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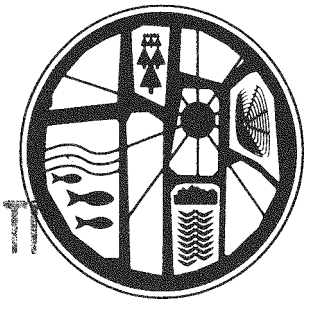
OAK RIDGE NATIONAL LABORATORY

MARTIN MARIETTA

Biological (Molecular and Cellular) Markers of Toxicity

Semi-annual Technical Progress Report (No. 2) April 1, 1989 to September 30, 1989

J. F. McCarthy



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MARTIN MARIETTA ENERGY SYSTEMS, INC.
FOR THE UNITED STATES
DEPARTMENT OF ENERGY

ORNL/M--948

DE90 002746

ENVIRONMENTAL SCIENCES DIVISION

BIOLOGICAL (MOLECULAR AND CELLULAR) MARKERS OF TOXICITY

Semi-annual Technical Progress Report (No. 2)
April 1, 1989 to September 30, 1989

John F. McCarthy

Date Published - October 1, 1989

Prepared for

U.S. Army Biomedical & Development Laboratories
Fort Detrick, MD 21701-5010

Prepared by the

Oak Ridge National Laboratory
Oak Ridge, Tennessee 37831-6285

operated by

Martin Marietta Energy Systems, Inc.
for the
U.S. Department of Energy
under contract DE-AC05-840R21400

Report No.:

ORNL/M-948

Contract No.:

U.S. Army No. 88PP8861
Interagency Agreement No. 1016-B047-A1

Contract Title:

BIOLOGICAL (MOLECULAR AND CELLULAR) MARKERS OF TOXICITY

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Report Date:

October 1, 1989

Type of Report:

Semiannual Technical Progress Report (No. 2)
April 1, 1988 to September 30, 1989

Contracting Officer's Technical Representative:

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U.S. Army Biomedical Research and Development Laboratory
Fort Detrick, MD 21701-5010

I. PURPOSE AND SCOPE OF RESEARCH EFFORT

The objectives of this research are to evaluate the Japanese Medaka as a model for prediction of potential health effects from environmental contaminants. Progress to date has concentrated on efforts to describe the molecular and biochemical events resulting from exposure to a well-characterized model carcinogen, diethylnitrosamine (DEN). There are two goals underlying this approach. Fundamental understanding of the molecular and biochemical events underlying the carcinogenesis process in Medaka may make it possible to employ these responses as biomarkers estimate exposure and predict cellular effects of genotoxic chemicals. Thus, it may be possible to determine if an individual chemical, or waste effluent from Department of Defense facilities, poses a health threat based on exposure of Medaka to the contaminant for the short time required for the biomarker to respond, rather than for the six months required to evaluate a histopathological effect. More fundamentally, critical evaluation of the molecular carcinogenesis process in Medaka provides a foundation for using this convenient and cost-effective animal model for more directly extrapolating to human health effects, based on pharmacodynamic models. Before this approach could be accepted, it is necessary to demonstrate the molecular, biochemical and cellular events in carcinogenesis are similar in fish and humans. If the results of this research support such a conclusion, a later task of this project involves development of a pharmacodynamic model for extrapolating human health effects from bioassays using the Japanese Medaka.

This research has focused on two key biochemical processes: DNA alterations, and activity of enzymes that metabolize the chemicals to highly toxic intermediates and also to readily excreted polar compounds. Two phases of metabolism are recognized and enzymes representative of both phases have been examined in this research. In Phase I metabolism, catalyzed by the mixed function oxidase (MFO) enzyme system, a reactive functional group (such as -OH) is inserted in the parent compound. This enhances the water solubility, and hence excretability, of the substrate; moreover, these activities provide suitable substrates for Phase II conjugating enzymes described below. MFO-mediated reactions are considered important detoxification pathways. However, in a number of instances including key environmental contaminants, this pathway serves to activate substrates to more reactive, toxic products. For example, procarcinogens are metabolized to DNA-reactive carcinogens via MFO activities. Phase II enzymes catalyze the conjugation of highly polar endogenous compounds (generally based upon glucose, amino acids or sulfate) onto suitable substrates, such as Phase I metabolites of lipophilic contaminants. The reactions comprising Phase II metabolism serve to detoxify carcinogens via greatly enhanced excretability of the products. The enzyme receiving attention in this study is the glutathione S-transferases (GST) that catalyze conjugations with reduced glutathione (GSH).

Since alteration of the DNA is understood to be the initiating event in carcinogenesis, the effect of DEN on several types of DNA damage are being evaluated in the Medaka, including early events such as adduct formation and secondary modifications of DNA integrity, as well as later, generally irreversible effects such as cytogenetic aberrations.

