THE UNIVERSITY OF MANITOBA

ASPECTS OF THE UPTAKE, DEPURATION, AND METABOLISM OF FOREIGN ORGANIC COMPOUNDS DURING EARLY LIFE STAGES IN RAINBOW TROUT *Salmo gairdneri*

BY

ROBERT JOHN HIGGINS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

Department of Zoology

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ABSTRACT

An attempt was made to determine the relationship between the appearance of the cytochrome P-450 mediated isozyme(s) benzo(a)pyrene hydroxylase and the kinetics of uptake and depuration by eggs and sac-fry of rainbow trout (Salmo gairdneri) exposed to benzo(a)pyrene and Aroclor 1242 (a PCB mixture). In addition the benzo(a)pyrene hydroxylase assay was characterised and its inducibility by chronic exposure of rainbow trout eggs and sac-fry to Aroclor 1254 was examined.

Exposure of rainbow trout eggs to three concentrations (1.0, 10.0, and 100.0 PPB) of benzo(a)pyrene and Aroclor 1242 over three stages of embryonic development (Days 5-15, Days 16-26, Days 27-37 post-fertilization) established a correlation between observed bioconcentration factors and the ages of the eggs. Analysis by t-test for homogeneity of slope indicated a significant increase in uptake rates at days 27-37 compared to days 5-15 and 16-26 post-fertilization over all concentrations and both compounds tested, except for the exposure to Aroclor 1242 at 1.0 PPB. Depuration rates determined during all egg stages were low but variable with half-lives ranging from 231 to 4.3 days.

Exposure of rainbow trout sac-fry to benzo(a)pyrene and Aroclor 1242 at three concentrations resulted in uptake and

depuration rates greater than those observed in the eggs by 5 to 100 fold. Sac-fry bioconcentrated these compounds between 10 and 100 times more effectively than eggs.

Characterisation of the benzo(a)pyrene hydroxylase assay suggested that the low water solubility of the benzo(a)pyrene substrate was a factor limiting the rate of enzymatic hydroxylation. The concentration of protein in the assay mixture was shown to be directly correlated to enzymatic activity. In addition an unanticipated effect of the substrate carrier solvent was observed.

Chronic exposure of rainbow trout eggs to Aroclor 1254 at 100 and 200 PPB was shown to induce benzo(a)pyrene hydroxylase. Activity of the isozyme(s) appeared at low levels two days prior to hatch then doubled to tripled during the hatch period. Activity after hatch decreased but remained higher than pre-hatch levels.

Although benzo(a)pyrene hydroxylase activity increased after hatch it could not be correlated with the increase in depuration observed in eggs and sac-fry with age. The equal elimination of benzo(a)pyrene and Aroclor 1242 (which cannot be metabolised by trout) indicated that the rate controlling step in depuration was not the enzymatic activity measured here.

ACKNOWLEDGMENTS

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CONTENTS

INTRODUCTION

Toxicology is usually defined as the study of poisons, with poison being regarded as any substance that exerts a deleterious effect on an organism (Hodgson and Guthrie, 1980). The boundaries of the science of Toxicology can therefore be considered arbitrary as most substances exert some deleterious effect at some concentration. Loomis (1974) believed that Toxicology was composed of three principle sub-disciplines; these were Environmental, Economic, and Forensic. This study would claim membership within the environmental branch of Toxicology.

The work presented in this report has investigated aspects of the interactions of benzo(a)pyrene (a polyaromatic hydrocarbon created in trace quantities by the combustion of hydrocarbons) and Aroclor 1242 and 1254 (polychlorinated biphenyl mixtures, previously of economic importance as dielectric heat exchangers and plasticisers) with the early life-stages of rainbow trout (Salmo gairdneri). Both of these materials have been shown to be deleterious to organisms at low concentrations. Benzo(a)pyrene, while not acutely toxic, has been found to be a carcinogen. Kang et al. (1982) reported an increase in tumors in rats given benzo(a)pyrene at 50 mg/kg/day for three days. Hose et al. (1982) reported that chronic exposure to 0.1 PPB benzo(a)pyrene in sand sole increased

the rate of mutagenesis. Polychlorinated biphenyls have been reported to be both acutely toxic and carcinogenic. A 96 hour LC-50 in fathead minnows (Pimephales promelas) of 27.7 PPB was determined by Nebecker et al. (1974) for Aroclor 1254. As a carcinogen, Kanechlor 500 (a PCB mixture) induced mice hepatomas after exposure to 500 PPM in food for 32 weeks (Ueda, 1976).

Toxicologists interested in assessing the risk of foreign organic compounds to organisms in the environment have attempted to determine an exposure parameter known as the Maximum Acceptable Toxicant Concentration (MATC) (McKim et al., 1977; Woltering et al., 1984). To avoid the lengthy and expensive chronic exposures necessary to determine MATC, toxicologists targeted the embryo-larval stage that was generally the most sensitive, and used the determined detrimental threshold exposure levels to estimate MATC (Mauck et al., 1978; Weiss and Weiss, 1978). Considerable research has therefore been performed on the physiology, pathology, and lethality of foreign organic compounds to the early life-stages of animals. Unfortunately little research has been directed into the kinetics of uptake, depuration, and metabolism of foreign organic compounds during these early life-stages.

Metabolism of foreign organic compounds has been demonstrated indirectly by Atchinson and Johnson (1975) who found that some DDT was degraded to DDD and DDE during the egg and sac-fry stages of development in brook trout

(Salvelinus fontinalis). Binder and Stegeman (1980; 1982; 1983; 1984) have directly assayed the isozyme(s) of the cytochrome P-450 mediated hydroxylation system known as benzo(a)pyrene hydroxylase (BPHase) and demonstrated its inducibility by PCBs in brook trout and an estuarine fish (Fundulus heteroclitus). This present work has assessed activity of the same isozyme(s) in rainbow trout prior to and after hatch.

The uptake and depuration rates of foreign organic compounds during the early life-stages of fish has seldom been addressed in the literature. Broyles and Noveck (1979) have assessed uptake and depuration of a tetrachlorobiphenyl in lake trout (Salvelinus namaycush) eggs and sac-fry. Similar work has been performed by Korn and Rice (1981) on coho salmon (Oncorhynchus kisutch) eggs, however, there are no reports of determinations of uptake and depuration kinetic constants at different stages during egg embryogenesis.

This is the first report, to the knowledge of the author, which has attempted to correlate depuration kinetics with metabolic enzymes. during the early life-stages of trout. By determining the kinetics of uptake and depuration of a readily metabolised compound (benzo(a)pyrene) and an poorly metabolised compound (Aroclor 1242) this report has tried to assess the significance of the development of benzo(a)pyrene hydroxylase during the early ontogeny of rainbow trout.

METHODS AND MATERIALS

1.0 CHEMICALS AND REAGENTS

1.1 Solvents

Hexane: Caledon Laboratories, distilled in glass. Acetone: Fisher brand, greater than 99.5% pure. Dimethylsulphoxide: BDH Chemicals, analytical reagent 99.0% pure. Methanol: Caledon Laboratories, distilled in glass, 99.8% pure. Water: double distilled in glass at the Freshwater Institute. Xylenes: Fisher brand, purified grade, mixture of ortho-meta-para. Atomlight scintillation fluor: New England Nuclear. PCS (phase combining system) scintillation fluor: Amersham. CO2-mMet (4-methoxy ethanolamine): Amersham.

1.2 Radiolabelled compounds

Carbon-14 standards for oxidation sample analysis: Amersham, 30,000 bq. Universally labelled 14-C Aroclor 1242 was a gift from Dr. A. Niimi at the Canadian Center for Inland Waters, Burlington Ontario. Upon arrival it was evaporated to dryness under nitrogen and a stock solution of total activity of 3.7×10^7 bq containing 8.28 mg Aroclor 1242 in 100 ml of hexane prepared. Universally labelled 14-C

benzo(a)pyrene (specific activity 6.03×10^8 bq/mmol) and 2,2',4,4',5,5'-hexachlorobiphenyl was purchased from New England Nuclear. 3-H(1,3,6) benzo(a)pyrene, specific activity 2.9×10^9 bq/mmol, was also obtained from New England Nuclear (at 97% purity). This was purified by TLC upon arrival to a purity of 98.5 and then diluted with cold benzo(a)pyrene to a specific activity of 7.27×10^5 bq/umol.

