This report was prepared 4/1/91.

ORNL-DWG 91M-7472

Cost Summary Graph

Project Title / No.: Biological (Molecular and Cellular) Markers of Toxicity / U.S. Army No. 88PP8861
Organization / PI: Oak Ridge National Lab / L. R. Shugart
Reporting Period: October 1, 1990 to January 15, 1992
Total Allocation: $174,490



APPENDIX A

Analysis of DNA Adducts, Strand Breaks, and Chromosomal Proteins in the Japanese Medaka*

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December, 1990

Prepared in partial fulfillment of the requirement of the ORNL-GLCA/ACM Science Semester under the direction of Dr. Stephen J. D'Surney, Research Supervisor, in the Environmental Sciences Division.

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ABSTRACT

Work in this lab has focused on using DNA damage in fish as a biological marker to quantify environmental insult, with benzo[a]pyrene as a test contaminant. Damages studied include adducts formed by the diol-epoxide metabolite of BaP, and single strand breaks. In exposure studies conducted on the Japanese Medaka, adducts were detected after exposure of 42 hours, but none were detected after 10 day exposure. Strand breaks were detected after 10 days, but not after 42 hours. In conducting strand break studies, it was observed that Medaka DNA resisted unwinding from alkalinity and heat, leading to studies investigating this phenomenon. Exposure to BaP in water was conducted for 51 days, at a concentration of 4 μg/L. From blood samples of control and exposed Medaka, along with control Bluegill, nuclear chromatin was isolated, and chromosomal/nuclear proteins were studied by SDS polyacrylamide gel electrophoresis. Major differences were observed in the histone fractions between Medaka and Bluegill extracted with NaCl, and between exposed and control Medaka, this may account for resistance of medaka DNA to alkaline unwinding. There was a noticeable decrease in non-histone protein in 51d BaP exposed medaka suggesting that this phenomenon is a consequence of chronic exposure to PAH's and may affect the expression or integrity of the genetic information.

INTRODUCTION

In screening aquatic systems for the presence of contaminants, it is useful to have a model organism for which the toxicological, carcinogenic, teratogenic, and mutagenic effects have been well characterized. The Japanese Medaka (Oryzias latipes) has been proposed as such an organism (Donaldson and Scherer, 1983). A typical method of quantifying the effects of contaminant exposure has been to focus on carcinogenesis, with the identification of neoplastic legions serving as a biological endpoint (Shugart et al., 1990). However, because there is frequently a long time period between exposure and the appearance of such lesions, an effort has been undertaken to identify endpoints which would detect exposure to xenobiotic compounds within a shorter latent period. This has the advantage of early detection and determination of the severity of the exposure, taking into account physiological factors which may serve to moderate intake, with the intent of beginning preventive measures before irreparable damage has been done to the ecosystem. One promising endpoint has proven to be the interactions of contaminating compounds with DNA of the model organism.

One of the primary groups of contaminants that these studies are concerned with are polycyclic aromatic hydrocarbons (PAH's). It is known that these chemicals are ubiquitous pollutants, and that fish accumulate them through their water and diet. It is also known that some PAH's can be metabolized to more toxic metabolites (Varanasi et al., 1982, 1983). A number of metabolites can react

covalently with DNA to form adducts (Phillips and Sims, 1979), one of the primary interactions that has been studied. It has also been proposed that the cellular concentration of adducts can be used as an approximation of the "biologically effective" dose of the contaminant, and can therefore be used effectively as a measure of exposure (Hoel et al., 1983).

A technique was introduced for the detection and quantitation of the stable adducts formed by the carcinogenic diol epoxide metabolites of benzo[a]pyrene (BaP) (Shugart et al., 1982, 1983). The technique involves the removal of the BaP-diol epoxide tetrols by acid hydrolysis, and analysis by HPLC/fluorescence. This is an improvement over previously used methods, since it allows for detection of fmol quantities of adducts, and it detects only those adducts formed from the diol epoxide metabolite of BaP.

In addition to the stable adducts that can be formed, the diolepoxide metabolites of BaP are also capable of forming alkali-labile adducts with DNA that result in single strand breaks (Shugart, 1988). The alkaline unwinding assay has been developed as a technique for determining the concentration of single strand breaks (Rydberg, 1975; Kanter and Schwartz, 1979; Ahnstrom and Erixon, 1980). The technique is based on the principle that, under conditions of alkaline pH and heat, DNA will begin to unwind at single strand breaks. Thus, upon exposure to these conditions for a standard length of time, assuming the DNA is not allowed to renature, the amount of double stranded DNA remaining will be inversely proportional to the concentration of strand breaks. This

technique can be used to conduct in vivo assays of cells in culture (Kanter and Schwartz, 1982; Daniel et al., 1985), and the procedure of Downs and Wilfinger (1983) has been modified by Shugart (1988) to allow for the isolation of DNA from the liver of fish, for the purpose of conducting the alkaline unwinding assay.

In performing this analysis, it was observed by Shugart et al. (in press) that while in Bluegill (Lepomis macrochirus), a species of fish used for comparison, a nearly linear relationship exists between temperature of incubation and fractional decrease in fluorescence, this pattern is not observed in medaka (Fig. 1). In fact, little if any DNA unwinding is observed at temperatures as high as 50° C. Further data showed that this effect was even more pronounced in groups exposed to BaP and other xenobiotics (Fig. 2). A possible explanation for the observed resistance to unwinding is some natural factor associated with the DNA that is induced in response to xenobiotic exposure. This factor could function to shield the DNA, and/or possibly to aid in DNA repair.

Because it was also observed that proteinase K treatment releases, to a large extent, the effect of the resistance to unwinding (Shugart et al., in press), it was concluded that protein is the most likely candidate for the factor(s) causing resistance, although polyamines (Bachrach, 1973), Poly-ADP-ribose or DNA sequence structures could account for this observation. Research was begun to analyze the proteins associated with medaka chromatin to determine the identity of this factor(s), with particular emphasis on the histones as a starting point. Because the histones

are intimately involved in determining the structure of chromatin, this group of proteins is believed to be a logical starting point to study the factor(s) responsible for resistance to alkaline unwinding of medaka DNA.

MATERIALS AND METHODS

BaP Exposure I:

Exposure to BaP and BaP + verapamil was conducted identically to that of exposure III as described by Shugart et al. (in press). Two consecutive exposure experiments used eight fish per treatment group per 5 liter tank. For the BaP exposed groups, BaP was equilibrated in the tanks by continuous diffusion from a BaP generator at a concentration of 4 μg/L. The generator consisted of BaP coated on glass beads. After 42 hours, the fish were sacrificed by spinal scission, and DNA was extracted by the procedure described by Shugart et al., (in press). This procedure was designed, by the use of Sarkosyl, DNAase-free RNAase, and proteinase k, to remove protein and yield an exceptionally pure sample of DNA. Analysis of adducts was performed according to the procedure of Shugart et al., (1986). Strand break analysis was also conducted by the procedure of Shugart (1988).

BaP Exposure II:

Exposure was similar to that of exposure I, with the only difference being the length of time of exposure, 10 days instead of 42 hours. At the end of the exposure period the fish were

sacrificed. Strand break and DNA adduct analyses were conducted similarly to Exposure I.

BaP Exposure III:

Exposure was similar to I and II, but for 51 days, but minus the BaP + verapamil treatment group. At the end of that time, chromosomal and nuclear protein samples were obtained from isolated blood cell chromatin. Isolation of nuclei was done by a modification of a procedure of Hewish and Burgoyne (1973). Two medaka were placed in a small beaker containing 4 mls of buffer 1 (15 mM Tris, 15 mM NaCl, 15 mM ß-mercapto ethanol, 60 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 0.34 M sucrose, 2 mM EDTA, 0.5 mM EGTA, pH 7.4). The fish were decapitated, and blood was collected by filtration through glass wool using a Pasteur pipet. The solution was transferred to a test tube, and centrifuged for 5 minutes at 1000 rpm on a Dynac tabletop centrifuge. The supernatant was drawn off, and the cellular pellet was resuspended in 2 mls of buffer 1. This washing procedure was repeated a total of 3 times, after which the pellet was resuspended in buffer 2 (identical to buffer 1 but made up to 0.5% NP-40). Samples were washed twice, using 5 minute, 1500 rpm centrifugation in order to pellet nuclei. Samples were then washed twice in buffer 3 (15 mM Tris, 15 mM NaCl, 15 mM ß-mercapto ethanol, 0.5 mM spermidine, 0.15 mM spermine, 0.34 M sucrose, 1 mM PMSF, pH 7.4). After the second wash, the pellet was resuspended in 500 μL of distilled water, with 20 μL of 2.0% SDS in buffer 3. Samples were spun on an Eppendorf

Microfuge at 13,000 rpm for 2 minutes. The supernatant containing chromatin was collected and frozen for later use. Some samples, instead of freezing immediately, were ethanol precipitated using 2 volumes of ice-cold 100% ethanol.

Chromatin samples were analyzed using SDS polyacrylamide gel electrophoresis (SDS/PAGE) with a 13% acrylamide concentration by the procedure of Laemmli (1970), for chromatin and nuclear protein molecular weight analysis. Precipitated samples were redissolved in pure sample solution (1.52 g Tris base, 20 ml of glycerol, 2.0 g of SDS, 2.0 ml of 2-mercaptoethanol, 1 mg of Bromphenol Blue in 100 ml of water, pH 6.8), while raw chromatin samples were dissociated in 2 volumes of sample solution. A weaker dissociation was performed by making the nuclear lysates up to 2 M NaCl and dissolving in an equal volume of sample solution. All samples were then boiled for 5 minutes, and centrifuged on an Eppendorf centrifuge for 2 minutes at 13,000 rpm before application to the gel which were electrophoresed at 25-35 ma for 4 hours.

The gels were stained, either by Coomassie Blue, as described by Ausubel et al. (1989), or by Silver Stain, according to the procedure described by Ausubel et al. (1989) modified from a procedure described by Oakley et al. (1980). The gels were scanned on a LKB Ultroscan Laser Densitometer.