II. OVERALL PROGRESS

1. Methods

a. Exposure Protocol

Approximately 70 adult Medaka (both sexes) were exposed by USABRDL to a solution of 200 mg/L of DEN for 24 hours, followed by transfer to clean water for 6 d. This exposure protocol was repeated three additional times for a total of four incremental exposures to DEN. A control population of 70 fish was similarly treated, but without exposure to DEN. At the end of the exposure, 10 fish from each group were retained by USABRDL for histopathological evaluation. The remaining animals were sent to Oak Ridge National Laboratory for analysis. Fish in the exposed and control groups were pooled in groups of 10 animals (approximately equal numbers of each sex in each replicate). Livers were removed and pooled for evaluation of detoxication enzymes and measurement of DNA alterations (strand breaks and abnormal DNA distribution). The remainder of the animals (termed "carcass") in each replicate were pooled for additional measures of DNA alteration (strand breaks, adducts, and minor nucleoside content).

b. DNA Alteration

Several measures of DNA alteration were evaluated.

(1) DNA Strand Breaks

DNA isolation was accomplished by homogenizing the intact Medaka in 1 N NH_4OH/0.2% Triton X-100. The DNA was further purified by differential extraction with chloroform/isoamyl alcohol/phenol (24/1/25-v/v), and passage through a molecular sieve column (Sephadex G50).

DNA strand breaks were measured in the isolated DNA by an alkaline unwinding assay as modified by Shugart [Aquatic Toxicol.,13:43(1988)]. The technique is based on the time-dependent partial alkaline unwinding of DNA followed by determination of the duplex:total DNA ratio (F value). This procedure has been further modified to accommodate the isolation and detection of strand breaks in the DNA from a single Medaka liver.

The relative number of strand breaks (N value) in DNA of Medaka from an exposed population can be compared to those from a control population as follows:

$$N = (\ln F_s / \ln F_r) - 1$$

where F_s and F_r are the mean F values of DNA from the exposed and control populations respectively. N values greater than zero indicate that DNA from the exposed population has more strand breaks than DNA from the control population; an N value of 5, for example, indicates five times more strand breakage.

(2) Minor Nucleoside Content (5-methyl deoxycytidine, 5m-dCyd)

Deoxyribonucleoside analysis was performed by a modification of the procedure of Uziel, et al.[Anal. Biochem.,25:145(1968)] on DNA isolated from one intact Medaka . To approximately 25 ug of DNA in buffer is added 10 ug each of pancreatic DNase, snake venom phosphodiesterase and bacterial alkaline phosphatase. The contents are mixed, and incubated at 37° C for one hour. The mixture is injected onto a 0.6 X 45 cm glass column packed with the cation exchanger Aminex A-6 (Bio-Rad Labs, Richmond, CA) equilibrated with 0.45M ammonium formate, pH 4.5. The column is maintained at a constant temperature of 50°C and the sample is eluted isocratically in the same buffer at a flow rate of 1.0 ml/min. The column eluent is monitored by an absorbance detector at 260 nm and the chromatographic data recorded.

(3) DNA Adducts (Monoclonal Antibodies)

This work was performed in collaboration with Dr. James M. Parry, University College of Swansea, Wales, United Kingdom.

O^6-Ethyl guanine adducts in DNA are detected by a noncompetitive solid-phase immunoassay (Adamkiewics et al., Environ. Health Perspec., 62:49-55 1985). Approximately 3 micrograms of single-stranded DNA, produced by heat-denaturation, are immobilized on nitrocellulose filters and any O^6-ethylguanine residues are detected with monoclonal antibodies.

(4) Abnormal DNA Distribution of Hepatocytes (Flow Cytometry)

This work was conducted in collaboration with Dr. Joseph E. Fuhr, Memorial Research Hospital, University of Tennessee, Knoxville, Tennessee.

Liver tissue is prepared for flow cytometric analysis according to the procedure of McBee and Bickham (Bull. Environ. Contam. Toxicol., 40:343-349 1988). DNA histograms indicating the frequency of DNA distribution within the hepatocytes are recorded for each preparation.

c. Detoxication Enzymes

(1) Phase I Activity (Ethoxyresorufin-O-deethylase, EROD)

Livers of fish were pooled, weighed and homogenized in sucrose 0.25M, 0.1M Tris buffer at pH 7.4. Homogenates were centrifuged twice and microsomes obtained by differential centrifugation at 106,000 x g for 2 hrs. Microsomes and cytosolic supernatants were stored at -120° C until used for the enzyme activity assays.