1.3 Reagents

Lowry protein reagents: reagent A-2% sodium carbonate (Fisher) in 0.1 N sodium hydroxide (Fisher); reagent B-0.5% cupric sulphate (Fisher) in 1.0% sodium potassium tartate (Fisher); phenol reagent (BDH Chemicals). Benzo(a)pyrene hydroxylase cofactors: isocitric dehydrogenase(Sigma)-Type 4, 100 units per ml; DL-isocitric acid (Trisodium salt)-Sigma, Type 1, 98% pure; NADP (Disodium salt) (Boehringer Manheim)- 98% pure; benzo(a)pyrene hydroxylase killing solution: prepared from 0.15 M potassium hydroxide (Fisher) in 85% DMSO. Buffers: TRIS/HCl pH 7.5-prepared from 55.8 mM solution of Tris (Hydroxymethyl)-aminomethane (BDH Chemicals) and mixed with 0.5 N HCl to a pH of 7.5 . HEPES/KCL pH 7.4-prepared from 0.02 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Boehringer Manheim) and 0.15 M KOH. Non-radiolabelled benzo(a)pyrene was obtained from Aldrich at 98% purity. Technical grade (non-labelled) Aroclor 1242 and 1254 were

obtained from Chem-Service.

2.0 INSTRUMENTATION

2.1 Liquid scintillation counting

A Beckman 7500 liquid scintillation counter was used to measure tritium (3-H) and carbon-14 (14-C) activity. The instrument was programmed to cover the entire 3-T or 14-C energy windows and automatically compensate for quenching, dependant on the fluor used. Radioisotopes in an aqueous medium were counted in Atomlight while samples generated by oxidation were dissolved in a 2:1 mixture of PCS:xylenes. Sample counting continued for 10 minutes or until a 2% sigma error factor was achieved (Beckman LS 7500 operator's manual). The compton edge of each sample was determined to assess the degree of quenching. The compton spectra was generated by exposing samples to 137-Cs. By measuring the upper energy edge of this spectrum an H number was assigned to each sample after comparision to quenched standards.

2.2 Oxidation

A Packard 306 sample oxidiser was used to recover 14-C from fish eggs and sac-fry. Samples were burned in an oxygen atomsphere to oxidise carbon to carbon dioxide. This was trapped in three ml of CO2-mMet and then flushed into a

scintillation vial by 12 ml of (2:1) PCS:xylenes. Carbon-14 standards were oxidised with 98 % efficency. Benzo(a)pyrene and Aroclor 1242 were oxidised with 94 and 93 % effiency respectively. Samples were oxidised in paper thimbles with a small piece of Kim-Wipe and weighed from 400 to 900 mg. Eggs were crushed before they were oxidised.

2.3 Spectrophotometry

2.31 Protein analysis

A Carl Zeiss PMQ II spectrophotometer was used for analysis of protein in crude homogenate samples. Analysis was performed at 600nm using a 6V 30W incandescent lamp with a diffraction grating. Samples were tested in 0.5 ml borosilicate cuvettes.

2.32 Difference spectrophotometric analysis of cytochrome P-450

A Hewlett-Packard 8450 UV/VIS spectrophotometer was used to quantitate levels of cytochrome P-450 in microsomal suspensions. Deuterium and tungsten-halogen light sources were used with a holographic grating to impinge a spectrum on a photodiode array. The spectrum generated from controls could be stored and subtracted from experimental solutions to generate a difference spectrum.

2.4 Centrifugation

2.41 Sub-cellular isolation of microsomal fractions

Sub-cellular isolation of microsomes was accomplished by a Beckman Airfuge. Post-mitochondrial fractions (150 ul) were spun in cellulose nitrate tubes (19.9X34.8mm) for one hour at 20 pounds per square inch (¬ 105,000 Gav). Temperature was maintained at two degrees Celsius in the controlled environment room in which the centrifuge was housed.

2.42 Sub-cellular isolation of post-mitochondrial fractions

Tissue homogenates greater than five ml were fractioned by a Sorvall RC2-B refrigerated centrifuge at zero degrees Celsius. Homogenates were pipetted into 15 ml Corex high speed centrifugation tubes. These were spun in a Sorvall SS-34 34 degree angle rotor with inserted rubber adaptors for the tubes. The homogenates were spun at 10,000 rpm (¬12,400 Gmax) for 15 minutes.

Tissue homogenates less than five ml were fractionated on a Fisher brand table top centrifuge. Homogenates were pipetted into one ml polystyrene centrifuge tubes, capped and then spun at 7,000 Gmax for 20 minutes on a swinging bucket rotor. Centrifugation was done within a two degree Celsius controlled environment room.

2.43 Exraction centrifugation

Seperation of aqueous from organic phases during liquid-liquid extractions for determination of BPHase activity was accomplished by a Fisher brand table top centrifuge. Extraction solutions in Kimax (13X100mm) screw top tubes were centrifuged at 2000 Gmax for five minutes in a swinging bucket rotor at room temperature.

3.0 BIOLOGICAL UPTAKE AND DEPURATION

3.1 Chemicals and Water

All water used in biological uptake and depuration experiments came from the City of Winnipeg water supply. This water was dechlorinated either by ultraviolet light or by addition of thiosulphate.

All radioisotopes used in biological uptake experiments have been identified in section 1.2 of the Methods and Materials. It was necessary, however, to modify the specific activity of these compounds for the experiments. The specific activity of the radioisotopes used in all egg uptakes were: 6.03×10^9 bq/mmol, 1.21×10^8 bq/mmol, and 1.81×10^7 bq/mmol for 1.0, 10.0 and 100.0 PPB of benzo(a)pyrene respectively. They were (for eggs): 4.5×10^6 bq/mg, 8.93×10^5 bq/mg, and 8.92×10^4 bq/mg for 1.0, 10.0, and 100.0 PPB of Aroclor 1242. For sac-fry they were: 1.9×10^8 bq/mmol, 1.9×10^7 bq/mmol, and 1.9×10^6 bq/mmol for 1.0, 10.0,

100.0 PPB of benzo(a)pyrene respectively and for Aroclor 1242 they were: 4.5×10^6 bq/mg, 8.9×10^5 bq/mg, and 8.9×10^4 bq/mg for 1.0, 10.0, and 100.0 PPB respectively.

3.2 Bioconcentration and depuration in rainbow trout eggs

Rainbow trout eggs (Salmo gairdneri Richardson) were brought into the lab from the Rockwood Hatchery* within 36 hours of fertilization and placed in a floating egg tray within a 144-litre flow-through tank at 10 degrees Celsius. This tank was within a controlled environment room maintained at the same temperature. In this room two spill trays were set out, each with the bottom half of six petri dishes attached to them by silicon sealant. To each petri dish 50 eggs were added and the dish filled with water. The petri dish tops were put in place such that no headspace existed. The total volume of the petri dish covered was 100 +/- 5 ml. The dishes were labelled (Fig. 1) and allowed to sit. Six of the petri dishes were used in bioconcentration and depuration experiments over days 5 to 15 post-fertilization while the remaining six were scheduled for identical experiments over days 16 to 26 post-fertilization. Bioconcentration and depuration experiments consisted of five days of static exposure to benzo(a)pyrene or Aroclor 1242 at concentrations of 1.0, 10.0, and 100.0 PPB

