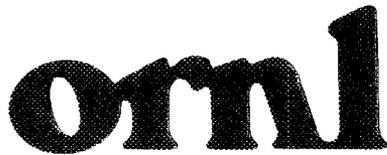




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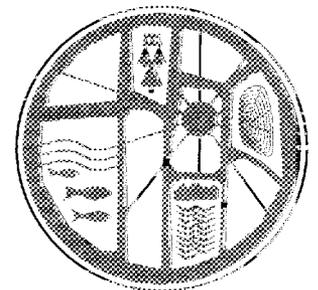
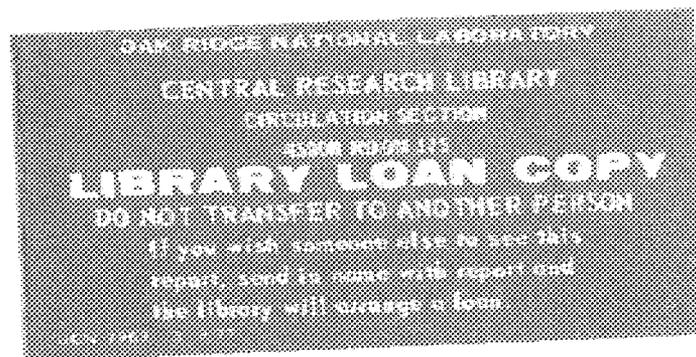
**OAK RIDGE
NATIONAL
LABORATORY**

MARTIN MARIETTA

**Biological (Molecular and Cellular)
Markers of Toxicity**

**Semi-annual Technical
Progress Report (No. 1)
October 1, 1988, to March 31, 1989**

J. F. McCarthy



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ORNL/M-829

ENVIRONMENTAL SCIENCES DIVISION

BIOLOGICAL (MOLECULAR AND CELLULAR) MARKERS OF TOXICITY

Semi-annual Technical Progress Report (No. 1)
October 1, 1988 to March 31, 1989

John F. McCarthy

Date Published - April 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 37831-6258

operated by

Martin Marietta Energy Systems, Inc.
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U.S. Department of Energy
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Report No.:

ORNL/M-829

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:

April 1, 1989

Type of Report:

Semiannual Technical Progress Report (No. 1)
October 1, 1988 to March 31, 1989

Contracting Officer's Technical Representative:

Mr. Hank Gardner
Health Effects Research Division
U.S. Army Biomedical Research and Development Laboratory
Fort Detrick, MD 21701-5010

I. PURPOSE AND SCOPE OF RESEARCH EFFORT

The overall objective of this study is to evaluate the use of a small aquarium fish, the Japanese Medaka, as a predictor of potential health effects following exposure to the carcinogen diethyl nitrosamine (DEN).

Research efforts will investigate the dose response relationship that exists between a suite of molecular parameters and the carcinogenic agent with respect to external dose, dose at the target organ/tissue, appearance of preneoplastic lesions and foci, and the occurrence of tumors. The overall project is divided into several Tasks (see original Proposal), however, two Tasks that are being pursued this fiscal year are:

- Task 1) Evaluate, in the liver of the Japanese Medaka exposed to the carcinogen DEN, the integrity of DNA and the status of the detoxification system (biotransformation capabilities).
- Task 2) Evaluate the kinetics of formation of various liver cell types subsequent to exposure to DEN.

II. OVERALL PROGRESS

1. Task 1.

a. Exposure protocol.

An exposure protocol has been worked out and will be performed at UABRDL - Approximately two hundred (200) adult Japanese medaka (Oryzias latipes) will be exposed to N,N-diethyl nitrosamine (DEN) at a concentration of 200 ppm (200 mg/L) in their water at 22^o for 24 hrs followed by no exposure in clean water for 24 hrs. This exposure protocol will be repeated three additional times for a total of four incremental exposures to DEN. Two hundred (200) fish, to act as a control population, will be similar treated, but with out exposure to DEN. At the end of the fourth exposure one hundred and eighty (180) fish each of the exposed and non-exposed groups will transferred to clean water and shipped immediately by UPS to The Oak Ridge National Laboratory (ORNL) for analyses concerned with DNA damage. Twenty (20) fish each of the exposed and non-exposed groups, will be retained by USABRDL for histopathological analyses. The above exposure protocol will be duplicated, or repeated one additional time at the convenience of the personnel at USABRDL.

b. DNA integrity.