The activity of 7-ethoxyresorufin O-deethylase (EROD) from the low speed supernatant of the liver homogenate and from the purified hepatic microsomes were measured fluorometrically at 30°C [Drug Metab. Dispos.,2:583(1974)] and expressed as pmoles of resorufin min^{-1} mg^{-1} of microsomal and homogenate protein respectively.

Concentrations of cytochrome P450 in microsomes were determined spectrophotometrically by a modification of the methods of Omura and Sato [J. Biol. Chem.,239:2370(1964)]. Cytochrome P450 was oxidized with carbon monoxide and reduced with sodium dithionite. The concentration of cytochrome P450 is expressed as nanomoles of cytochrome P450 per mg of microsomal protein.

(2) Phase II Activity (Glutathione-S-Transferase, GST)

GST activity was determined on cytosol fractions according to the methods of Habig [J. Biol. Chem., 249:7130(1974)]. GST activity was estimated with glutathione reduced form (GSH, 1 mM) and 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate in a spectrophotometer at 340nm. The results were expressed in units of enzyme activity per mg of protein.

Microsomal and liver homogenate proteins were determined using a centrifugal fast analyzer (Cobas Fara) with the Bio Rad protein assay reagent. Bovine serum albumin was used as standard. The protein concentration was expressed as mg/ml.

2. Results

a. Evaluation of Detoxication Enzymes

Assay conditions for the enzyme assays were optimized with respect to substrate concentration, pH and temperature using adult fish that had not been exposed to any chemicals. Optimal conditions are listed in Table 1. The Km for the EROD activity was determined, and was in the same range as has been reported for the rat and for a number of other fish species (Table 2).

EROD purification and recovery were evaluated by comparing activity assayed in the low speed supernatant (referred to as the "homogenate" fraction) and the activity recovered in the purified high speed supernatant (the microsome fraction). As indicated in Table 3, although the differential centrifugation purified the protein 3-fold, EROD activity in the microsomes was only sightly greater than that observed in the low speed supernatant. The budget of activity and amount of protein showed that over half of the total EROD activity was lost during the purification. This could be due to several reasons, including enhanced denaturation of the protein because of the small volumes and dilute protein concentrations dictated by the size of Medaka livers. Cytochrome P450 spectra indicated a large peak at 420 nm, relative to the peak at 450 nm, suggesting degradation of the cytochrome P450 during purification. The possibility that the cytosol contains an endogenous inhibitor of EROD activity was also tested. Microsomes were isolated and diluted with an equal volume of either the high speed supernatant (the cytosol) or with an equal volume of assay buffer. Results of three EROD assays are shown in Table 3. EROD activity was 18-38% lower in the presence of the cytosol, suggesting the possibility of a cytosolic inhibitor. These results suggest that for this study, measurements of EROD activity be measured in the low speed supernatant until problems of microsomal purification can be resolved.

EROD and GST activity were measured in livers of the Medaka exposed to the DEN and in the control group. EROD activity decreased significantly in the DEN-exposed group (Table 4); the magnitude and direction of the decrease was similar regardless of whether the enzyme activity was measured in the low speed supernatant or in the purified microsomes. In contrast, GST activity increased over 40%, although the statistical significance of that increase was marginal (Table 4). The general pattern of depressed Phase I activity and enhanced Phase II activity following exposure to genotoxic agents such as DEN has also been observed in rodents.

TABLE 1
OPTIMUM CONDITIONS
FOR VARIOUS ENZYME ASSAYS

EROD FOR LIVER HOMOGENATES

Buffer	pH	[7-ethoxyresorufin]	Assay temp.	[NADPH]
Tris 0.1M	8.0	1.5 uM	30°C	0.3mM

EROD FOR LIVER MICROSOMES

Buffer	pH	[7-ethoxyresorufin]	Assay temp	[NADPH]
Tris 0.1M	8.0	1.5 uM	30°C	0.3mM

GSH-T FOR CYTOSOLIC FRACTIONS

Buffer	pH	[GSH]	[CDNB]	Assay temp.
Phosphate 0.1M	7.4	1mM	1.5mM	30°C

TABLE 2
EROD

Species	Km (uM)
Medaka	0.11
Croaker	0.36
Hogchoker	0.19
Toadfish	0.16
Scup	0.24
Rainbow Trout	0.14
Rat	0.13 - 0.16

TABLE 3

EROD PURIFICATION AND RECOVERY

Step	EROD (Activity)	EROD (Units)	EROD (Recovery)	Protein (Recovery)
Low Speed	8.65	32.48	100%	100%
Microsomes	11.47	13.73	42%	33%

TEST FOR EROD INHIBITOR IN CYTOSOL

Sample	EROD (Microsomes)	EROD (+Cytosol)	Inhibition (%)
A	12.67	10.41	17.8
B	34.52	23.33	32.4
C	19.91	12.34	38.0