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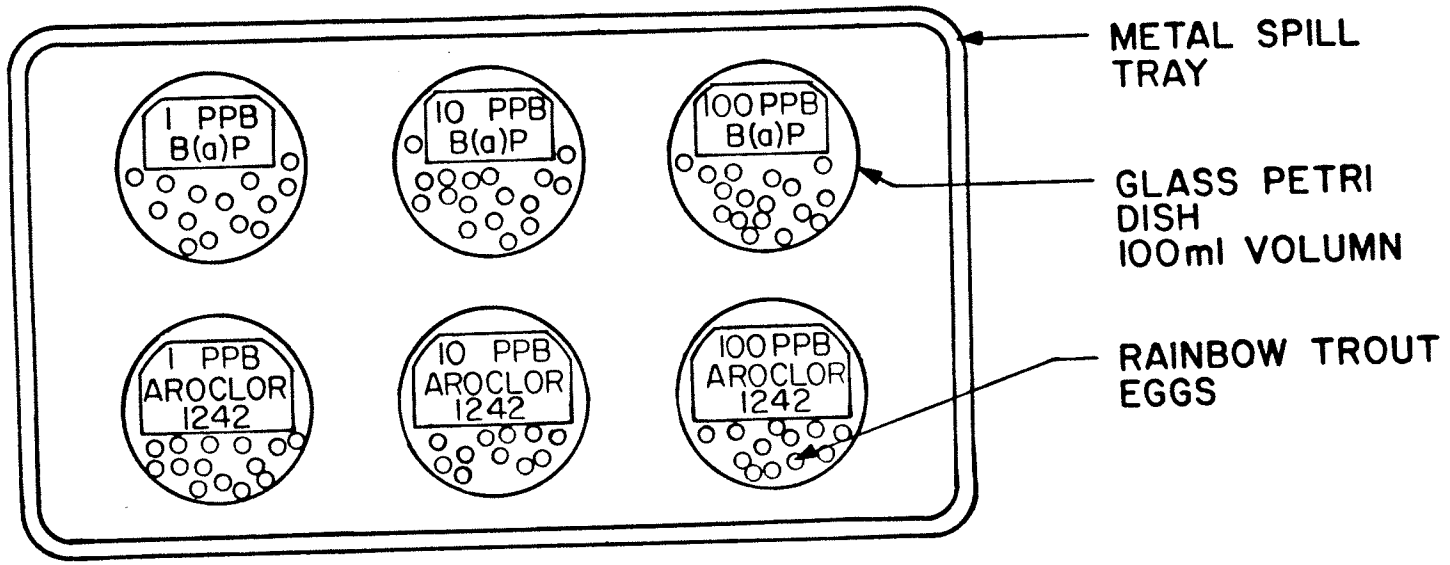
*Federal Department of Fisheries and Oceans, Gunton Manitoba, Canada

Figure 1. Experimental apparatus used to expose rainbow trout eggs to benzo(a)pyrene and Aroclor 1242 at 1.0, 10.0, and 100.0 PPB.


1 PPB B(a)P
10 PPB B(a)P
100 PPB B(a)P
1 PPB AROCLOR 1242
10 PPB AROCLOR 1242
100 PPB AROCLOR 1242
METAL SPILL TRAY
GLASS PETRI DISH 100ml VOLUMN
RAINBOW TROUT EGGS

followed by five days of clearance in uncontaminated water. Every 24 hours all petri dish tops were briefly removed, the water gently drawn off and replaced with clean well oxygenated water using a 50-ml disposable syringe. Bioconcentration and depuration experiments were also performed on eggs at 26-36 days post-fertilization. These eggs were held in egg trays in flow-through water (as noted previously) and subdivided into petri dishes only at the time of the experiments. The eggs eventually used in this specific experiment were obtained from the Mount Lassen Trout Farm*.

3.21 Bioconcentration and depuration in eggs at days 5-15 and 16-26 post-fertilization

The 100 ml of water within each petri dish was replaced every 24 hours. During the five-day exposure period sampling of eggs and water was performed daily at the time when water was replaced with freshly treated water. After removal of petri dish tops two one-ml water samples were taken using a Socrex automatic pipette with a plastic disposable tip. The was water ejected into a scintillation vial containing Atomlight fluor and the 14-C activity determined on the Beckman LS 7500. Three eggs were then removed from each experimental dish, rinsed with water and rolled dry on paper towelling. Each egg was then placed in

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*Mount Lassen Trout Farm, Mount Lassen, California, U.S.A.

an oxidation paper thimble, crushed with a Kim-Wipe and weighed. The eggs were then either oxidised immediately or refrigerated in plastic bags until oxidation. Contaminated water was then removed from the petri dishes and they were refilled with fresh water and then dosed again with the appropriate compound and concentration. All doses were dissolved in 50 ul of acetone and dispensed in glass Lang-Levy pipettes. After dosing, the petri dish tops were replaced and the compounds allowed to equilibrate by diffusion. At the end of the five day exposure period the contaminated water was removed as usual but then the eggs were gently washed with water ten times. After the last wash a water sample was taken, then the water was removed and the tray placed within a 20 litre glass aquarium. The aquarium was then slowly filled with 10 litres of water. The eggs were incubated for five days, during which three eggs were removed from each dish for analysis every 24 hours.

3.22 Bioconcentration and depuration in eggs at days 27-37 post-fertilization

The procedure followed was the same as that given for eggs of earlier ages with the following modifications. Water was replaced and dosed every 12 hours instead of 24. Water samples were taken every 12 hours but egg samples were still taken every 24 hours.

3.3 Bioconcentration and depuration in sac-fry

Sac-fry were brought from the Rockwood hatchery and held in a flow through tank at 10 degrees Celsuis for two weeks prior to experimentation. The sac-fry were held in glass battery jars (4,250+/-50ml) during exposures. These were covered with plexi-glass sheets such that little or no headspace existed. The experimental procedure followed that of the egg exposures with the following changes. Water removal from the tanks was accomplished by siphon and dosing occurred just prior to complete refilling of the tanks (to facilitate mixing). Compounds were delivered in 400 ul of acetone using Lang-levy pipettes. After the five-day exposure period the sac-fry were placed in plastic mesh buckets within the aquarium used in the egg clearance experiments.

3.4 Localisation of benzo(a)pyrene and hexachlorobiphenyl within rainbow trout eggs

Eyed rainbow trout eggs were set up for exposure to 14-C radiolabelled benzo(a)pyrene and hexachlorobiphenyl (HCBP) as done for bioconcentration and depuration experiments in eggs 27-37 days old. Two sets of 50 eggs were placed in two petri dishes. One set was exposed to benzo(a)pyrene at 0.2 PPB while the other was exposed to HCBP at a concentration of 1.0 PPB. The rate of uptake was measured over six days in the same manner as before. On the sixth day samples were taken as done normally but another

set of five eggs were removed from each dish for dissection to seperate chorion, embryo, and yolk. The eggs were placed individually in 0.9% NaCl solution then, with a syringe and fine forceps, the chorion and embryo were shaken free of the yolk. Each chorion and embryo was oxidised seperately using the Packard Sample Oxidizer (see section 2.2, Methods and Materials) to determine radioisotope content. These levels were then compared to radioisotope levels in the whole egg. The yolk was not analyzed but its activity levels could be estimated by subtracting chorion and embryo contamination from contamination in the whole egg.

3.5 Analysis of biological uptake and depuration data

Radioactivity within eggs was expressed as grams of benzo(a)pyrene or Aroclor 1242 (as appropriate) using the known specific activity (see section 3.1; Methods and Materials) and then expressed as parts per billion with the egg on a weight to weight basis. Depuration rate constants (K2) were calculated directly as the slope of a regression line fitted to logarithmic (natural) clearance data (Zitko, 1979). Half-lives (t1/2) of depuration data were calculated using the equation LN 2.0=K2 x t1/2. Uptake rate constants (K1) were calculated from the equation K1=CF/(CW*(1-EXP(-K2*T))/K2 from Hamelink (1977) where: CF is the concentration of the compound of interest in the fish, CW is the concentration in the water, and T is the time of exposure. Bioconcentration factors (observed) (BCF)

were calculated as the ratio of the maximum concentration in the fish to the concentration in the water (Esser and Moser, 1978).