The purpose of the work reported in this section is to evaluate certain measures of DNA integrity as a useful indicator of exposure of the organism to genotoxic agents. It is the inability (permanent or transient) of an organism to cope with DNA damage and to maintain DNA integrity that provides the investigator the opportunity to test for genotoxicity.

Covalent modification of DNA (adduct formation), strand breakage, and minor nucleoside composition are three measures of DNA integrity being investigated.

(1) DNA adduct formation. It is anticipated that the DNA of the Japanese Medaka exposed to carcinogenic amounts of DEN will experience damage, especially since DEN is a potent ethylating agent. Analytical HPLC techniques exist for the detection of ethylated moieties of DNA [Carcinogenesis, 1:595(1980)]. This capability is currently in place and will be used to detect and quantify ethylated bases in the DNA of DEN-exposed medaka.

(2) DNA strand breaks. Another potential source of damage to DNA subsequent to exposure to DEN is strand breakage. Alkaline unwinding is a sensitive analytical technique which has previously been used in cells in culture to detect and quantify DNA strand breaks induced by physical and chemical carcinogens [Anal.Chem., 144:390(1985)]. To assess the level of strand breaks of DNA in Medaka, existing methods were modified to allow for the isolation of intact, highly polymerized DNA and the subsequent estimation of strand breaks. Medaka currently on hand in the laboratory were used to verify the protocols listed below.

DNA isolation was accomplished by homogenizing the intact Medaka in 1 N NH₄OH/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 (E value). Since DNA unwinding takes place at single-strand breaks within the molecule, the amount of double-stranded DNA remaining after a given period of alkaline unwinding will be inversely proportional to the number of strand breaks present at the initiation of the alkaline exposure, provided renaturation is prevented. The amounts of these two types of DNA are quantified by measuring the fluorescence that results with bisbenzimidazole - Hoechst dye #33258.

This procedure has been further modified to accommodate the isolation and detection of strand breaks in the DNA from a single Medaka liver.

Rydberg [Radiat. Res., 61:274(1975)] has established the theoretical background for estimating strand breaks in DNA by alkaline unwinding, which is summarized by the equation:

$$\ln E = -(K/M)(t^b)$$

where K is a constant, t is time, M is the number average molecular weight between two breaks, and b is a constant less than 1 which is influenced by the conditions for alkaline unwinding.

Once DEN-exposed fish are in hand 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:

$$N = (\ln E_S / \ln E_R) - 1$$

where E_S and E_R are the mean E 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.

(3) $^5\text{m-dCyd}$ content of DNA. $^5\text{m-dCyd}$ content of DNA - 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^o 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^oC 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.

Analyses indicate the $^5\text{m-dCyd}$ content of unexposed Medaka DNA to be 4% of the total dCyd content ($^5\text{m-dCyd}$ + dCyd) of the DNA.

(4) Conclusions. Preliminary experiments to date indicate that, using the modified alkaline unwinding procedure, the amount of DNA isolated from the liver of one Medaka is sufficient to perform the alkaline unwinding assay for strand breakage. However, it is estimated that from two to three livers would be needed to provide an adequate amount of DNA for the determination of the $^5\text{m-dCyd}$ content. The number of Medaka needed to perform the adduct analyses will be determined on DEN-exposed animals. One confounding factor is that the DNA must be free of proteins and RNA's before HPLC analysis. See Section III. below for additional discussion.

b. Detoxification system.

(1) Introduction. Aquatic organisms living in polluted environments are constantly being exposed to a wide variety of anthropogenic compounds, many of which are of organic nature. As a response to the incorporation of many organic chemicals in tissues, organisms will induce a family of enzymes better known as the Mixed-Function Oxidase (MFO) System or Cytochromes P-450. These enzymes aid in the elimination of xenobiotic chemicals by increasing the polarity of these compounds so they can be excreted through the bile and kidney.

An enzyme representative of the MFO system is 7-ethoxyresorufin O-deethylase (EROD). This enzyme has been shown to be induced in fish livers

by polycyclic hydrocarbon such as benzo(a)pyrene and is presently being employed as a biological indicator of pollution at the Oak Ridge National Laboratory (ORNL) in their Biomonitoring Program [Comp. Biochem. Physiol.,91:11(1989)].