TABLE 4
SUMMARY OF RESULTS

Detoxication Capacity

Group	EROD[a]	GSH-t[b]
Control	8.22 ± 3.76 (5)	534 ± 88 (5)
DEN-Exposed	2.70 ± 1.16 (5)	724 ± 213 (6)

DNA Alterations

Group	Strand Breaks[c]	m^5d-Cyd	DNA Distribution
Control	0.40 ± 0.04 (10)	4.8	normal
DEN-Exposed	0.21 ± 0.12 (30)	5.5	tetraploidy

"t" test:

a alpha > 0.05 < 0.001

b alpha > 0.01 < 0.05

c alpha > 0.001

b. DNA Alterations

(1) DNA Strand Breaks

Because of the limited amount of liver tissue, an attempt was made to isolate DNA from the low speed pellet in the same samples being used to measure EROD and GST activity. Unfortunately, the conditions of the homogenization were sufficiently severe to shear the DNA polymer and make it impossible to quantify the number of strand breaks caused by the exposure to DEN.

Strand breaks were evaluated in DNA isolated from the Medaka carcasses, and are reported as the F-value in Table 4. There is a slight, but statistically significant decrease in the duplex:total DNA ratio of the DEN-exposed group, indicating an increased number of strand breaks. Based on these F-values, the relative number of strand breaks (N) in the DEN-exposed fish is 0.7, that is, the DNA of the DEN-exposed animals has 70% more strand breaks than the DNA from the control animals. This is a relatively small difference in DNA integrity, compared to fish collected from contaminated streams or exposed to low (1 ug/L) concentrations of benzo(a)pyrene (BaP; Shugart, L.R. in 13th Symposium on Aquatic Toxicology and Risk Assessment, Amer. Soc. Testing Materials, Philadelphia [1989]), in which N values can reach levels of 6-10 (6 to 10-fold increases in numbers of strand breaks).

(2) Minor Nucleoside Content

The only methylated deoxynucleoside in eukaryotic DNA is 5m-dCyd. It has been demonstrated in cell culture that chemical carcinogens and mutagens alter the normal patterns of DNA methylation by interfering with the fidelity of the normal post-replicative modification of the DNA. The hypomethylation of the DNA has been shown to lead to inheritable abnormalities in gene expression. The effect of carcinogens on DNA methylation patterns has been demonstrated in vivo in fish. Bluegill sunfish exposed to BaP (1 ug/L) for 40 d experienced a 50% decline in 5m-dCyd content in their DNA.

Analyses of 5m-dCyd content of DNA from Medaka carcasses is in progress. The results of the single pair of samples that has been analyzed does not reveal any substantial difference in exposed versus control fish (Table 4).

(3) DEN-DNA Adducts

DNA adducts refers to a chemical or its metabolite that is covalently bound to DNA. Modification of the DNA by adducts is understood to be a critical event in carcinogenesis and mutagenesis. A wide range of specific ethylated adducts form on DNA following exposure to DEN, and protocols for isolating and quantifying these

adducts using HPLC have been well described. Because these procedures are time-consuming and labor-intensive, we have explored an alternate method for detection of DEN adducts of DNA, the use of monoclonal antibodies to the adducts. The advantages of the antibody approach include (1) adequate sensitivity (adduct levels of 1 adduct per 10^6 to 10^8 can be readily detected), (2) cost-effectiveness since large numbers of samples can be analyzed simultaneously, and (3) selectivity (levels of a specific adduct can be quantified in the presence of other adducts). Potential problems include concerns that antibody specificity may be compromised because the DNA structure may sometimes be recognized by the antibody, but this can be addressed by enzymatic degradation of the DNA prior to introduction of the antibody.

Samples of purified DNA were sent from ORNL to Dr. Parry for analysis of O^6-ethyl guanine adducts, using the monoclonal antibodies he has developed. Of the array of ethylated DNA adducts formed by exposure to DEN, the O^6-ethyl guanine adduct was focused on because its concentration and persistence has been correlated with tumor formation in several species. Three replicate samples of DNA from the DEN-exposed animals, and four samples from the control animals were assayed, and no O^6-ethyl guanine adducts were detected in any of the samples (detection limits are estimated to be approximately 1 adduct per 10^6 to 10^7 nucleotides).