The statistical relationship of uptake rates between ages at specific concentrations was analysed by using a small sample t-test to assess for homogeneity of slopes (Kleinbaum and Kupper, 1978).

4.0 CHARACTERIZATION OF THE CYTOCHROME P-450 ISOZYME(S)

4.1 Determination of protein concentration

The Lowry technique was used to determine the concentration of protein in homogenates prepared for benzo(a)pyrene hydoxylase analysis (Lowry et al, 1951). Normally the protein was diluted 20 to 40 fold prior to the protein assay. Duplicate 30-ul aliquots of each sample were pipetted into test tubes. These were then mixed with 500 ul of Lowry Reagent C (freshly prepared from mixing 10 ml of Lowry Reagent A* with 200 ul of Lowry Reagent B*). After 10 minutes 50 ul of standard phenol reagent were added while the sample was vortexed. All samples were then allowed to stand for 30 minutes and then absorbances were read spectrophotometrically on the Ziess PMQ II at 600 nm. These were compared to a blank prepared in the same manner

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*see section 1.3 of Methods and Materials

in which double distilled water was used in place of homogenate. Absorbance values were related to protein concentration by a standard curve prepared from aqueous solutions of Bovine Serum Albumin (BSA) using regression analysis.

4.2 Induction of cytochrome P-450 in adult arctic charr by benzo(a)pyrene-Spectral analysis

4.21 Induction and isolation of microsomal fraction

Adult arctic charr (Salvelinus alpinus) were exposed to benzo(a)pyrene to induce levels of cytochrome P-450. Four adult charr were selected from holding tanks at the Freshwater Institute. Exposure to BaP occurred through interperitoneal injection of non-labelled BaP dissolved in corn oil at a concentration of 10 mg/ml (20 mg BaP/kg fish). Prior to injection the animals were anaesthetized in MS-222 and weighed. Two fish received injections of BaP in corn oil while two controls received equal injections of pure corn oil. The fish were then replaced into the holding tanks. After 24 hours the fish were removed and sacrificed by a blow to the head. The livers were excised and weighed. Hepes/KCl buffer was added at four times tissue weight and the liver chopped then homogenized via a motorized potter homogenizer at 400 rpm. Sub-cellular fractions were then prepared by centrifugation. The post-mitochondrial fraction was separated on a Fisher brand table top centrifuge. The supernatants were drawn off and then spun on a Beckman

Airfuge to pellet the microsomes (see section 2.42 and 2.41 respectively; Methods and Materials). The pellets were washed in 50 ul of Hepes/KCl, pooled, and then resuspended to a protein concentration of three mg/ml in the same buffer. This was then stored on ice. All isolation steps were carried out at two degrees Celsius.

4.22 Spectral analysis

Analysis of Cytochrome P-450 content in microsomes by difference spectroscopy was done on the Hewlett-Packard 8450 UV/VIS spectrophotometer (see section 2.32; Methods and Materials) according to the technique of Klingenberg (1958). The technique involves simultaneous analysis of two cuvettes filled with the same microsomal suspension. To both cuvettes a few milligrams of dithionite were added and gently mixed. The spectrophotometer was programmed to balance the spectrum (300-550 nm) obtained from both cuvettes such that a baseline was generated. Carbon monoxide (Linde Co.) from a lecture bottle was then bubbled into one cuvette through a 12 gauge syringe needle for two minutes. Simultaneous analysis of the two cuvettes then generated a difference spectrum of reduced against carboxy-complexed cytochromes which was plotted and specific absorbance values recorded.

4.3 Assay conditions for benzo(a)pyrene hydroxylase (BPHase) activity

4.31 The benzo(a)pyrene hydroxylase assay

Addition of a non-polar aromatic hydrocarbon substrate like benzo(a)pyrene to a microsomal suspension will result in formation of polar metabolites if isozymes collectively known as Cytochrome P-450 are active. The activity of the enzyme is determined by the rate at which BaP metabolites are produced. The metabolites are separated from the non-polar parent compound by solvent extraction and counted for radioactivity. The general technique followed in this work was that of DePierre et al., 1975 with an extraction modification described by Van Cantford et al., 1977.

Livers used in assessing optimal BPHase conditions were obtained from six adult male rainbow trout in pre-spawning condition. The trout were sacrificied and their livers excised. Two samples of three livers were separated and placed in plastic bags which were frozen at -80 degrees Celsius. When required, a sample of liver was obtained by taking approximately 0.5 gram subsamples of each liver. The unused portions were then replaced in the -80 degree Celsius freezer. The subsamples were pooled and then homogenized in four times volume Hepes/KCl buffer by a motorised potter homogenizer (400 rpm). The post-mitochondrial (PM) fraction was isolated on the Sorvall RC2-B (see section 2.42; Methods

and Materials). Following centrifugation the supernatants were collected, pooled, and stored on ice for a maximum of two hours before assaying. Typically five 10 ul aliquots were removed from the PM homogenate for protein analysis (See section 4.10).

Benzo(a)pyrene hydroxylase assays were conducted in Kimax screw top test tubes (100x13mm). All assays were run in triplicate. Controls, also in triplicate, were prepared in exactly the same manner as the experimental assays except that the killing solution (noted below) was added prior to the addition of substrate. Assays contained PM homogenate, cofactors, BaP substrate, and Tris/HCl buffer (pH 7.5) in a total volume of 500 ul. Cofactors were added dissolved in Tris/HCl (pH 7.5) in a volume of 10 ul. Cofactors were added to generate incubation concentrations of: NADP, 1 mM; isocitric acid, 6 mM; isocitrate dehydrogenase, 0.36 U; MgCl2 5 mM. Post-mitochondrial homogenate was added to attain an incubation protein concentration of four to five mg per ml. This usually required 150-200 ul of PM homogenate. Tris/HCl was added to bring the volume up to 480 ul. After addition of cofactors, PM homogenate and Tris/HCl the assay suspensions were placed in a 25 degree Celsius water bath for 30 minutes. This pre-incubation was necessary to generate NADPH. Following this 3-H BaP (specific activity $7.3X10^5$ bq/umol) was added in 20 ul of acetone for an incubation concentration of 90 uM. The tubes were incubated for 30 minutes at 25 degrees Celsius then the

reaction terminated by adding one ml of 0.15 M KOH in 85% DMSO. The test tubes were mixed on a Vortex shaker after addition of each incubation component. Extraction of metabolites then occurred either immediately or after storage at two degrees Celsius for periods up to a few days.

Benzo(a)pyrene substrate was separated from polar metabolites by three extractions with three volumes of hexane. After initial addition of hexane the tubes were tightly capped, shaken, and partially opened briefly to release pressure. The tubes were placed within a Whirl-pacR bag which was then nested inside a screw top cardboard tube. This was then shaken on a wrist action shaker for three minutes. The tubes were removed and centrifuged on a clinical table top centrifuge for five minutes to increase phase separation. The upper hexane phase containing unreacted BaP was aspirated off. This was repeated twice. Metabolites remaining in the aqueous phase were then assayed by counting three 200 ul aliquots in Atomlight fluor.

4.32 Post-mitochondrial homogenate storage effect

As noted in section 4.31 (Methods and Materials) postmitochondrial homogenates were stored on ice until required for BPHase analysis. To assess the effect of storage on activity the PM homogenate was tested over a period of two days. A liver PM homogenate was prepared as outined in section 4.31 (Methods and Materials). A

subsample of this homogenate was assayed for BPHase activity within one hour of completing the preparation, and again after storage on ice for 12, 24, and 48 hours.

4.33 Effect of incubation time on benzo(a)pyrene hydroxylase activity

The incubation time was defined as the time between addition of substrate and the termination of the assay. Hepatic PM homogenates were assayed for BPHase activity as outlined in section 4.31 (Methods and Materials) with only the time of incubation modified. Incubations were performed for durations of 1, 2, 5, 10, 15, 30, 60, and 90 minutes.