RESULTS

BaP Exposure I:

After a 42h exposure, no DNA strand breaks were observed (Fig.

3). Alkaline unwinding analyses were performed at 4°C, 38°C, and 52°C, none of which showed any difference among the three treatment groups. The BaP metabolite Tetrol I-1, which is the predominate form, was observed at a level of 0.5-1.25 ng/mg of hydrolysed DNA in BaP and (BaP & verapamil) exposed fish (Fig. 4).

BaP Exposure II:

After 10 day exposure, a low level strand breaks were detected (Fig. 5). F values were .225 ± .020 for the control group, .180 ± .017 for the BaP exposed group, and .167 ± .030 for the BaP and verapamil exposed group. This corresponds to a 15% increase in strand breaks for BaP exposed, and a 20% increase in strand breaks for the (BaP and verapamil) exposed group ($N= [\ln F_{exp}/\ln F_{control}]-1$). F values in this experiment were at the lower optimal range for DNA and may have resulted from DNA shearing during purification or is characteristic of DNA that has been highly purified with Proteinase K and RNAase treatment. No adducts were detected in this exposure (Fig. 6).

BaP Exposure III:

A comparison of chromosomal proteins extracted from precipitated chromatin for bluegill, medaka, and 51d BaP exposed medaka is shown in Figure 7. Both the core and H1-H5 histone profiles are very similar for control bluegill (Fig. 7A) and medaka (Fig. 7B) with medaka H1-H5 histone groups showing a slightly greater electrophoretic mobility than the bluegill H1-H5 histone

group. The H5 histone is found in the nucleated erythrocytes of both birds and fish, but is absent in other tissues. There is a greater amount and variety of non-histone proteins present in medaka chromatin relative to bluegill. These proteins are predominately much higher in molecular weight than the histone groups and have a regulatory function on DNA. In medaka exposed to BaP for 51d (Fig. 7C) there is a loss of most of the non-histone proteins, changes in histone profiles, and an increase in a protein(s) with an apparent mass of around 14-15 kD. The H1-H5 histone group shows a wide variable response in BaP exposed fish with enhancement or loss of the prominent peak in that group. In the core histone group the protein peak with the least mobility (more + charged protein band) is consistently reduced relative to the other core histone bands in BaP exposed fish.

A comparison of bluegill, medaka, and BaP exposed medaka nuclear (chromosomal) proteins extracted under partial dissociating conditions is shown in Figure 8. The H1-H5 histone group does not dissociate as readily from the DNA as the core histones. The core histones of bluegill (Fig. 8A) are extracted from DNA more easily than from both control and BaP exposed medaka DNA (Fig. 8B). Protein(s) with an apparent mass of 14-15 kD is detected in greater amounts in BaP exposed medaka nuclei.

DISCUSSION:

The data from Exposures I and II show that adducts are detectable after 42 hour exposure, but are not detectable after 10

days. In contrast, strand breaks are not detectable after 42 hours, but do appear in a 10 day exposure. It should be noted that the increase in strand breaks is approximately 15-20%, whereas in similar studies conducted on Bluegill, strand breaks increased by as much as 300% (Shugart, 1988). The factors causing a disappearance of adducts between these two time points concurrent with a rise in strand breaks are not conclusively known. However, this could be explained by a model based on the excision-repair mechanism for repair of damaged DNA (Hanawalt et al., 1979). In this model, adducts are repaired by a mechanism which temporarily creates strand breaks. An endonuclease nicks the DNA strand near the damaged site (adducts, dimers), a DNA polymerase synthesizes new DNA in place of the damaged portion and also excises the damaged portion from the remaining attached end simultaneously, and ligase finally seals the newly-repaired DNA. This model is only one of several known mechanisms of DNA repair, and is one possible explanation for the data. An additional theory is that instead of or in concert with DNA repair, a proteinase K sensitive protective mechanism is being induced which serves to shield the DNA from damage or maintain DNA structure. This could also account for the relatively small increase in strand breakage compared to similar studies in Bluegill. The high incidence of strand breaks in Bluegill may result from a greater initial sensitivity to DNA damaging agents but a decrease in strand breaks during chronic BaP exposure may result from induced repair processes (Shugart, 1988).

Fig. 7 shows that, under strong dissociating conditions,

medaka blood cells have more non-histone proteins than bluegill. Non-histone proteins comprise around 40% of the chromosomal protein in most cells (Van Holde, 1989), except nucleated erythrocytes where histone comprise over 80% of chromosomal protein. Non-histone proteins contain more than 100 different proteins each in low abundance relative to the 5-6 main histone types which are abundant in eukaryotic cells. The loss of non-histone proteins in 51d BaP exposed medaka may reflect an overall decrease in DNA regulatory/gene expression activity in chronic exposed fish.

Current studies in our lab exposing bluegill to heavy metal (Pb, Hg) and PAH contaminated sediment has shown a considerable increase in non-histone proteins at two weeks exposure and a near complete loss of these proteins at eight weeks exposure (unpublished observations). Many organ-tissue cells are in a constant state of differentiation and regeneration such as blood and liver tissue and changes in non-histone proteins may signal subsequent changes in the state of differentiation. These cellular changes are known to occur in pathological conditions (cancer, anemias, etc.). Additionally, changes in non-histone protein composition may follow the induction of the various enzymes responsible for DNA repair functions.

Histones comprise most of the chromosomal protein in eukayotic cells (Van Holde, 1989). The nucleosomal core structures contain two each of H2A, H2B, H3 and H4 histones common to all eukayotic cells while H1 histone serves to bind adjacent nucleosomes together to facilitate higher orders of chromosomal folding. Erythrocyte

nuclei of birds reptiles and fish contain an additional H5 histone whose concentration increases with red cell differentiation. The H1-(H5) histones are quite variable between and within species as well as in different tissues. The different electrophoretic mobility between bluegill and medaka H1-H5 histone groups denote different functional variants within this group. Changes in the quantitative profiles seen in BaP exposed fish may reflect a change in chromatin structure and cell differentiation.

Differences in the core histones between bluegill and control-exposed medaka under conditions of differential protein extraction (Fig. 8) indicate a nucleosomal structure more resistent to dissociation in medaka relative to bluegill. The protein(s) of apparent molecular weight 14-15 kD which is induced and easily extracted from BaP exposed medaka may be modified histone or represent new protein synthesis.

While these histones are highly evolutionarily conserved, they are known to possess modifications which can affect structure, function, and electrophoretic mobility (Van Holde, 1989). This is primarily because modifications, such as phosphorylation, acetylation, and poly-ADP-ribosylation decrease the positive charge of the histone, and serve to increase gel mobility. Poly-ADP-ribosylation of histone (Van Holde, 1989) is a possible explanation of resistance of nucleosomes to dissociation. Nucleosome binding by this polymer could confer some degree of resistance to protease treatment. Another modification that could be important is histone phosphorylation, which may be associated with a tightening of

chromatin structure (Van Holde, 1989). The increased migration distance associated with the negative charge on the phosphate group could change the histone densitometric profile.

It is clear that considerably more data need to be amassed before any conclusions concerning the phenomenon of observed resistance to unwinding in medaka DNA can be drawn. This work provides some basis for future study. It is apparent that there are differences in the protein content of chromatin, both between Bluegill and medaka, and between control and BaP exposed medaka. These differences may function to protect DNA and/or to be responsible for resistance to unwinding in medaka. Future studies could involve isolation and characterization of particular protein fractions, with the intent of identifying factors involved in DNA protection and resistance to unwinding. Experimentally, fractions could be tested for ability to bind to DNA, which may give some indication as to capacity to confer resistance to deproteination. Also, future studies could focus more extensively on the phenomenon of Histone modification. Poly-ADP-ribosylation and phosphorylation can be quantified in histone fractions. Employing 2D gel electrophoresis and western blot analysis with antibody probes can identify specific protein species which maintain DNA structure such as repair enzymes.

LIST OF REFERENCES

Ahnstrom, G. and K. Erixon, 1980. Measurement of strand breaks by alkaline denaturation and hydroxyapatite chromatography. In: DNA Repair, Vol. 1 part A, edited by E.C. Friedberg and P.C. Hanaweldt, Dekker, NY, pp. 403-419.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. (1989). Current Protocols in Molecular Biology. John Wiley and Sons, New York, NY.

Bachrach, U. 1973. Function of Naturally Occurring Polyamines. Academic Press, New York.

Daniel, F.B., D.L. Haas and S.M. Pyle, 1985. Quantitation of chemically induced DNA strand breaks in human cells via an alkaline unwinding assay. Analytical Biochemistry 144: 390-402.

Donaldson, E.M., and E. Scherer. 1983. Methods to test and assess effects of chemicals on reproduction in fish. In Vouk and Sheehan (eds.) Methods of Assessing the Effects of Chemicals on Reproductive Functions. Wiley, New York.

Downs, T.R., and W.W. Wilfinger, 1983. Fluorometric quantification of DNA in cells and tissue. Analytical Biochemistry 131: 538-547.

Hewish, D.R., and L.A. Burgoyne. 1973. Chromatin Sub-structure. The digestion of chromatin DNA at regularly spaced sites by a nuclear deoxyribonuclease. Biochemical and Biophysical Research Communications 52: 504-510.

Hanawalt, P.C., P.K. Cooper, A.K. Ganesan, and C.A. Smith. 1979. DNA repair in bacteria and mammalian cells. Annual Review of Biochemistry 48: 783-836.

Hoel, D., N. Kaplan and M. Anderson, 1983. Implication of nonlinear kinetics on risk estimation in carcinogenesis. Science 219, 1032-1037.

Kanter, P.M. and H.S. Schwartz, 1979. A hydroxylapatite batch assay for quantitation of cellular DNA damage. Analytical Biochemistry 97: 77-84.

Kanter, P.M. and H.S. Schwartz, 1982. A fluorescence enhancement assay for cellular DNA damage. Molecular Pharmacology 22: 145-151.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680-685.