Another group of enzymes involved in the detoxication process in fish are the conjugation enzymes. These enzymes are known to add polar groups (such as glutathione, sulfates and glucuronic acids) to organic molecules which aid in their removal from organ tissues. Conjugation with glutathione does not always lead to detoxication of xenobiotic compounds, but may cause bioactivation of a compound (for example, 1,2-dibromoethane) leading to the formation of DNA adducts [Environ. Health Perspec., 76:15(1987)]. We are particularly interested in the effects of organic pollutants on the glutathione S-transferase (GST) enzyme since elevated levels of this enzyme system have been found in organisms exposed to hepatotoxic agents [Oikari and Jimenez unpublished data], and are shown to be induced by other organic pollutants. For example, fish injected intraperitoneally with 3-methylcholanthrene (Comp. Biochem. Physiol.,83C:37(1986)) or benzo[a]pyrene [Arch. Environ. Contam. Toxicol.,15:257(1986)] produce a significant increase in GST activity.

The two enzyme activities previously mentioned have been shown to be useful indicators of organic pollution in many fish species. However, in small fish (guppies, fathead minnows and Japanese medaka) these enzymes analyses are very difficult to perform due to the limited amount of tissue obtained from these small organisms. We have developed a methodology to examine the activity of both these enzymes in medaka by pooling the livers of 12 medaka fish. The MFO activity (EROD) in liver homogenates as well as in hepatic microsomes has been optimized for this species.

(2) Methodology. Medaka fish were obtained from the Environmental Protection Agency at Gulf Breeze, Florida and Duluth, Minnesota. They were kept in 15 gallon aquaria under flow-through conditions at 25°C. All fish were grouped according to their sex. Groups of 20 to 40 male or female fish were sacrificed by cervical dislocation and their livers removed without bursting the gallbladder. Livers of fish from the same sex 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.

Enzyme assays: The activity of 7-ethoxyresorufin O-deethylase (EROD) from hepatic microsomes and liver homogenates was 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 P-450 and cytochrome b-5 in microsomes were determined spectrophotometrically by a modification of the methods of Omura and Sato [J. Biol. Chem.,239:2370(1964)]. Cytochrome P-450 was oxidized with carbon monoxide and reduced with sodium dithionite. The concentration of cytochrome P-450 is expressed as nanomoles of cytochrome P-450 per mg of microsomal protein. Cytochrome b5 was quantified obtaining a NADH

difference spectrum (424-413nm) and assuming an extinction coefficient of 185/cm/mM and the concentration expressed as nanomoles of cytochrome b-5 per mg of microsomal protein. The cytochrome b5 assays were performed prior to cytochrome P-450.

Glutathione S-transferase (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.

(3) Results. EROD activity for fish liver Homogenates: The assay was optimized with respect to pH, temperature, substrate concentration and cofactor using liver homogenates. Two different types of buffers: 1) sucrose 0.25M, Tris 0.1M (This is the same buffer system in which the livers are homogenized) and 2) Tris 0.1M. The optimum conditions for these two buffer systems are summarized on the following table.

Buffer	pH	[EROD]	Assay Temp.	[NADPH]
0.25M Sucrose, Tris	7.6	1.5uM	30°C	0.3mM
Tris 0.1M	8.0	1.5uM	30°C	0.3mM

Of the two buffers tested, the best results were obtained using 0.1M Tris buffer. Different volumes of homogenate sample were tested and enzyme rate linearity was obtained under these conditions indicating a proper homogenate protein range for the assay. The amount of homogenate protein used in this assay ranges from 200 to 400ug. The advantage of using liver homogenate in this assay is that of generating more replicates from fewer fish with less effort. Only four fish livers are pooled to obtain enough homogenate for two EROD assays.

EROD activities for fish microsomes: Hepatic microsomal EROD activity in medaka fish were first optimized for pH, then for [NADPH] and finally for substrate concentration. Female and male microsomes were used for the assay optimization. Results are summarized on the following table.

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

Different volumes of microsomal sample were tested and enzyme rate linearity was obtained under these conditions indicating a proper microsomal protein for the assay. The amount of protein used in this assay varies with the microsomal protein concentration of the sample and ranged from 200 to 375ug. A total of twelve livers are needed for four enzyme assays.