4.34 Effect of post-mitochondrial homogenate protein concentration on benzo(a)pyrene hydroxylase activity

Benzo(a)pyrene hydroxylase activity was assessed as outlined in section 4.31 (Methods and Materials) with only the concentration of the PM homogenate protein varied. Protein concentrations of 0.2, 0.4, 1.0, 2.0, 3.0, 4.0, 8.0, and 10.0 mg/ml were tested. Protein concentrations were altered by addition of differing volumes of PM homogenate (pre-analysed for protein) to incubation mixtures. The appropriate volume of Tris/HCl was added to maintain a total incubation volume of 500 ul.

4.35 Effect of cofactor concentration on benzo(a)pyrene hydroxylase activity

Benzo(a)pyrene hydroxylase assays were performed as given in section 4.31 (Methods and Materials) with only the concentration of cofactors altered. Triplicate BPHase assays were analysed comparing the effect of halving and doubling the concentration of cofactors. The volume in which cofactors were added to the incubation mixtures was not altered.

4.36 Effect of buffers on benzo(a)pyrene hydroxylase activity

The buffers Hepes/KCl (pH 7.4) and Tris/HCl (pH 7.5) were routinely used together during BPHase assays. To determine the relative effects of these buffers, assays were run in which the typical buffer mixture (see section 4.31; Methods and Materials) was compared to assays modified such that only one buffer was present.

4.37 Effect of substrate carrier solvent on benzo(a)pyrene hydroxylase activity

4.371 Dimethylsulphoxide versus acetone

Benzo(a)pyrene Hydroxylase assays were carried out as given in section 4.31 (Methods and Materials) with the BaP substrate delivered in 10 ul of either DMSO or acetone solvents.

4.372 Effect of the concentration of the substrate carrier acetone on benzo(a)pyrene hydroxylase activity

The effect of the amount of acetone substrate carrier used to deliver BaP substrate on BPHase activity was addressed. The BaP substrate was dissolved in differing amounts of acetone such that it was added to the incubation mixture in 10, 20, 40, 60, and 80 ul of acetone. A second test was then run on a specific area of interest at 10, 15, 20, 25, and 30 ul. To maintain a constant 500 ul incubation the volume of Tris/HCl buffer was adjusted as needed.

5.0 BENZO(a)PYRENE HYDROXYLASE IN EMBRYONIC RAINBOW TROUT

5.1 Induction by Aroclor 1254

Rainbow trout eggs were exposed to Aroclor 1254 in an effort to assess the inducibility of the BPHase isozyme(s) as has been noted by Binder and Stegeman, 1980, 1982, 1983, and 1984.

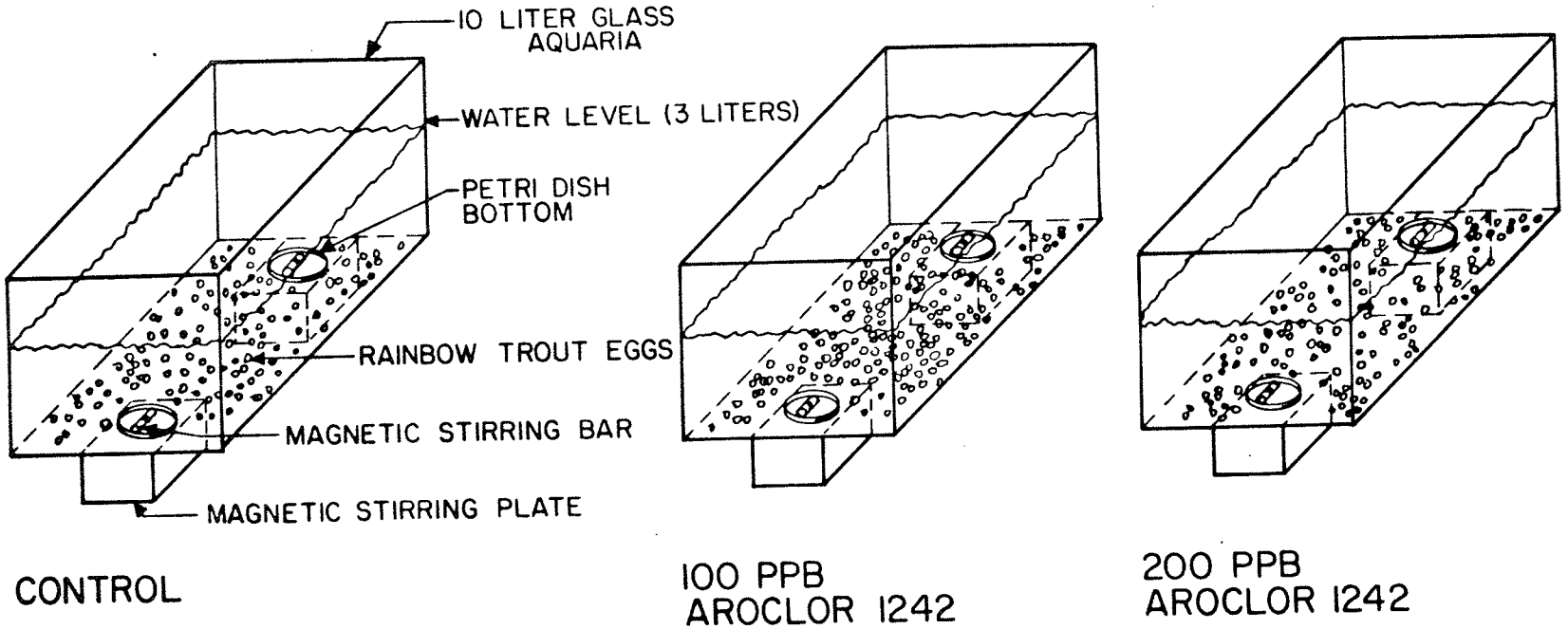
Twelve thousand non-fertilized rainbow trout eggs with milt were obtained from the Mount Lassen Trout Farm. They were fertilized upon receipt then divided into four sets of 3,000 eggs each. One set was placed within an egg tray (as in section 3.3; Methods and Materials). The remaining three sets were added to three 20 litre glass aquaria containing three litres of ultraviolet dechlorinated water. The

aquaria were contained within a 10 degree Celsius controlled environment room. Each aquarium was set upon two magnetic stirring plates. Within each aquarium were the lower halves of two 25 mm diameter plastic petri dishes each containing a magnetic stirring bar (Fig. 2). The stirring bars were used to circulate water within the tanks while the petri dishes stopped the magnetic bars from disturbing the eggs. Every 24 hours the water from each aquarium was siphoned out and three litres of fresh water siphoned in. On day five (post-fertilization) two of the aquaria were exposed to non-radiolabelled Aroclor 1254. One recieved Aroclor 1254 at a concentration of 100 PPB while the other received 200 PPB. The third set of eggs were maintained in non-contaminated water to act as controls. The two induction tanks were dosed with their appropriate concentration of Aroclor 1254 dissolved in 200 ul of acetone each day as the water was replaced. This occurred each day until day 20 post-hatch.

5.2 Benzo(a)pyrene hydroxylase activity in young rainbow trout

Benzo(a)pyrene hydroxylase assays were performed on rainbow trout embryos as they developed in water contaminated with Aroclor 1254 and compared to BPHase activity levels from eggs raised in non-contaminated water.

Figure 2. Experimental apparatus used to expose rainbow trout eggs to Aroclor 1254 at 100.0 and 200.0 PPB during their development.


10 LITER GLASS AQUARIA
WATER LEVEL (3 LITERS)
PETRI DISH BOTTOM
RAINBOW TROUT EGGS
MAGNETIC STIRRING BAR
MAGNETIC STIRRING PLATE
CONTROL
100 PPB AROCLOR 1242
200 PPB AROCLOR 1242

As previously noted exposure to Aroclor 1254 began five days after fertilization. Analysis of BPHase activity was initiated 10 days after fertilization. Assays were continued every two days until three days before hatch (29 days post-fertilization) at which time assays were performed every day until after hatch. From this date until day 49 post-fertilization assays were again performed every two days.