Oakley, B.R., D.R. Kirsch, and N.R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Analytical Biochemistry 105: 361-363.

Phillips, D. and P. Sims, 1979. PAH metabolites: their reaction with nucleic acids. In: Chemical Carcinogens and DNA, Vol. 2, edited by P.L. Grover, CRC Press, Inc., Boca Raton, FL, pp. 9-57.

Rydberg, B. 1975. The rate of strand separation in alkali of DNA or irradiated mammalian cells. Radiat. Res. 61: 274-287.

Shugart, L.R., R. Rahn and J. Holland, 1982. Quantifying BaP binding to DNA by fluorescent analysis. In: PAHs: 7th International Symposium on Formation, Metabolism, and Measurement, edited by M. Cooke and A. Dennis, Battelle Press, Columbus, OH, pp. 1087-1097.

Shugart, L.R., J. Holland, and R. Rahn, 1983. Dosimetry of PAH carcinogenesis: covalent binding of BaP to mouse epidermal DNA. Carcinogenesis 4: 195-198.

Shugart, L.R., J. McCarthy, B. Jimenez, and J. Daniels. Analysis of adduct formation in the Bluegill sunfish (Lepomis macrochirus) between benzo[a]pyrene and DNA of the liver and hemoglobin of the erythrocyte. Aquatic Toxicology 9: 319-325.

Shugart, L.R. 1988. Quantitation of chemically induced damage to DNA of aquatic organisms by alkaline unwinding assay. Aquatic Toxicology 13: 43-52.

Shugart, L.R., J.F. McCarthy, S.J. D'Surney, M.S. Greeley, and C. Gettys-Hull. 1990. Biological (Molecular and Cellular) Markers of Toxicity in the Japanese Medaka Oryzias latipes. 3rd Annual Carcinogenicity Research Review, August 1990, Frederick, MD. In Press.

Van Holde, K.E. 1989. Chromatin. Springer-Verlag, New York, NY.

Varanasi, U., J.E. Stein, M. Nishimoto and T. Hom, 1983. BaP metabolites in liver, muscles, gonads and bile of adult English sole (Parophrys vetulus). In: PAHs 7th International Symposium on Formation, Metabolism and Measurement, edited by M. Cooke and A.J. Dennis, Battelle Press, Columbus, OH, pp. 1221-1234.

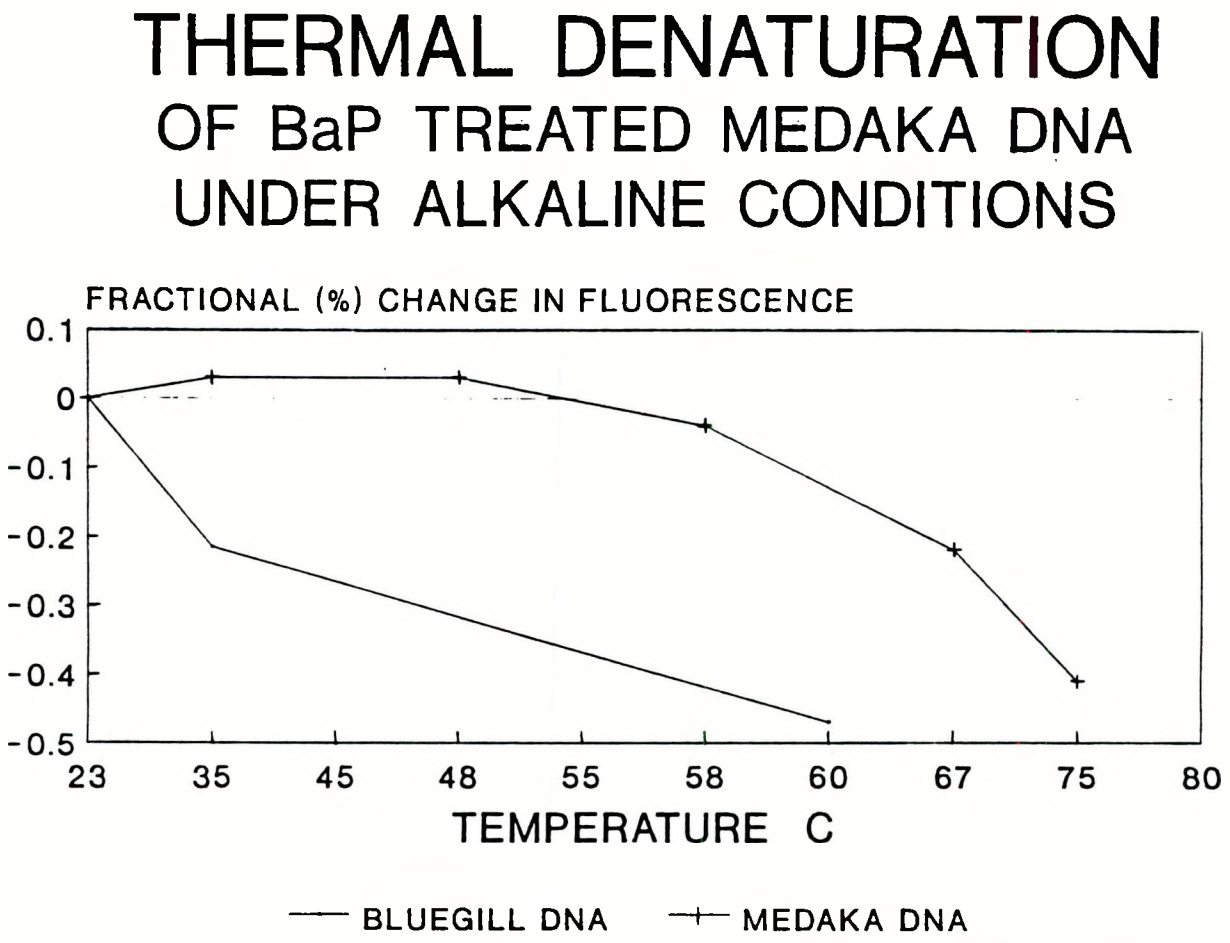


Figure 1. From Shugart et al., 1990 (in press). Thermal denaturation of BaP treated Medaka DNA under alkaline conditions. The extent of DNA unwinding was measured as the percent decrease in fluorescence readings of DNA + Hoechsh dye # 33258 with increasing temperature. Medaka DNA came from fish exposed to BaP in water. Data from Bluegill was obtained under similar conditions.

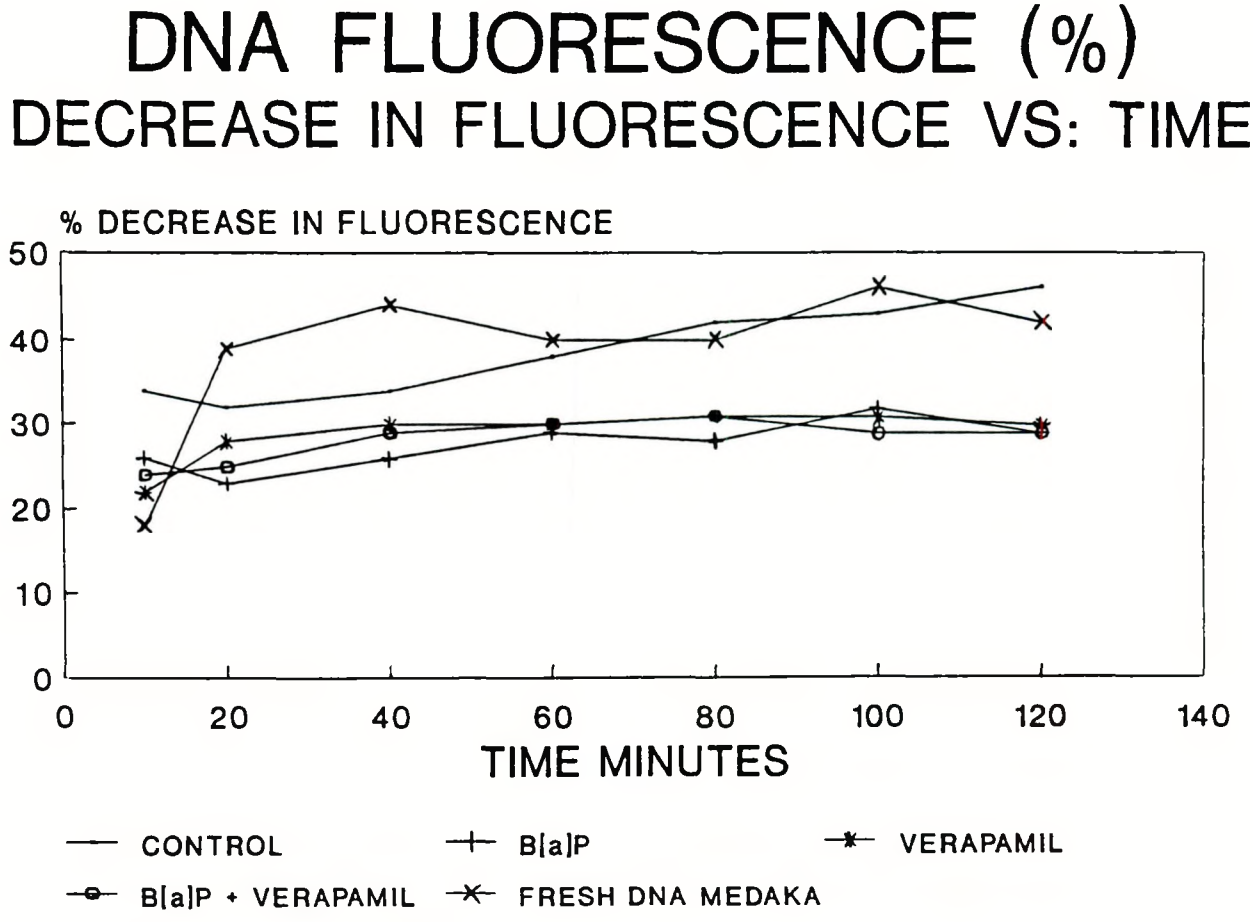


Figure 2. From Shugart et al., 1990 (in press). Comparison of alkaline unwinding of Medaka DNA from control, BaP and verapamil exposed fish. The degree of alkaline unwinding was determined as the percent decrease in fluorescence over time at 60°C. DNA extraction and alkaline unwinding assays were performed according to the methods of Shugart, 1988.