GST for cytosolic fraction: This assay is an unusual assay because it requires two different substrate Glutathione (GSH) and CDNB and it therefore very difficult to optimize for both substrate. We decided to keep the GSH concentration at 1mM because higher concentrations would increase the turbidity of the sample. The optimum conditions are summarized on the following table.

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

Linearity of the reaction was confirmed for different volumes of cytosolic sample.

Cytochromes P-450 and b5: Hepatic microsomes were isolated from unexposed medaka fish and their cytochrome P-450 and cytochrome b5 concentration determined. The cytochrome P-450 content in these fish ranged from 0.3 to 0.4 nmoles/mg microsomal protein and 0.15 nmoles/mg for cytochrome b5.

(4) Conclusions. Preliminary experiments show that detoxication enzymes could be measured in hepatic tissues of small fish, in both liver homogenate and hepatic microsomes. Although EROD activity was obtained from liver homogenates the variability between homogenates from fish obtained under similar conditions during the same time interval needs to be evaluated. A minimum of twelve adult fish livers must be pooled for the isolation of sufficient microsomal sample. At least 36 fish are required for each time interval to permit isolation of a sufficient amount of microsomal fraction for three enzyme assays, the minimum number needed for valid statistical comparisons. Although EROD activities can be determined on the liver homogenate fractions, other information such as the cytochrome P-450 concentrations can not be obtained from the homogenates.

2. Task 2.

An investigation into the kinetics of formation of various liver cell types over the life span of Medaka will commence in April 1989 with the first DEN exposure of the organism. The preparation of various tissues for histology will be performed at the U.S. Army Biomedical Research and Development Laboratory, Ft. Detrick, Md. under the supervision of H. S. Gardner. The evaluation of data on preneoplastic lesions and tumors will be at the Oak Ridge National Laboratory under the supervision of Dr. C. C. Travis.

III. PROBLEM AREAS

Several preliminary concerns must be addressed.

First, what is the amount of DNA or tissue needed to perform the necessary analyses? By definition, this constraint will define the number of animals when designing an exposure protocol. To address this problem, preliminary experiments to determine the sensitivity of the various analytical methodologies, as they apply to the Japanese medaka, have been and will continue to be performed until these problems are resolved.

Second, what ethylated adducts should be measured? Alkylating agents such as DEN are known to modify all the nucleic acid bases in DNA at several different sites and to different extents. Current information, from studies with rodents, indicates that it would be counterproductive to attempt to measure the concentration of every adducts in DNA as the presence of only a few have been shown to correlate with subsequent tumor formation. Initially, analysis for O⁶-ethylguanine and O⁴-ethylthymidine will be attempted.

Third, should the experimental population be limited to one sex? Preliminary exposure experiments will be with both sexes and should determine whether subsequent exposure experiments will be limited to a particular sex.

IV. WORK TO BE PERFORMED DURING NEXT SIX MONTHS

At the end of this research period sufficient data should be obtained to determine whether it is feasible to expect a dose response relationship between external dose and the amount of DEN metabolite(s) bound to DNA (adducts). Assuming adduct formation is proportional to the biologically available (*in vivo*) concentration of DEN and also to the time this concentration is maintained, then the amount of adduct(s) found should provide a reliable basis upon which to assess exposure and possible subsequent effects. Data on more subtle changes observed in DNA integrity (strand breaks and ⁵md-Cyd content) and detoxification capacities of the liver will be analyzed and interpreted within the framework of the experimental design.

Data collected on the kinetic formation of various liver cell types will be related to initial genotoxic damage and to subsequent tumor formation.

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.

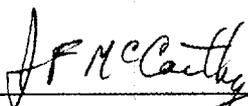
VII. COST SUMMARY REPORT

1. PROJECT COST SUMMARY

Attached.

2. COST SUMMARY GRAPH

Attached.