Given these results, it is perhaps significant to note that this adduct can be enzymatically cleaved by the activity of the enzyme, O^6-alkylguanine-DNA alkylase, and that malignant transformation may be associated with activation of this enzyme. For many, if not most, adducts, including well-characterized carcinogens such as BaP, adducts are removed by excision repair; a long segment of DNA is removed along with the chemically modified nucleotide and the excised region is then repaired by the activity of DNA polymerase and ligase. In this excision repair process, then, removal of adducts generates strand breaks in the DNA which will be detected by the alkaline unwinding procedure. In the case of the O^6-ethyl guanine adducts, however, adducts can be dealkylated enzymatically without causing strand breaks. Thus, it is possible that exposure of the Medaka to DEN activated the alkyltransferase which removed the adducts but did not result in increased levels of DNA strand breaks (Table 4).

(4) Abnormal DNA Distribution in Hepatocytes

The distribution of DNA content in hepatocytes of exposed and control Medaka were analyzed using a flow cytometer. The DNA content of cells is known to be altered by mutagens, carcinogens and ionizing radiation. The use of a flow cytometer to measure the DNA distribution of cells is recommended by the rapid sampling and low cost of the analyses, and by the large number of cells that can be analyzed. A typical result of an analysis of normal hepatocytes is

illustrated in Figure 1a. The majority of the cells are in the G1, or "resting" stage of the cell cycle. The DNA content of the cells represents the normal diploid compliment of DNA in each cell. Cells in G2, the stage of cell cycle in which the DNA has replicated, but before the cell divides, are tetraploid and have twice the DNA content per cell as a G1 cell.

On a quantitative basis the average number (percentage) of cells with DNA content beyond diploid Go/G1 was higher in the exposed medaka livers (18.9%) than was found in the medaka livers (12.8%).

Beyond quantitation, however, there were qualitative differences in the histograms in the two groups of fish (Figure 1b). The descending side of the diploid Go/G1 population in the exposed fish was more diffuse, did not descend to baseline as clearly as in the control samples. This pattern change could be attributable to more cells moving into early S phase of the cell cycle, or more likely represents the presence of reactive or inflammatory cells in the samples. Because the stain utilized in the analyses is an intercalating dye, it has been proposed that as cells become more metabolically active, their DNA unwinds exposing more sites for dye to bind. This gives the appearance of increased DNA within the nucleus. For this reason, we believe the most significant difference, based on very few numbers, is the apparent increase in inflammatory cells in the exposed fish specimens.

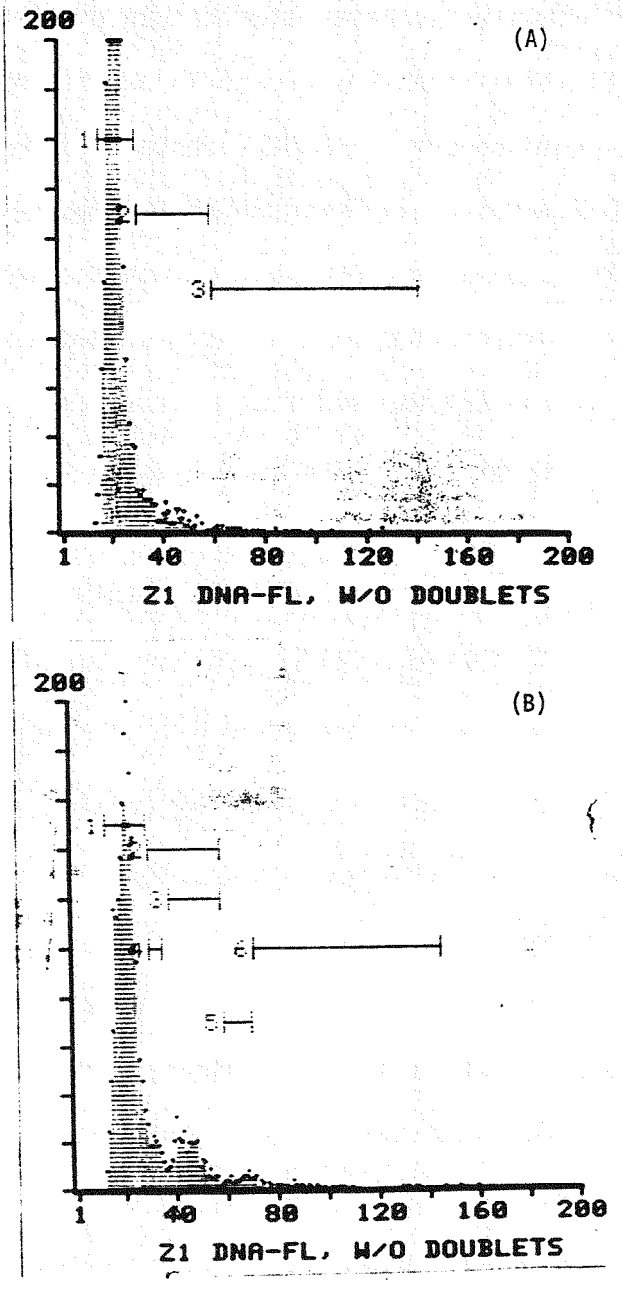
The numbers of cells in the diploid G2 region also appear to be increased. This difference, however, will require additional studies for confirmation. Some species normally accumulate cells in G2, and the loss of cells in that region represents an abnormality. More studies would be necessary to evaluate this observation in fish.