Between days 10 and 49 post-fertilization thirty eggs were sampled from each of the three rearing aquaria for BPHase analysis. The eggs were rinsed in dechlorinated water then stored on ice for a maximum of one hour as the embryos were removed from the eggs. Each egg was transferred to a petri dish containing ice-cold 0.9 % NaCl. Using a syringe needle (12 gauge) and a pair of fine forceps the egg chorions were punctured and the embryo shaken free into the solution. Any attached yolk sac was torn away from the embryo. The embryo was then drawn into a disposable glass pipette and ejected into fresh 0.9 % NaCl solution. The embryo was then pipetted into one of three test tubes according to its experimental group (Control, 100 PPB Aroclor 1254, or 200 PPB Aroclor 1254).

Embryos were transported to a two degree Celsius controlled environment room on ice for homogenisation. A pipette was carefully used to withdrawn any NaCl solution remaining from preparation. Following this, each treatment tube was washed in Hepes/KCl buffer (pH 7.4) to complete

removal of NaCl and aid in removal of oil globules originating from the yolk. Hepes/KCl buffer was then added at a volume of 450 ul and the embryos and buffer transfered to one ml homogenisation tubes. The embryos were homogenised by 20 strokes of a micro hand held Potter homogeniser and the homogenate transfered to one ml polystyrene centrifuge tubes. A post-mitochondrial fraction was then isolated as outlined in section 2.42 (Methods and Material). Analysis of BPHase activity then proceeded as noted in section 4.31 (Methods and Materials) with the following modifications. Incubation reaction volumes of 250 ul were used. In addition, the time of incubation was 60 instead of 30 minutes.

Radioactivity levels associated with sub-samples taken from extracted incubation mixtures, corrected by subtraction of the activity in blanks, were converted to pg of benzo(a)pyrene using the known specific activity. This value was then converted to BPHase activity as pg per minute per mg of protein and then adjusted to represent the activity within the whole volume of the incubation medium.

The statistical relationship of the egg treatments were then determined using a t-test of appropriately pooled data (Huntsberger and Billingsley, 1977). See section 4.0 of Results for details.

RESULTS

1.0 BIOLOGICAL UPTAKE AND DEPURATION

Exposure of trout eggs and sac-fry to benzo(a)pyrene and Aroclor 1242 at three different concentrations over four different stages of development yielded consistent patterns of biological uptake. In all exposures linear regression lines were fitted to uptake data and the slopes of regression equations were analysed for homogeneity using a small sample t-test. In all exposures, at similar concentrations, uptake rates by sac-fry differed from uptake rates by eggs (alpha=0.005). In all exposures, except 1.0 PPB Aroclor 1242, the uptake by eggs at days 27-37 post-fertilization differed from uptake by eggs at days 5-15 post-fertilization (alpha=0.05). In no exposure, except that of 100 PPB Aroclor 1242, could the uptake of chemicals by eggs at days 16-26 be resolved statistically from either uptakes at 5-15 or 27-37 days post-fertilization. In the 100 PPB Aroclor 1242 exposures the day 16-26 post-fertilization uptake could be statistically resolved from days 27-37 post-fertilization to a significance of alpha=0.05.

1.1 Benzo(a)pyrene

1.11 1.0 PPB exposure

Exposure of rainbow trout eggs and sac-fry to water containing 1.0 PPB benzo(a)pyrene over five days resulted in accumulation of this compound to concentrations in eggs 7-10 times greater than that of the water and 800 times greater in sac-fry (Fig. 3A and B). Uptake rate constants (K1) and observed bioconcentration factors (BCF) (see section 3.5, Methods and Materials for determination of these factors) show a clear difference in biological uptake between eggs and sac-fry (Table 1). The sac-fry uptake rate constant (K1) was over 35 times that of the latest egg stage and the BCF was more than 70 times the level observed in the eggs. Although not as strong, there was an also a consistent trend of increasing BCF within the egg during embryonic development.

Depuration of benzo(a)pyrene by trout eggs at this concentration was slight (Fig. 3A and B). Elimination rate constants (K2) (see section 3.5, Methods and Materials for determination of this factor) for the eggs at days 5-15 and 16-26 were actually positive, appearing to indicate an increase in benzo(a)pyrene concentration in the eggs during clearance (Table 1). Eggs at the last stage showed some elimination allowing calculation of a depuration half-life of 23.1 days. Sac-fry, however, rapidly lost benzo(a)pyrene with a calculated half-life of 2.5 days (Fig. 3B). Uptake rates were linear over the exposure period indicating that

equilibrium between water and embryos had not been reached by the end of the exposure period.

1.12 10.0 PPB exposures

Exposure of eggs and sac-fry to benzo(a)pyrene at 10.0 PPB, followed by a clearance period of 5 days, resulted in uptake and depuration of the compound (Fig. 4A and B) in a manner similar to the 1.0 PPB exposure. Again, the most striking difference within the exposure data, was the uptake rate constant (K1) and bioconcentration factor (BCF) of the sac-fry when compared to those of egg stages (Table 1). The sac-fry uptake rate constant (K1) was greater than 60 times higher than egg constants and the BCF was over 90 times larger. As with the 1.0 PPB exposure there was a trend to an increasing BCF with age. This was also observed with the uptake rate constants (K1). Bioconcentration factors, as a whole, for this group were approximately 30-50 percent lower than those of the 1.0 PPB exposure experiment.

Each egg stage eliminated some benzo(a)pyrene (Fig. 4A), but the depuration rate constants (K2) were slight, yielding calculated half lives ranging from 231 to 21 days (Table 1). The elimination rate constant (K2) in the sac-fry (Fig. 4B) was considerably greater producing a half-life of 3.76 days.

1.13 100.0 PPB exposure

Exposure of trout eggs and sac-fry to 100 PPB

benzo(a)pyrene in water for five days followed by a clearance period of five days produced uptake and depuration data showing the same trends noted within the data from 1.0 and 10.0 PPB exposures (Fig. 5A and B). The uptake rate constants (K1) and bioconcentration factors (BCF), increased with age of the embryo (Table 1). Again, there was a significant increase in the K1 and BCF of the sac-fry when compared to the eggs. The sac-fry K1 was 15 times greater than that observed in the eggs while the BCF was just less than 10 times larger. Although these differences were significant they were almost one order of magnitude lower than the analogous data observed in the 1.0 and 10.0 PPB exposures.

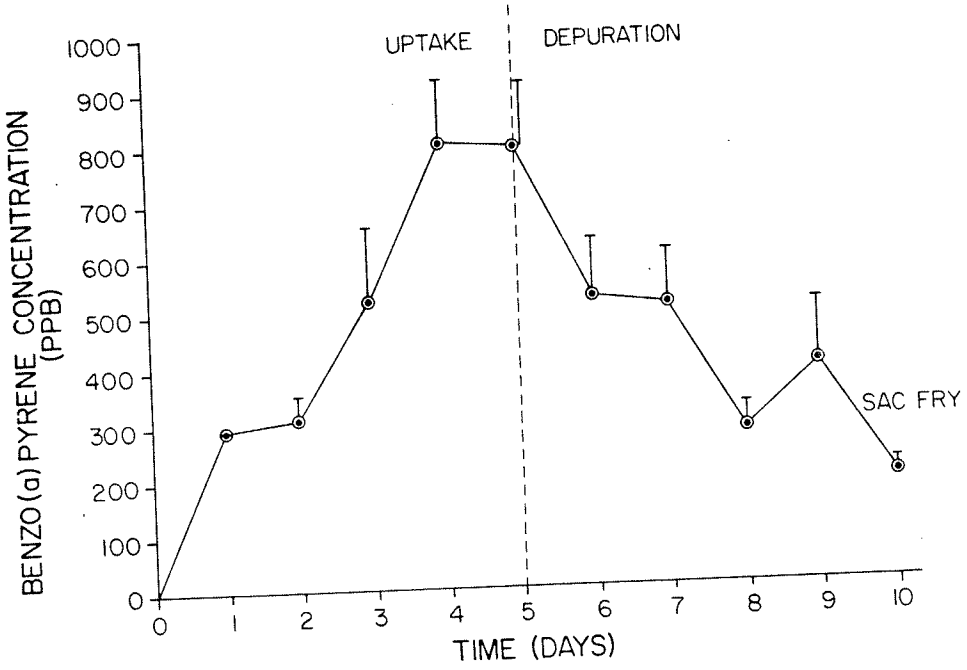
Depuration rate constants (K2) were consistently greater for the 100.0 PPB exposures than noted in 1.0 or 10.0 PPB experiments. Calculated half-lives ranged from 8.88 to 4.33 days in the eggs. Clearance data from the sac-fry yielded a half-life of 2.69 days.