J.F. McCarthy April 1, 1989

PROJECT COST SUMMARY

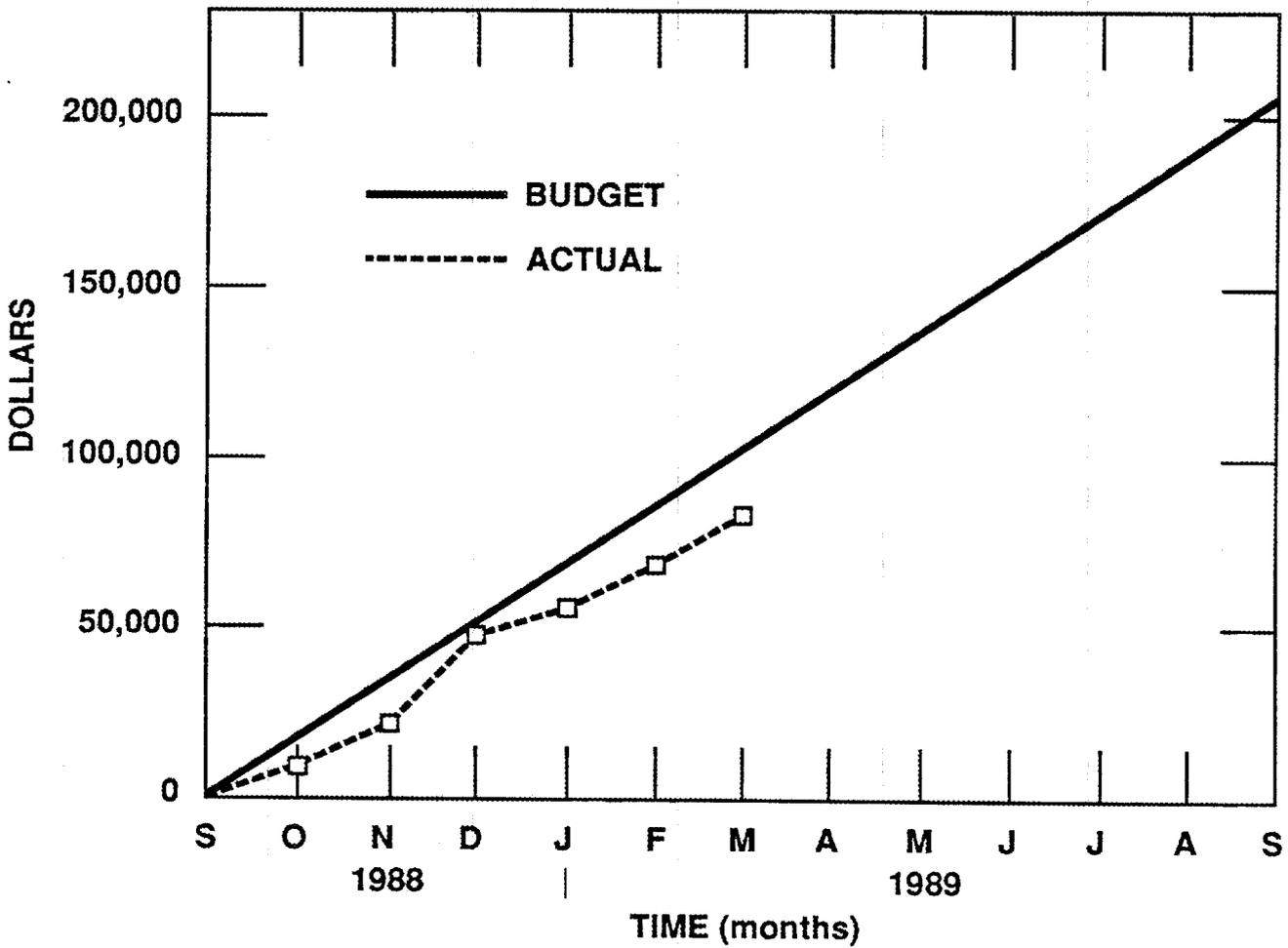
Project Title No.: Biological (Molecular and Cellular) Markers of
Toxicity/U.S. Army 88PP8861
Organization/PI: Oak Ridge National Laboratory/J.F. McCarthy
Reporting Period: October 1, 1988 to March 31, 1989
Total Allocation: \$203,000

	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	179,167
Dec	16,916	23,040	+6,124	50,748	46,275	- 4,473	156,727
Jan	16,916	10,371	-6,545	67,664	56,646	-11,018	146,356
Feb	16,916	13,727	-3,189	84,580	69,918	-14,662	132,629
Mar	16,916	14,642	-2,274	101,496	84,560	-16,936	117,987
Apr	16,916			118,496			
May	16,916			136,328			
Jun	16,916			152,244			
Jul	16,916			169,160			
Aug	16,916			186,076			
Sep	16,916			203,000			

Note: Cost data as of end of each month.
This report was prepared 4.1/89.