c. Histopathology of DEN-Exposed Medaka

The exposed and control Medaka preserved for histopathological evaluation have not been analyzed at this time. These data will be useful in interpreting the possible causes of the abnormal DNA distribution and other data. It is unclear, for example, if the effects we have described are related to a carcinogenic effects, as evidenced by formation of preneoplastic foci or lesions, or whether the effects reflect a non-cancerous cellular toxicity resulting from exposure to the very high levels of DEN. Cytotoxic effects have been shown to alter some of the molecular and biochemical responses measured in this study; for example, treatment of sunfish with hepatotoxic agents reduces contaminant-associated induction of EROD activity and results in increased levels of DNA strand breaks (McCarthy et al. Mar. Environ. Res. 1989).

FIGURE 1

Frequency histograms of DNA content in hepatocytes. The results of analyses of Medaka hepatocytes from (A) control animals and (B) DEN-exposed animals is shown. The major peak represents cells in the G1 (resting) stage of the cell cycle.



d. Summary

Several molecular and biochemical markers of toxicity have been adapted for measurement in Medaka, and have been applied to describe the effects of treatment of adult Medaka to a high dose of DEN. As summarized in Table 4, DEN treatment inhibited Phase I enzyme activity and increased the activity of a Phase II enzyme; this pattern of response has been described in preneoplastic rodents. No O^6-ethyl guanine adducts were detected, and only a slight (but statistically significant) increase in DNA strand breaks was observed. Limited numbers of samples have not yet revealed any effects of DEN exposure on 5m-dCyd content of the DNA. These results are consistent with a hypothesis that the prolonged exposure and high levels of DEN induced alkyltransferase activity which enzymatically removed any adducts that formed but did not result in strand breaks or hypomethylation of the DNA such as might be expected from excision repair of chemically modified DNA. The DEN-exposed animals had a significantly greater fraction of hepatocytes in G2 phase of the cell cycle, suggesting increased cellular replication. Histological evaluation of the DEN-exposed fish should help determine whether these effects can be related to carcinogenic transformations or, alternately, may be attributed to direct cytotoxic effects of the high dose of DEN.

III. PROBLEM AREAS

One of the biggest problems we have encountered is obtaining sufficient numbers of adult Medaka for experimental work. Because of the small size of the Medaka and difficulties in removing livers for enzyme assays, we must use adult animals. We have attempted to use whole animals for some analyses, but enzyme activity cannot be measured in whole animal preparations; we suspect that this difficulty may be due to the presence of proteases in the gut. Furthermore, since the liver is the primary site of toxic action, measurement of the whole animal "dilutes" the biochemical responses we wish to study.

In order to address this problem, we started grow-out facilities for rearing Medaka fry to adulthood. Medaka fry have been supplied by Dr. William Walker of the Gulf Coast Research Laboratory, Ocean Springs, MS. This grow-out facility is beginning to provide animals of the size needed for biochemical experimentation. Dr. Walker continues to supply us with shipments of fry every few months so that we can have a continuing supply of adults. This facility is critical for productive research on this project, but does extract a price in terms of time and labor needed to maintain the animals.

IV. WORK TO BE PERFORMED DURING THE NEXT SIX MONTHS

1. Culture of Medaka at ESD/ORNL

As indicated in Section III, one of our biggest problems has been the availability of Medaka for experimental work. We are currently taking the necessary steps to establish a stock of adult Japanese Medaka (Oryzias latipes) to ensure sufficient numbers of animals for experimental purposes for this project.

In late August, Dr. Mark Greeley, our reproductive physiologist, and Christine Gettys, a graduate student at the University of Tennessee, who is assisting us in this project, visited the Gulf Coast Research Laboratory (GCRL) in Ocean Springs, MS to obtain first-hand information in the development and maintenance of a Medaka culture. Most of our procedures have been learned through our association with this institution.

At present we have approximately 500 adults and 900 fry. The fry are obtained from GCRL when they are approximately 10 days old and are gradually acclimated to 27^0 C, and are maintained in static aquaria (60 X 30 X 31 cm), with approximately 100 fry in each. The fish are thinned out to approximately 35 per tank as they mature. Tanks are cleaned 1-2 times per week by a concomitant 20% water change. Fry are fed 24 hour old Artemia daily, and fed dry food (Aquavet Stress Flakes) 3 times daily.