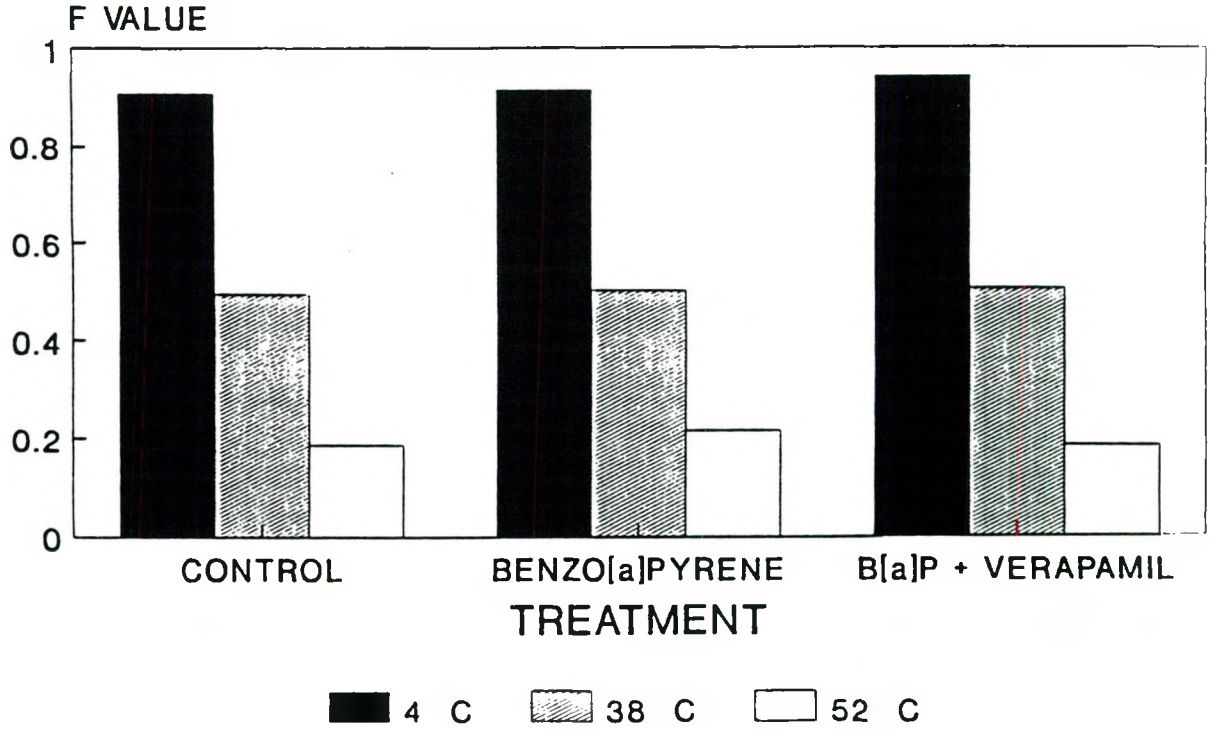


Figure 3. From Shugart et al., 1990 (in press). Comparison of alkaline unwinding F values from Medaka exposed to constant concentrations of BaP and BaP & verapamil for 42 hours. DNA was purified by a modified procedure and alkaline unwinding was performed according to the procedures of Shugart, 1988 at three temperatures.

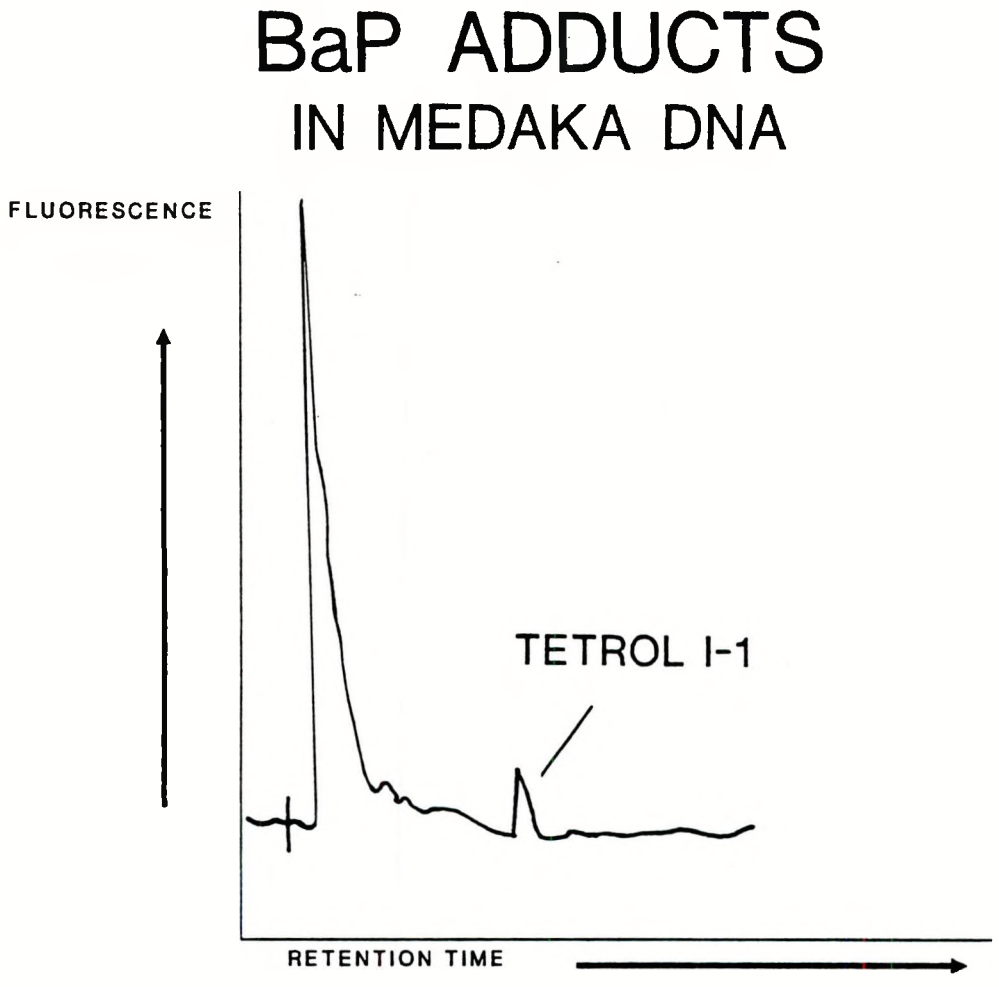
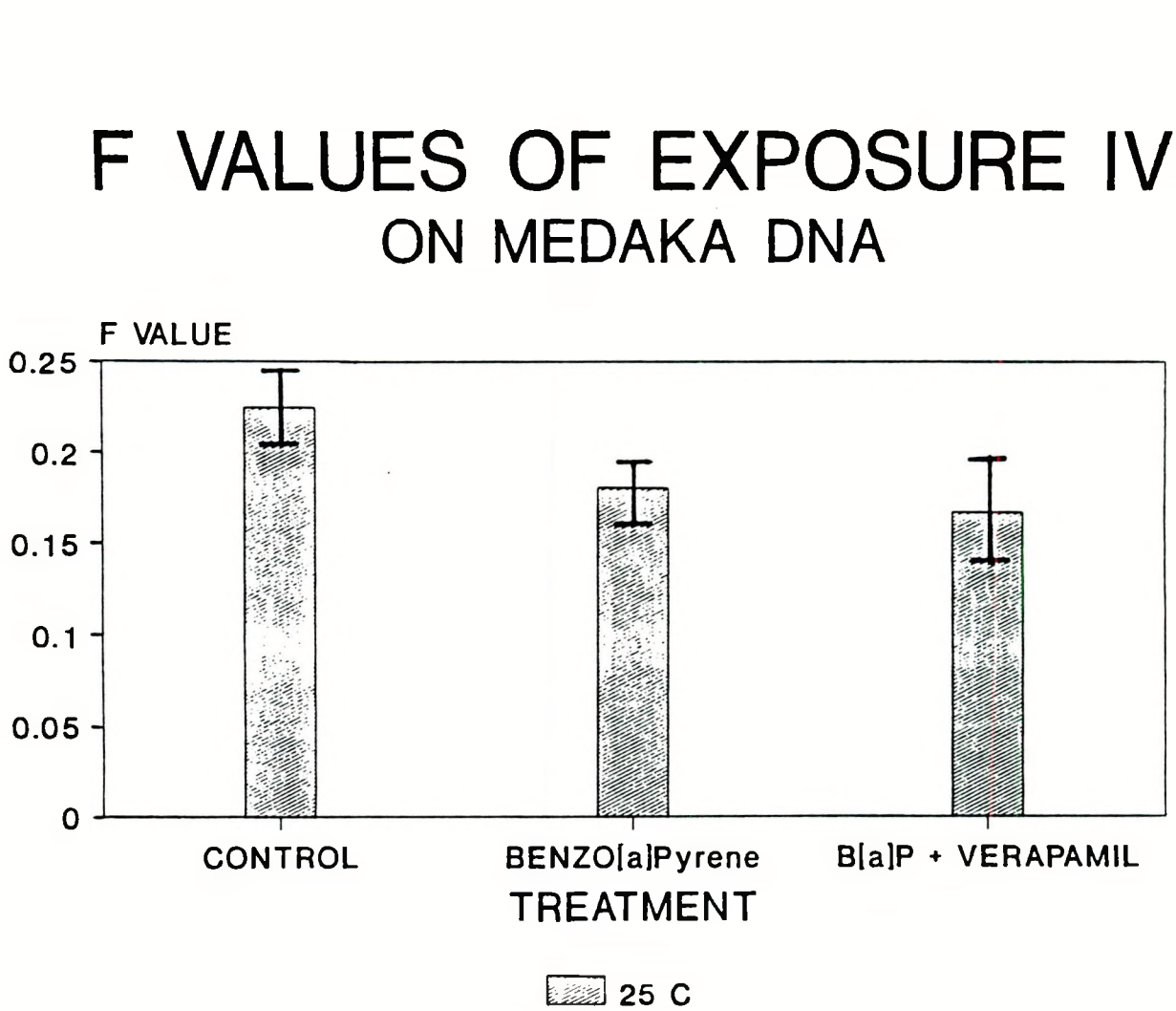


Figure 4. BaP adducts in Medaka DNA exposed to BaP for 42 hours, determined by HPLC/fluorescence analysis of free tetrols liberated from DNA by acid hydrolysis.