1.2 Aroclor 1242

1.21 1.0 PPB exposures

Exposure of trout eggs and sac-fry to 1.0 PPB Aroclor 1242 in water over five days resulted in accumulation of those compounds to concentrations greater than that present in the water (Fig.6A and B). Although the greatest bioconcentration factor during egg development was recorded for days 27-37 post-fertilization,

Figure 3. Biological Uptake and Depuration of Benzo(a)pyrene at 1.0 PPB over three embryonic stages (5-15, 16-26, and 27-37 days post-fertilization) (Lower) and one post-hatch stage (50-60 days post-fertilization) (Upper) in rainbow trout. Vertical bars represent standard deviations.


UPTAKE
DEPURATION
BENZO(a) PYRENE CONCENTRATION (PPB)
1000
900
800
700
600
500
400
300
200
100
0
SAC FRY
0 1 2 3 4 5 6 7 8 9 10
TIME (DAYS)

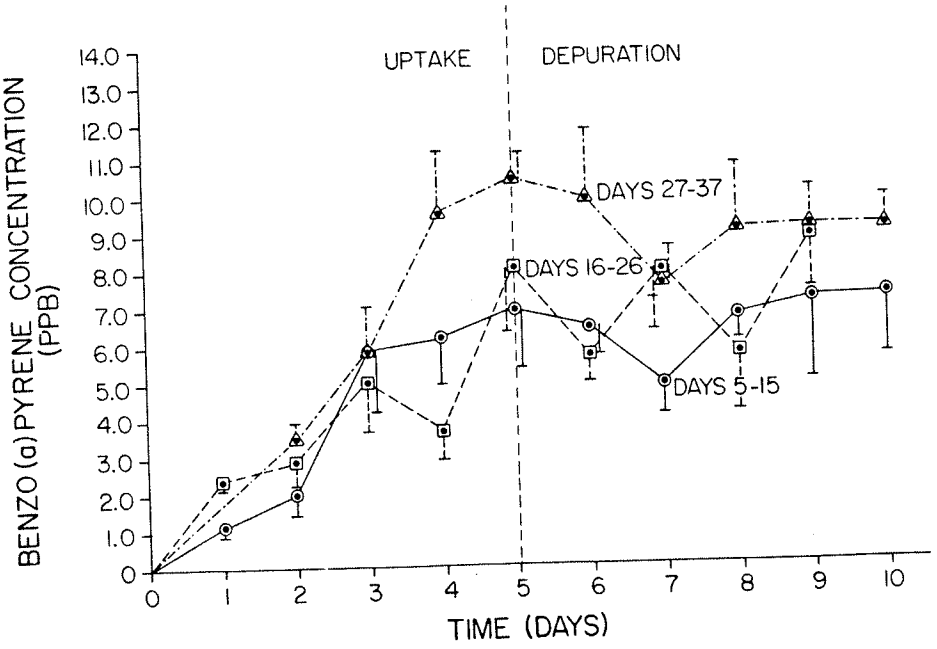
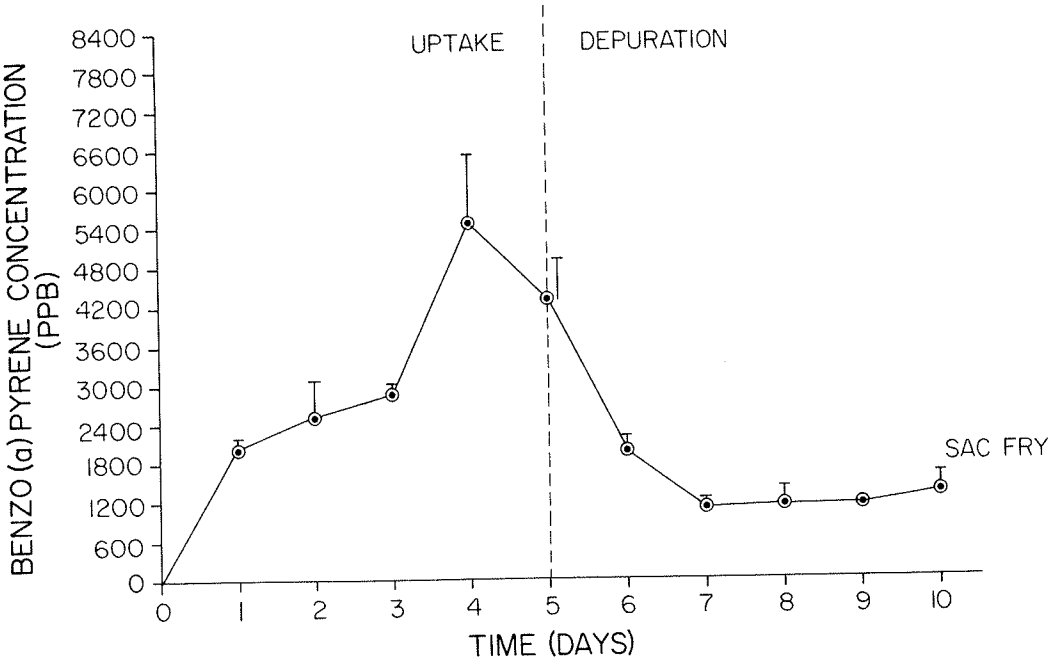

UPTAKE
DEPURATION
BENZO(a) PYRENE CONCENTRATION (PPB)
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
DAYS 27-37
DAYS 16-26
DAYS 5-15
0 1 2 3 4 5 6 7 8 9 10
TIME (DAYS)

Figure 4. Biological uptake and depuration of benzo(a)pyrene at 10.0 PPB over three embryonic stages (5-15 , 16-26 , and 27-37 days post-fertilization) (Lower) and one post-hatch stage (50-60 days post-fertilization) (B) in rainbow trout. Vertical bars represent standard deviations.