The Medaka are mature at roughly 3 months of age. At this time, they are slowly acclimated to $20\text{-}22^0$ C in large living stream tanks (200 X 57 X 57 cm), where they remain until used in experiments. We are also working to develop techniques used by the GCRL to culture Medaka from fertilized eggs. This technique will be used only for supplementing stocks and for reproduction experiments. We will continue to periodically receive 10 day old fry from GCRL to replenish our stocks.

2. DEN-Dose Response Studies

Studies will continue to focus on alterations to DNA integrity and changes in detoxication system of Medaka exposed to DEN, however, exposure protocols will be modified from that previously used (i.e., exposure to various concentrations of DEN for shorter durations). The documentation of a dose response relationship between the biochemical endpoints of interest and the concentration of DEN to which the Medaka are exposed will be the emphasis of this work.

Adult medaka cultured at ESD/ORNL will be shipped to USABRDL for exposure and then returned for biochemical analyses. More detailed exposure protocols will be submitted to USABRDL prior to exposures. Upon receipt at ESD/ORNL the animals will be sexed,

livers removed, and all tissues stored under liquid N_2 until analyzed.

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

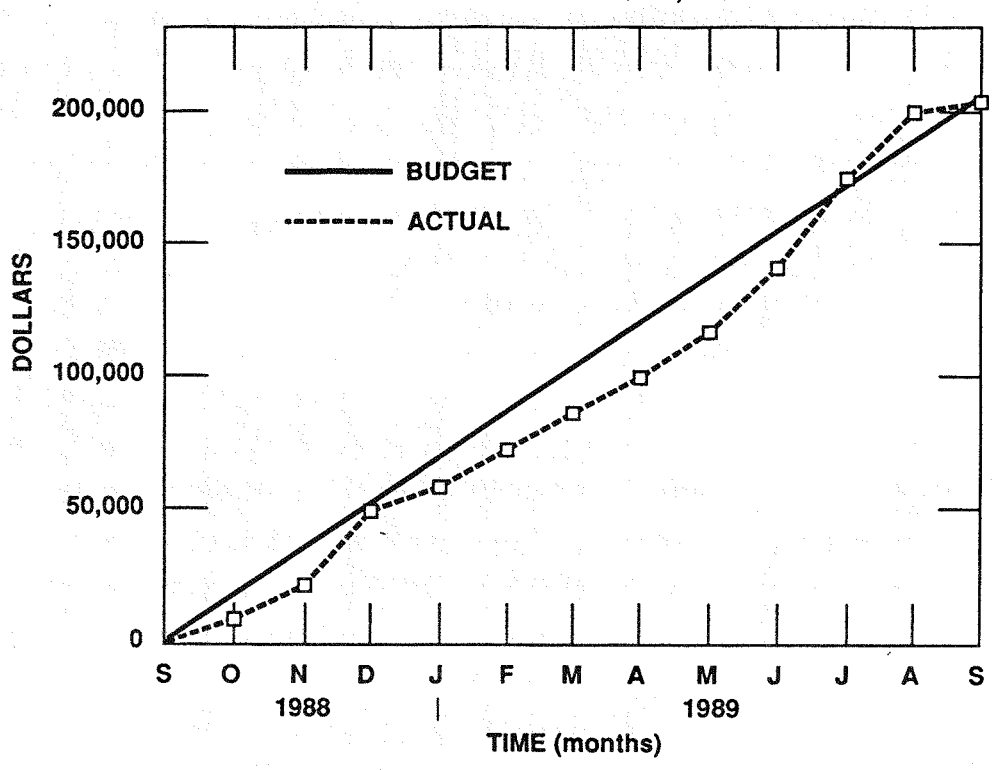
   Attached.

J.F. McCarthy, October 1, 1989

ORNL-DWG 89M-9487

Cost Summary Graph

Project Title / No.: **Biological (Molecular and Cellular) Markers of Toxicity / U.S. Army No. 88PP8861**
Organization / PI: **Oak Ridge National Lab / J. F. McCarthy**
Reporting Period: **April 1, 1989 to September 30, 1989**
Total Allocation: **$203,000**



PROJECT COST SUMMARY

Project Title No.: Biological (Molecular and Cellular) Markers of Toxicity/U.S. Army 88PP8861
Organization/PI: Oak Ridge National Laboratory/J.F. McCarthy
Reporting Period: April 1, 1988 to September 31, 1989