10d BaP exposure

Figure 5. F values of exposure IV on Medaka DNA. F values were determined by conducting an alkaline unwinding assay at 25°C for control, BaP exposed, and (BaP & verapamil) exposed samples.

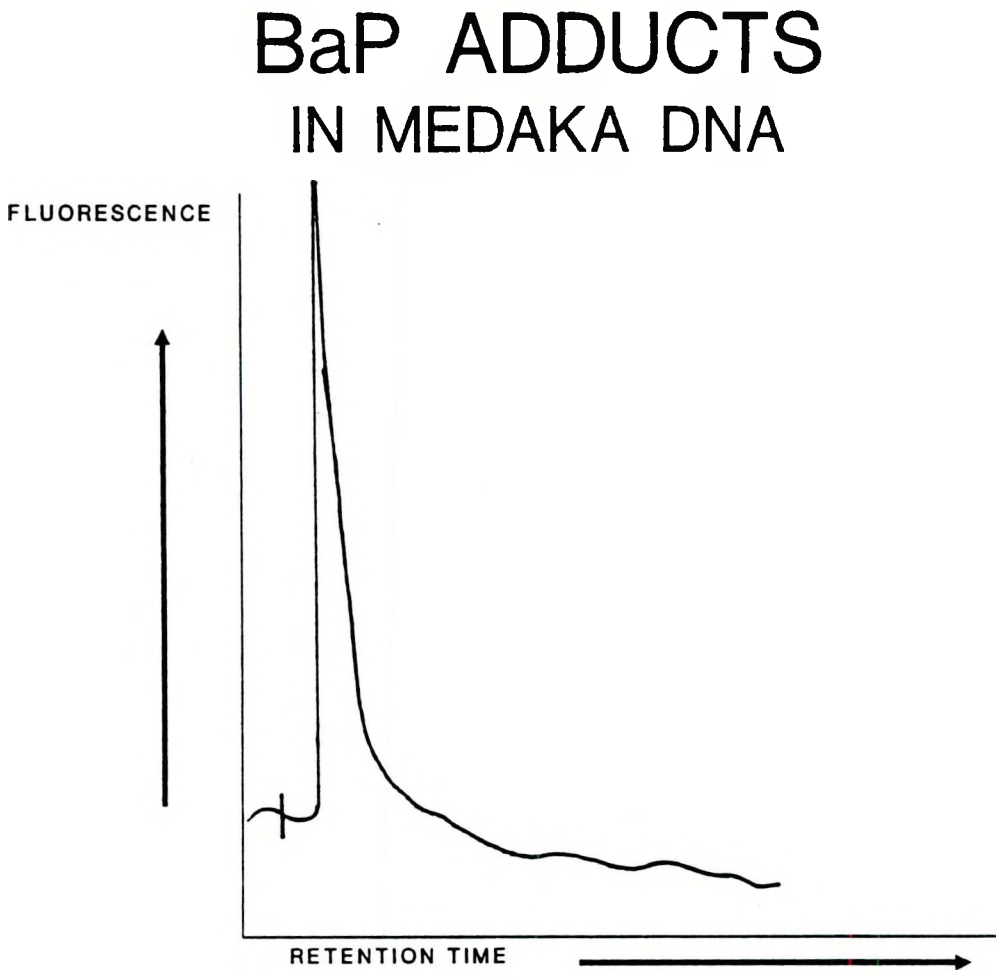


Figure 6. BaP adducts in Medaka DNA exposed to BaP for 10 days. Procedure of analysis was identical to that of the 42-hour exposure group. Adducts were not detected in this exposure.

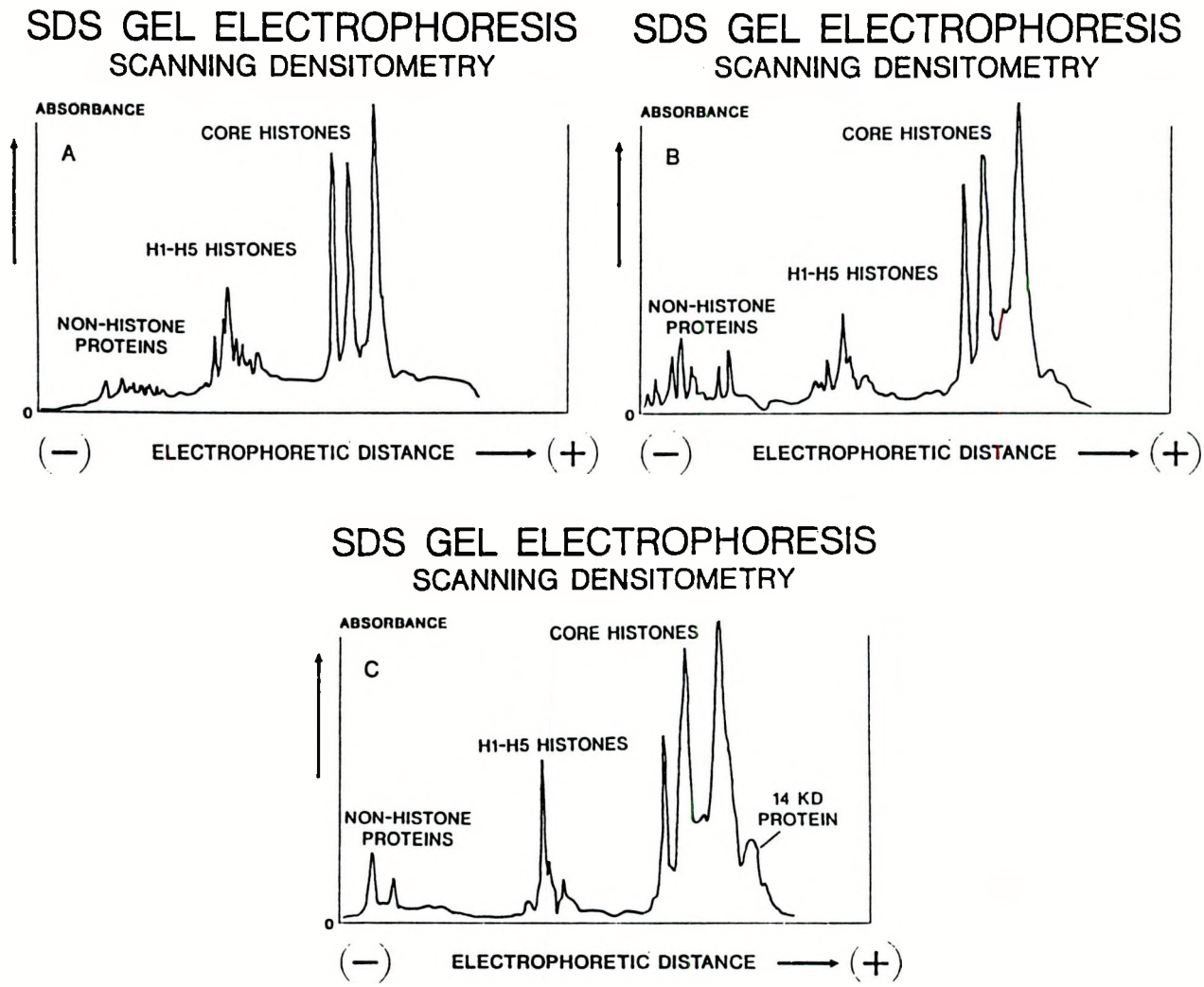


Figure 7. Scanning densitometric profiles of completely dissociated bluegill and medaka chromosomal proteins. Blood cell nuclei were purified, lysed, and the chromatin was precipitated in ethanol. Protein were dissociated from DNA by boiling in full strength SDS-beta-mercaptoethanol sample solution. 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