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: October 1, 1988 to March 31, 1989
Total Allocation: \$203,000



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

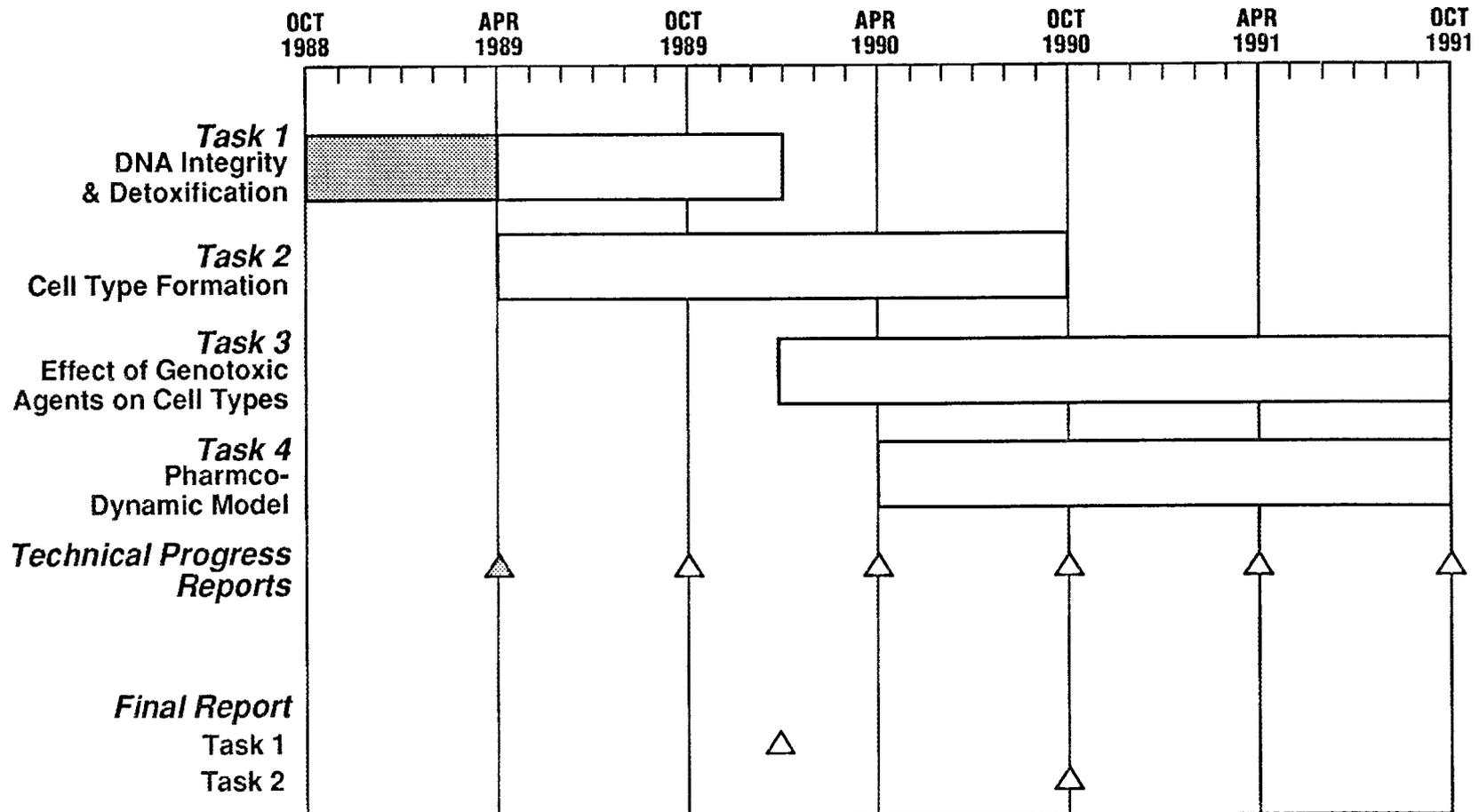
Contract No.: U.S. Army 88PP8861
IAG 1016-B047-A1

Reporting Period: October 1, 1988 to March 31, 1989

Performing Organization: Oak Ridge National Laboratory

Principal Investigator: J. F. McCarthy

Date: April 1, 1989



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