Total Allocation: $203,000

	Monthly Expenditures			Cumulative Expenditures			Available
	Budget	Actual	Variance	Budget	Actual	Variance	Balance
203,000							
Oct	16,916	9,268	- 7,648	16,916	9,268	- 7,648	193,732
Nov	16,916	13,967	- 2,949	33,832	23,235	-10,597	179,765
Dec	16,916	25,040	+ 8,124	50,748	48,275	- 2,473	154,725
Jan	16,916	10,371	- 6,545	67,664	58,646	- 9,018	144,354
Feb	16,916	13,727	- 3,189	84,580	72.373	-12,207	130,627
Mar	16,916	14,642	- 2,274	101,496	87,015	-14,481	115,985
Apr	16,916	11,252	- 5,664	118,496	98,267	-20,145	104,733
May	16,916	19,588	+ 2,672	136,328	117,855	-17,473	85,145
Jun	16,916	24,246	+ 7,330	152,244	142,101	-10,143	60,899
Jul	16,916	32,758	+15,842	169,160	174,859	+ 5,699	28,141
Aug	16,916	20,262	+ 3,346	186,076	195,121	+ 9,045	7,879
Sep	16,916	7,879	0	203,000	203,000	0	0

Note: Cost data as of end of each month.
This report was prepared 10/1/89.

REVISED SCHEDULE TABLE

Biological Markers of Toxicity

Project period: April 1, 1989 - September 30, 1989

Revision	Explanation/Impact
1.	Demonstrating the suitability of performing biochemical analyses in small quantities of tissue, and availability of adult medaka have extended completion of this task.
2.	Histopathological data from samples collected this period not available, furthermore, Task 2 depends upon dose dependency studies.

ORNL-DWG 89M-9499

Project Title: Biological (Molecular and Cellular) Markers of Toxicity

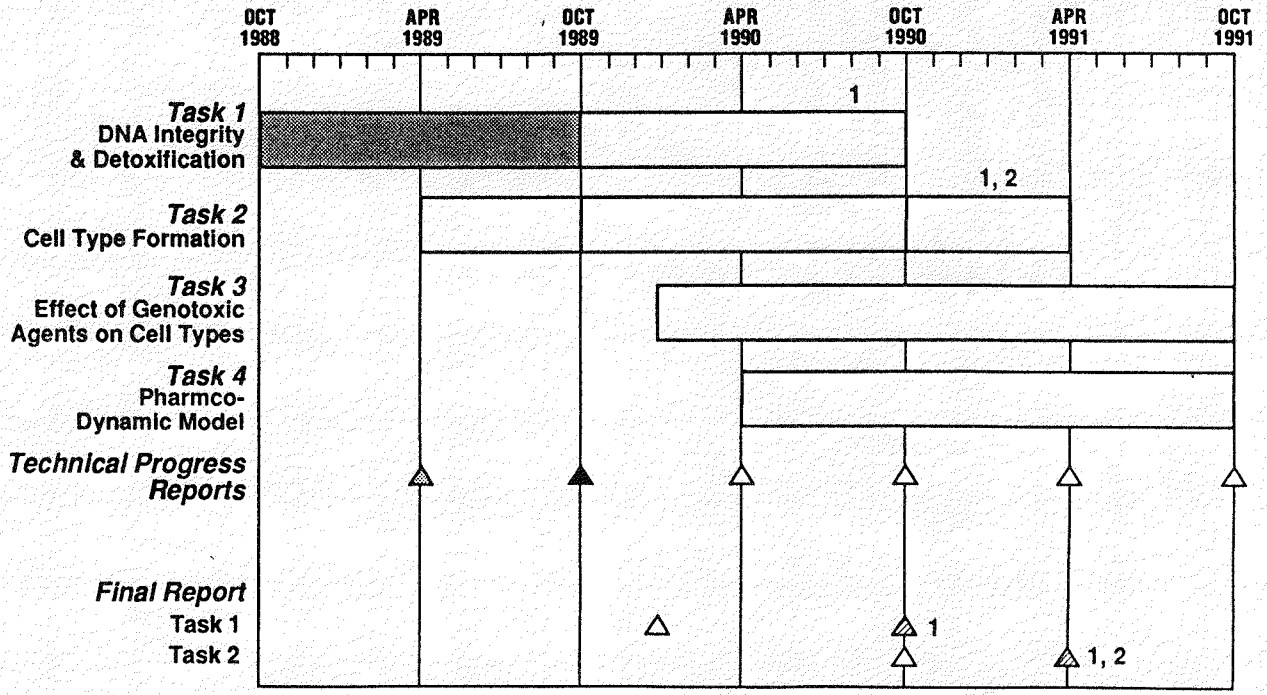
Contract No.: U.S. Army 88PP8861
IAG 1016-B047-A1

Reporting Period: April 1, 1989 to September 30, 1989

Performing Organization: Oak Ridge National Laboratory

Principal Investigator: J. F. McCarthy

Date: October 1, 1989



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