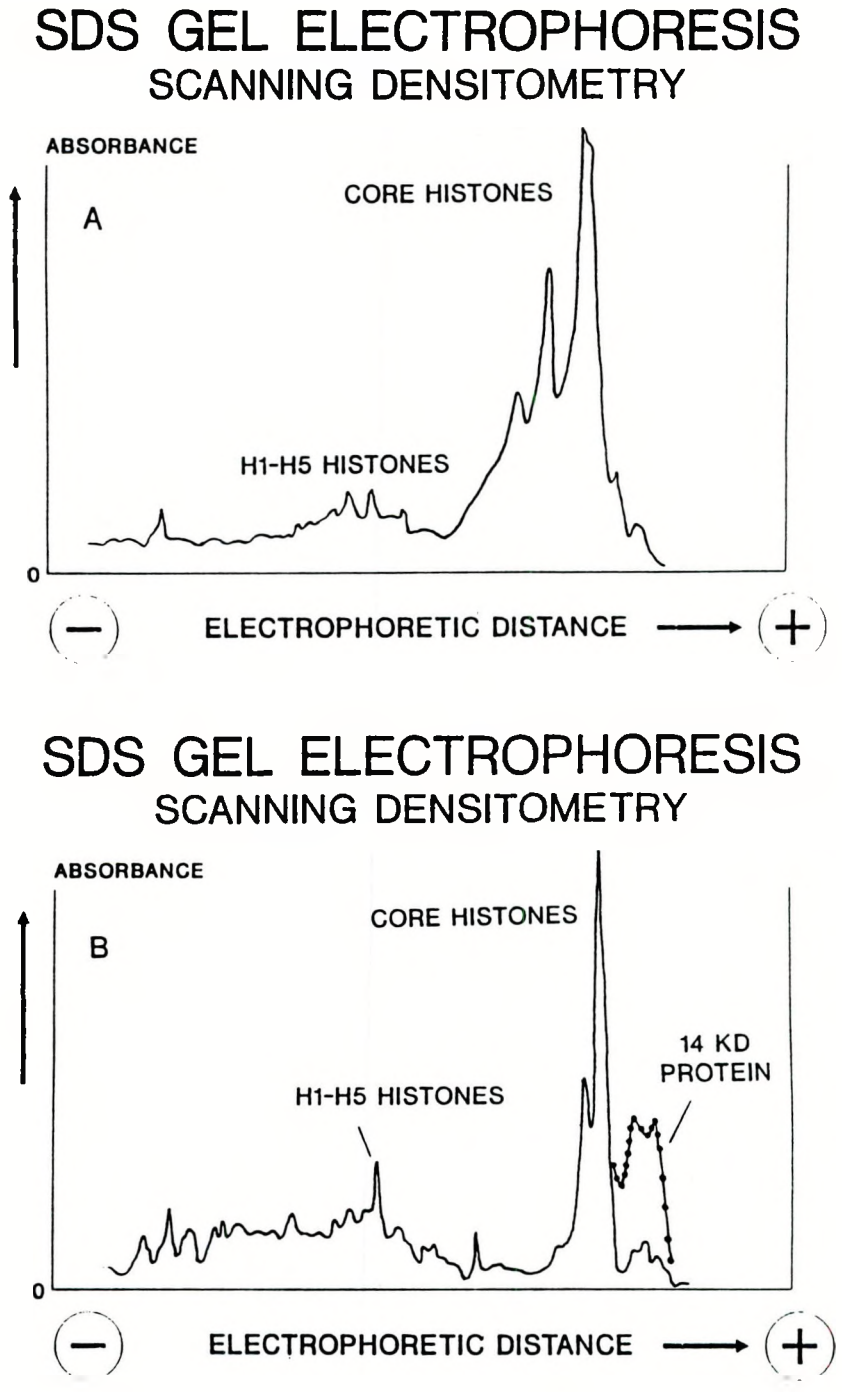


Figure 8. Scanning densitometric profiles of partially dissociated bluegill and medaka nuclear proteins. Blood cell nuclei were purified and lysed. The lysate was made up to 1M NaCl, mixed with an equal volume of SDS-beta mercaptoethanol sample solution, and boiled. Proteins were analyzed on 13% SDS-PAGE gels and silver stained.

A.Bluegill control

B.Medaka control and exposed to BaP for 51 days

APPENDIX B

Japanese medaka embryo assay for developmental toxicity: abnormalities observed after exposure to benzo[a]pyrene.

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Abstract

Medaka embryos (*Oryzias latipes*) were exposed to 10 µg/L BaP in embryo rearing medium (ERM) from 4 h post fertilization through yolk sac absorption. One group of embryos underwent complete ERM + BaP change every 24 h (24 h BaP) and the other group every 48 h (48 h BaP). Controls recieved complete ERM exchanges every 48 h. There were no obviously lethal effects observed in the embryos and fry. Both BaP treatments groups had significantly more circulatory abnormalities, with 14.29% ± 3.55 in 24 h BaP embryo and fry and 12.14% ± 3.33 in 48HBaP embryos. Only 4.55% ± 2.22 of control embyros were observed with circulatory abnormalities. Developmental abnormalities such as twinning, exophthalmia and altered pigmentation occurred in the exposed groups, but were not observed in the controls. BaP-exposed embryos required 48-72 h longer for 50% of the embyros to hatch, and exposed embryos continued to hatch for 192 h beyond the last successful control hatch. The absence of directly lethal effects was evident in the lack of significant difference in the hatching success, mortality, swimbladder inflation or length of time fry could swim between control and exposed groups.

Introduction

The Japanese medaka *(Oryzias latipes)* is a fish which is commonly used for embryo toxicity and developmental tests. Medaka embryo assays have been utilized in a number of attempts to determine the toxicity (TLm's and LC_{50}'s) and teratogenicity of various xenobiotics. However, there is currently no EPA protocol for the assay and there has been little consistency in its application between labs and researchers. For example, initial exposures of medaka embryos to tested xenobiotics have ranged from 2 hours post fertilization (Solomon and Weis, 1979; Dial, 1978) to organogenesis (Solomon and Faustman, 1987; Waterman, 1939; Shi and Faustman, 1979), and mode of exposure has varied from short-term

pulses (Siu-yin Leung and Bulkey, 1979; Hiraoka, et. al. 1989; Marty, et. al. in press) to exposure throughout the incubation period (Michibata, et. al. 1987; Stoss and Haines, 1979; Schreiweis and Murray, 1976; Heisinger and Green, 1975; Hiraoka and Okuda, 1983).

Currently, this lab is investigating various methods of conduction developmental toxicity tests utilizing the medaka, and it is our goal to develop a standard method for the embryo assay which is not labor intensive and can be easily adapted for various classes of chemicals. Ongoing experiments are designed to determine and contrast the effects of exposure of embryos to xenobiotics at several reproductive and developmental levels, including exposure to the parents, at the point of fertilization and during embryogenesis. Data from these experiments will complement existing carcinogenesis and adult toxicity data utilizing the Japanese medaka (Hatanaka et. al., 1982; Kyono and Egami, 1977; Hawkins et. al., 1988; Hawkins et. al., 1988; Hawkins et. al., 1985).

BaP has been shown to cause various developmental abnormalities in steelhead trout embryos after 24 h pulse exposure (2 and 20 μg/ml in DMSO) at specific developmental stages (Kocan and Landolt, 1984). This study demonstrated that the trout embryos were permeable to BaP, could metabolize the parent compound, and excreted some of the metabolites. Embryos exposed earlier in development were seen to both accumulate more and excreted less [^{14}C]BaP. This experiment described teratogenic effects, including

cephalic and spinal deformities, ocular abnormalities and fin reductions or eliminations. Others have shown that lesser amounts of BaP could both reduce the hatching success of sand sole (*Psettichthys melanostichus*) eggs (0.10 µg/L) and produce developmental abnormalities (Hose, et. al., 1982). Kocan and Landolt (1984) observed an extension of the hatching period of the steelhead trout as a result of BaP exposure (2-20 µg/ml on either day 15 or 25 post fertilization). BaP was found to produce similiar effects in Rainbow trout (Hannah et. al., 1982), where aqueous doses of BaP as low as 0.08 ppb in the hatching medium extended the hatching time of the embryos. Such results are not necessarily consistent in all species of fishes. Hose et. al.(1982) also reported that the survival of flathead and English sole was unaffected by exposure to BaP. The objective of the work described here was to determine if the polycyclic aromatic hydrocarbon, benzo[a]pyrene (BaP), could cause measurable developmental or lethal effects in medaka embryos exposed from 4 hours post fertilization through the yolk sac absorption stage. Other data on adult medaka BaP-exposures done in this lab have indicated that this species may have some resistance to BaP (unpublished data), thus this experiment will also provide data on the sensitivity to another life-stage of the medaka.

Methods and Materials:

Adult Care and Maintenance:

Japanese medaka (*Oryzias latipes*) fry obtained from the Gulf Coast Research Laboratory, Ocean Springs, MS., were raised to adulthood in 100 gal. flow-through aquaria supplied with dechlorinated tap water. The light cycle for breeding was 16 hour light:8 hour dark photoperiod at 25 °C. Medaka were offered Stress Flakes twice daily and 24 h brine shrimp once daily. Breeding groups of adult medaka (ratio 3 female, to 2 male) were placed in two 10-gal aquaria (Tanks A and B) for an overall density of 5 medaka/gal. These breeding aquaria were supplied with dechlorinated tap water which was purified by passage through an activated carbon filter cartridge, a UV sterilizer and a series of three 25-μm filters (Balston 54/50 LP-20 cartidge filters).

Egg Collection:

Eggs were collected beginning 1 h after the initiation of the light cycle. Females were removed from each breeding tank with a soft, nylon net. Eggs were gently brushed from the outer abdomen with gloved fingers or blunt forceps and placed into petri dishes containing embryo rearing medium (ERM). ERM was modified from Kirchen and West (1976), and contained 1 g NaCl, 30 mg KCl, 40 mg $CaCl_2.2H_2O$ and 163 mg $MgSO_2.7H_2O$ in 1 L of distilled Millipore-filtered water. Eggs from Tanks A and B were placed into separate petri dishes with approximately 150 eggs per dish. Eggs remain in clutches of 3-50 eggs, held together by chorionic filaments. Clutch separations and removal of these filaments were done under a binocular dissection microscope using fine-tipped watchmakers forceps. Damaged or unfertilized eggs, and any eggs which were

obviously remenants of a previous days spawns, were discarded.

Exposure to BaP:

Treatment with BaP was initiated within 3.5 to 4 h of egg collection. There were three treatment groups: a) controls, which were incubated in ERM alone and received exchanges of aerated ERM every 48 h until hatch, at which time they received 48 h exchanges of aerated distilled water, b) 24 h BaP, which were incubated in ERM + 10 μg/L BaP and received exchanges of this aerated solution every 24 h until hatch, at which time they received 24 h exchanges of 10 μg/L BaP in distilled water and c) 48 h BaP, which were incubated in ERM + 10 μg/L BaP and received exchanges of this aerated solution every 48 h until hatch, at which time they received 48 h exchanges of 10 μg/L BaP in distilled Millipore filtered water.

Each egg was placed individually into 7-ml scintillation vials containing 1 mL of either ERM or ERM + 10 μg/L BaP. Incubations were carried out at 24.5 - 25°C under low light conditions with the same photoperiod as the adults. Medaka eggs have a clear chorion, making microscopic observation of development easy. Embryos were observed at 24 h intervals for abnormalities, deaths or hatching. Once hatched, fry were observed daily for survival, swimbladder inflation, development of swimming ability, and post-hatch manifestation of any other obvious developmental abnormalities.

Preparation of Exposure Solutions:

ERM-BaP + 10 μg/L for exposures was made using BaP coated glass beads. The glass beads (10-20) which had been previously

coated with BaP were placed into a glass jar and ~250 mL of ERM added. The mixture was stirred for 24 h, following which a 10 mL aliquot was recovered for analysis on HPLC. This procedure was repeated every 48 h. Beads were replaced when chemical analysis showed any changes in BaP concentration of the ERM medium. For the analysis, the 10 mL aliquot was removed from the collection vial and passed over an equilibrated C18 Sep Pac. The collection vial was extracted twice with 2 mL of 20% HPLC-grade methanol, which was then passed through the Sep Pac. All BaP was removed from the Sep Pac with 3 mL of 100% methanol. This was taken to dryness under air at 85°C and resuspended in 100 μL of 100% methanol. The sample was injected onto an HPLC C18 column and eluted isocratically with 100% methanol. BaP quantities were calculated by comparison of peaks to known standards.

Statistics:

Data for mortality and hatching success for Tanks A and B within each treatment group were subjected to Chi-square analysis and pooled for further consideration if no significant differences were found ($P < 0.05$). Pooled treatment groups were then subjected to the same analysis. When two-by-two contingency tables were analyzed, corrections for continuity were included. Standard error for proportion (S_p) was calculated according to the formula ($S_p = \sqrt{p\,q\,/n-1}$), where p= the proportion of the population in that category and q= 1-p (Zar, 1984).

Results

Abnormalities in fry:

Both 24 h BaP and 48 h BaP groups showed significantly more developmental abnormalities during embryogenesis than the control group. The most commonly observed abnormalities were pooling of the blood along the veins of the yolk sac and within the developing embryo. Pooling generally occurred in the fore- and hindbrain and in the tail region. Pooling in the brain was most obvious at 168-192 h post fertilization; thereafter, increased pigmentation made continued observation difficult beyond this period. Incidence of circulatory problems were 14.29% ± 3.55 and 12.24% ± 3.33 in the 24 h BaP and 48 h BaP groups respectively, as compared with only 4.55% ± 2.22 of the control embryos (Table 1).

Several embryos in the BaP-treated group exhibited multiple abnormalities. There was one case of twinning in the 24 h BaP goup, with the second embryo apparent only as a heart and associated areas of pooled blood along with a urinary bladder. The heart of this smaller twin ceased beating prior to hatch, but the larger twin hatched and survived to yolk sac absorption. The remains of the second twin appeared to be incorporated into the abdominal area of the larger twin during yolk sac absorption. Also in this group, one embryo exhibited marked unilateral exophthalmia, severe circulatory problems and an overall yellow-green coloration. This embryo did hatch, but was never observed swimming and failed to inflate its swimbladder. In the 48 h BaP group, one embryo had a large mass of tissue within the yolk sac adjacent to the abdomen. The mass was gray and opaque in coloration. The embryo failed to

hatch and was observed dead within the chorion 456 h post fertilization.

Although not quantitated, it was observed that many of the BaP-exposed embryos and fry had the previously mentioned yellow-green coloration along the dorsal surface and around the outer edges of the mouth. The altered coloration was easily distinguished from the golden-brown coloration of the controls. These embryos did hatch and suffered no apparent problems through yolk sac absorption.

No abnormalities, other than previously mentioned circulatory problems, were observed in the control embryos and fry.

Mortality and hatching success and swimbladder inflation:

No significant differences were seen in the percent mortality of the three groups of embryos or in the ability of the fry to inflate their swimbladders (Table 1). All control embryos which survived to 288 h postfertilization hatched successfully; however, both 24 h BaP and 48 h BaP groups contained embryos which failed to hatch within 840 hours postfertilization. Unhatched embryos were pooled with mortalities for statistical purposes (Table 1). There were distinct differences in the time-to-hatch for the various treatment groups. BaP-exposed embryos did not complete hatching during the time period required for the controls (Figure 1). BaP-exposed embryos continued to hatch for 192 h after the last control embryo had successfully hatched. Control embryos hatched in 46.5% of the time required for the exposed embryos, leaving 15.3% of 24

h BaP and 13.3% of 48 h BaP embryos still unhatched. Another way to express time-to-hatch is the time at which 50% of the eggs have hatched (Leung and Bulkley, 1979). As seen in Table 2, the BaP-treatment groups required 48-72 h longer for 50% hatch.

No further abnormalities were observed in the fry and there was no significant difference in the time during which the fry were able to actively swim. This parameter was determined by noting the time period during which each fry was observed swimming and the time at which it ceased. Cessation of swimming usually preceeded death by 48-96 h.

Discussion

The primary goal of this study was to determine if BaP could produce an effect on medaka embryos when exposure was initiated within 4 h post fertilization and continued through yolk sac absorption. Marty, et. al. (in press) documented the uptake of labeled BaP into medaka embryos over time. The increased incidence of developmental abnormalities and prevalence of circulatory problems in both BaP-exposure groups provides evidence that the compound is entering the egg and affecting development. Further, although hatching success and mortality did not differ significantly from the controls, both exposure groups did exhibit extended hatching times. This corresponds with the data presented in Kocan and Landolt (1984) where the time to hatching for steelhead trout embryos was also extended when exposed to BaP. These researchers theorized that BaP could interfere with the

function of hatching enzyme, thereby altering the hatching dynamics of the treated embryos. It has been established that such enzymes exist and control hatching, both in the medaka and in other fish (Blaxter, 1969; Isida, 1944; Yamagami, 1981). Interference with the ability of the enzyme to function would account for both extended time to hatch and failure to hatch. Leung and Bulkey (1979) found that varying concentrations of benzene could alter the ability of medaka eggs to hatch, with premature hatching occurring at lower concentrations and delayed hatching occurring at higher concentrations.

The mortality and hatching success reported in this study agrees with other data reported for BaP-exposed embryos, including Hannah, et. al. (1982) and Kocan and Landolt (1988). One would expect fry survival time to be altered by the continued exposure to BaP, however, Nebeker, et.al. (1974) found that, in the case of one PCB mixture, fry produced by fathead minnow adults aqueously exposed to the commercial PCB Aroclor 1242 during spawning and subsequent embryonic development, showed excellent survival if kept at the same concentrations of the PCB as the adults and embryos. This could indicate a resistance developed toward specific compounds or chemical classes which the parents confer on the offspring or which is aquired via exposure during embryonic development. Exposure of fry which undergo embryonic development in control ERM would begin to elucidate the sensitive stage and possibility of resistance.

Additionally, this laboratory is in the process of documenting

the effects of different culturing techniques on medaka. Thus far, we have developed the above used technique, which enables us to incubate individual eggs. This aspect has two advantages. First, it allows the observer to follow the progress of individual eggs on a day to day basis, where culturing several eggs in a petri dish or vial does not. Second, it prevents possible interactions between eggs, such as stimulating hatching and, (more importantly in environmental samples) prevents the spread of bacteria or algae between eggs. When environmental water sources are tested, they are sterile filtered, but limited problems still occur with infection in the eggs. Since eggs may contain some microbes from the parents and the water in which they were spawned, it is possible that some water sources are providing nutrients for these endogenous infections. When distilled Millipore filtered water is used, there is no evidence of contamination in the eggs.

Another parameter which has been shown to affect hatching and time to hatch is aeration of the fresh ERM prior to each ERM exchange. Lack of aeration will extend hatching in controls, but not to the extent seen in the BaP-exposed embryos.

Further studies will include verification of these results, and exposure during of other reproductive and developmental times, including: 1) fry through yolk sac absorption, 2) gametes during fertilization and 3) parental exposure prior to spawning.

Acknowledgements

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References

Blaxter. J. H. S., 1969. Development: Eggs and Larvae. In: Fish Physiology. Ed. by W. S. Hoar & D. J. Randall. Acad. Press, New York, 3:178-252.

Dial, N. A. 1978. 1978. Methylmercury: some effects on embryogenesis in the Japanese medaka, *Oryzias latipes*. Teratology. 17 (1):83-92.

Hannah, J. B., J. E. Hose, M. L. Landolt, B. S. Miller. S. P. Felton, and W. T. Owaoka. 1982. Benzo[a]pyrene-induced morphologic and developmental abnormalities in Rainbow trout. Arch. Enbironm. Contam. Toxicol. 11:727-734.

Hatanaka, J., N. Doke, T. Harada, T. Aikawa and M. Enomoto. 1982. Usefulness and rapidity of screening for the toxicity and carcinogenicity of chemicals in the medaka, *Oryzias latipes*. Japan. J. Exp. Med. 52(5):243-253.

Hawkins, W. E., R. M. Overstreet, J. W. Fournie and W. W. Walker. 1985. Development of aquarium fish models for environmental carcinogenesis: Tumor induction in seven species. J. of Applied Toxicol. 5(4):261-264.

W. E. Hawkins, R. M. Overstreet, W. W. Walker. 1988. Carcinogenicity tests with small fish species. Aquatic Toxicol. 11:113-128

W. E. Hawkins, W. W. Walker, R. M. Overstreet, T. F. Lytle and J. S. Lytle. Dose-related carcinogenic effects of water-borne benzo[a]pyrene on livers of two small fish species. Ecotoxicol. and Environm. Safety. 16:219-231.

Heisinger, J. F. and W. Green. 1975. Mercuric chloride uptake by eggs of the ricefish and resulting teratogenic effects. Bull. Environm. Contamin. Toxicol. 14 (6):665-673.

Hiraoka, Y. and H. Okuda. 1983. Characteristics of vertebral abnormalities of medaka as a water pollution indicator. Hiroshima Journal of Medical Sciences. 32:261-266.

Hiraoka, Y., J. Tanaka and H. Okuda. 1989. Toxicity to medaka of solutions of fentrothion degraded by strong alkali. Environm. Pollution. 58:35-42

Hose, J. E., J. B. Hannah, D. DiJulio, M. L. Landolt, B. S. Miller, W. T. Iwaoka and S. P. Felton. 1982. Effects of benzo[a]pyrene on early development of flatfish. Arch. Environm. Contam. Toxicol. 11:167-171.

Ishida, J. 1944. Hatching enzyme in the freshwater fish, *Oryzias latipes*. Annot. Zool. Japon. 22:137-164.

Kirchen, R.V. and W.R. West. 1976. The Japanese medaka; its care and development. Carolina Biological Supply.

Kocan, R.M. and M. L. Landolt. 1984. Alterations in patterns of excretion and other metabolic functions in developing fish embryos exposed to benzo[a]pyrene. Helgoländer Meeresunter.37:493-504.

Kyono, Y. and N. Egami. 1977. The effect of temperature during the diethlynitrosamine treatment on liver tumorigenesis in the fish *Oryzias latipes*. Eur. J. Carcin. 13:1191-1194.

Leung, .T. S-y. and R. V. Bulkley. 1979. Effects of petroleum hydrocarbons on length of incubation and hatching success in the Japanese medaka. Bull. Environm. Comtam. Toxicol. 23:236-243.

Marty, G. D., J. Nunez, D. J. Lauren and D. E. Hinton. 1980. Age-dependent changes in toxicity of N-nitroso compounds to Japanese medaka (*Oryzias latipes*) embryos. Aquatic Toxicology. In press.

Michibata, H., Y.Nojima and M. K. Kojima. 1987. Stage sensitivity of eggs of the teleost *Oryzias latipes* to cadmium exposure. Environm. Res.42:321-327.

Nebeker, A. V., F. A. Puglisi and D. L. DeFoe. 1974. Effect of polychlorinated biphenyl compounds on survival and reproduction of the fathead minnow and flagfish. Trans. Amer. Fish. Society. 103:562-568.

Schreiweis, D. O. and G. J. Murray. 1976. Cardiovascular malformations in *Oryzias latipes* embryos treated with 2,4,5-trichlorphenoxyacetic acid (2,4,5-T). Teratology. 14:287-290.

Shi, M. and E. M. Faustman. 1989. Development and characterization of a morphological scoring system for medaka *Oryzias latipes* embryo culture. Aquatic Toxicol. 15:127-140.

Sin-yin Leung, T. and R. V. Bulkey. 1979. Effects of petroleum hydrocarbons on length of incubation and hatching success in the Japanese medaka. Bull. Environm. Contam. Toxicol. 23:236-243.

Solomon, F. P. and E. M. Faustman. 1987. Developmental toxicity of four model alkylating agents on Japanese medaka fish (*Oryzias latipes*) embryos. Environm. Toxicol. and Chem. 6 (10):747-754.

Solomon, H. M. and J. S. Weis. 1979. Abnormal circulatory development in medaka caused by the insecticides carbaryl, malathion and parathion. Teratology. 19 (1):51-62.

Stoss, F. W. and T. A. Haines. 1979. The effects of toluene on embryos and fry of the Japanese medaka *Oryzias latipes* with a proposal for rapid determination of maximum acceptable concentration. Environm. Pollut. 20 (2):139-148.

Yamagami, K. 1981. Mechanisms of hatching in fish: Secretion of hatching enzyme and enzymatic choriolysis. Amer. Zool. 21:459-471.

Zar, J. H. 1984. In: Biostatistical Analysis, 2nd Edition. Ed. Prentice-Hall, Inc. Englewood Cliffs, NJ. p. 377.

FIG. 1 BaP embryo hatching

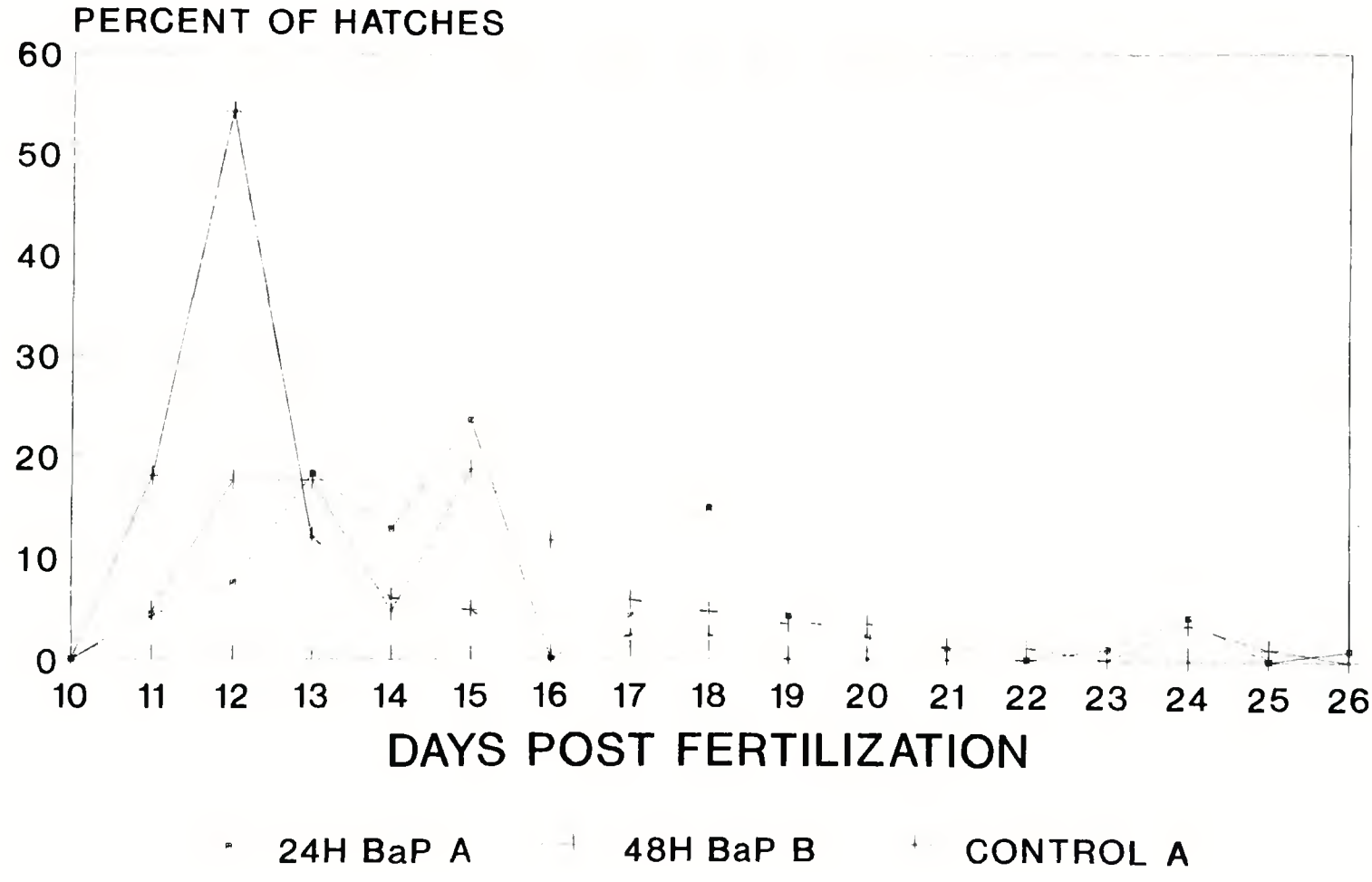

TABLE 2. The time at which 50% of the embryos within each group hatched (where 50% hatch = total number of successful hatches /2).

GROUP	TIME AT WHICH 50% HATCH OCCURRED
CONTROL	288 H
24HBaP	360H
48HBaP	336H

TABLE 1. Hatching success and mortality in the three BaP treatment groups.

	Control	24HBaP	48HBaP
N=	88	98	98
PERCENT EMBRYO MORTALITY	5.68±2.49	5.10±2.23*	13.27±3.40**
PERCENT EMBRYO HATCH	94.32±2.48	94.70±2.23	86.73±3.40
PERCENT FRY WHICH FAILED TO INFLATE SWIMBLADDER WITHIN 48 HOURS POST HATCH	2.41±1.69	2.30±1.62	3.70±2.11
PERCENT EMBRYOS & FRY WITH CIRCULATORY PROBLEMS	4.55±2.22	14.29±3.55	12.24±3.33

* 2 embryos failed to hatch by 648 h post fertilization
** 1 embryo failed to hatch by 648 h post fertilization

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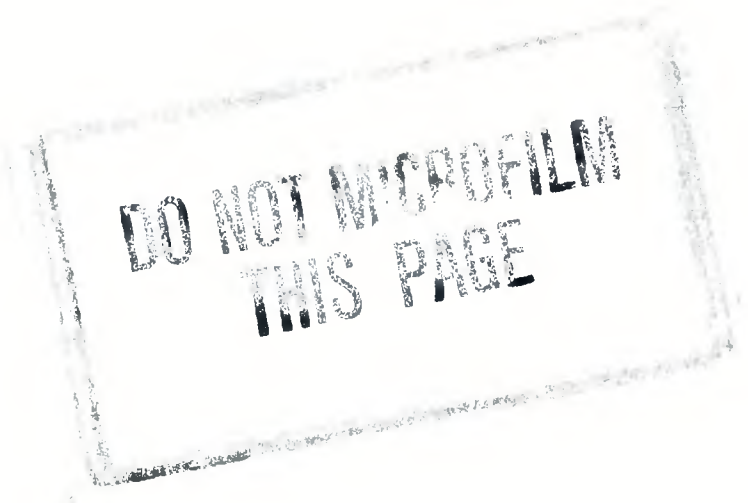

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