UPTAKE
DEPURATION
BENZO (a) PYRENE CONCENTRATION (PPB)
8400
7800
7200
6600
6000
5400
4800
4200
3600
3000
2400
1800
1200
600
0
SAC FRY
0 1 2 3 4 5 6 7 8 9 10
TIME (DAYS)

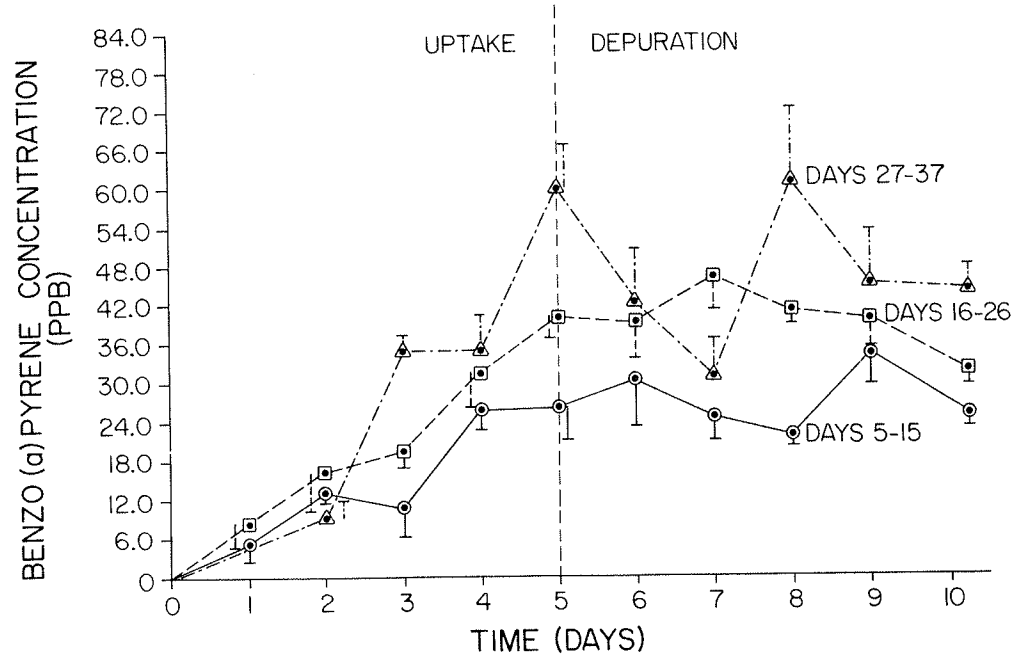
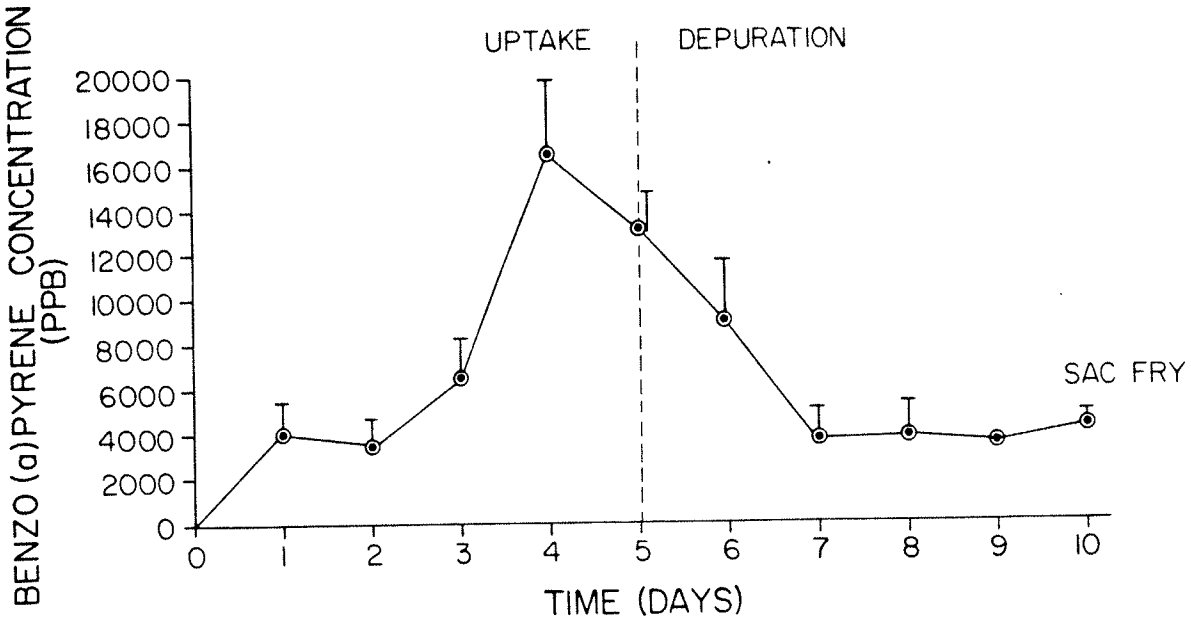

UPTAKE
DEPURATION
BENZO (a) PYRENE CONCENTRATION (PPB)
84.0
78.0
72.0
66.0
60.0
54.0
48.0
42.0
36.0
30.0
24.0
18.0
12.0
6.0
0
DAYS 27-37
DAYS 16-26
DAYS 5-15
0 1 2 3 4 5 6 7 8 9 10
TIME (DAYS)

Figure 5. Biological uptake and depuration of benzo(a)pyrene at 100.0 PPB over three embryonic stages (5-15, 16-26 , and 27-37 days post-fertilization) (A) and one post-hatch stage (50-60 days post-fertilization) (Upper) in rainbow trout. Vertical bars represent standard deviations.


UPTAKE
DEPURATION
BENZO (a) PYRENE CONCENTRATION (PPB)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0 1 2 3 4 5 6 7 8 9 10
TIME (DAYS)
SAC FRY

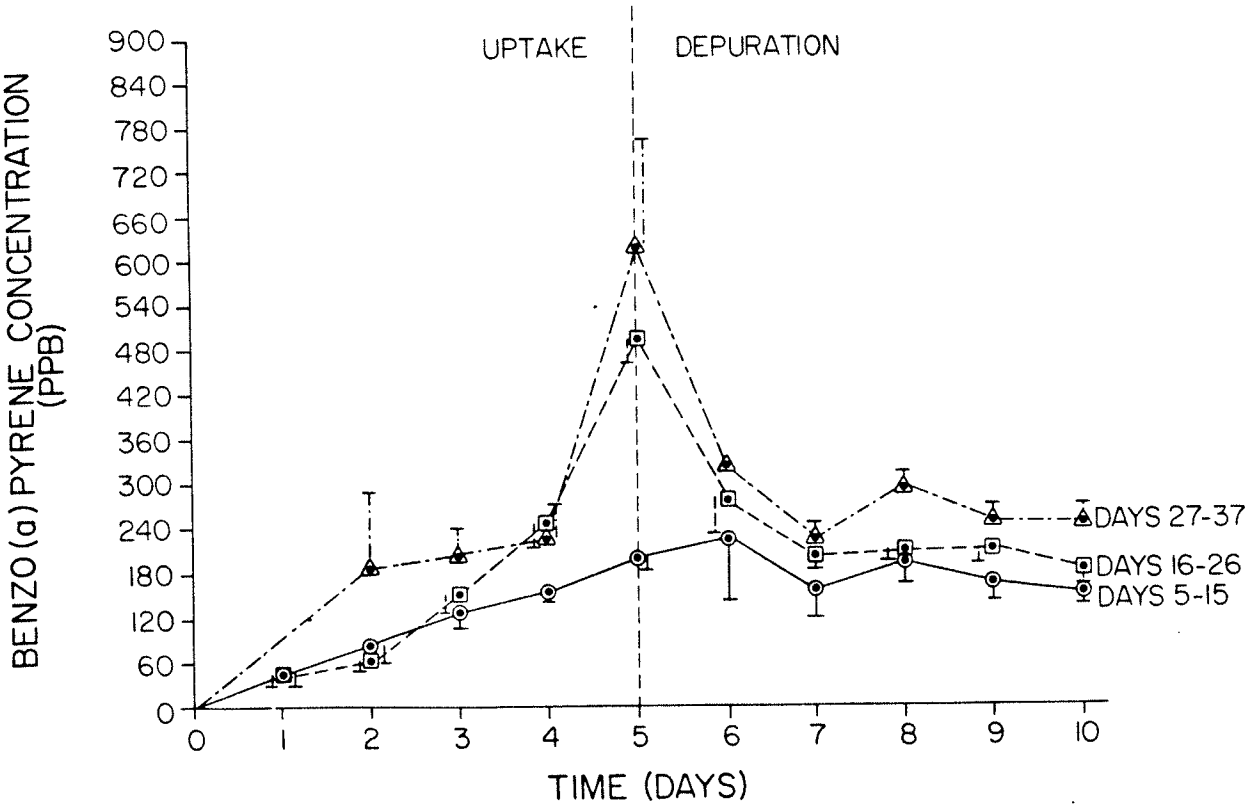
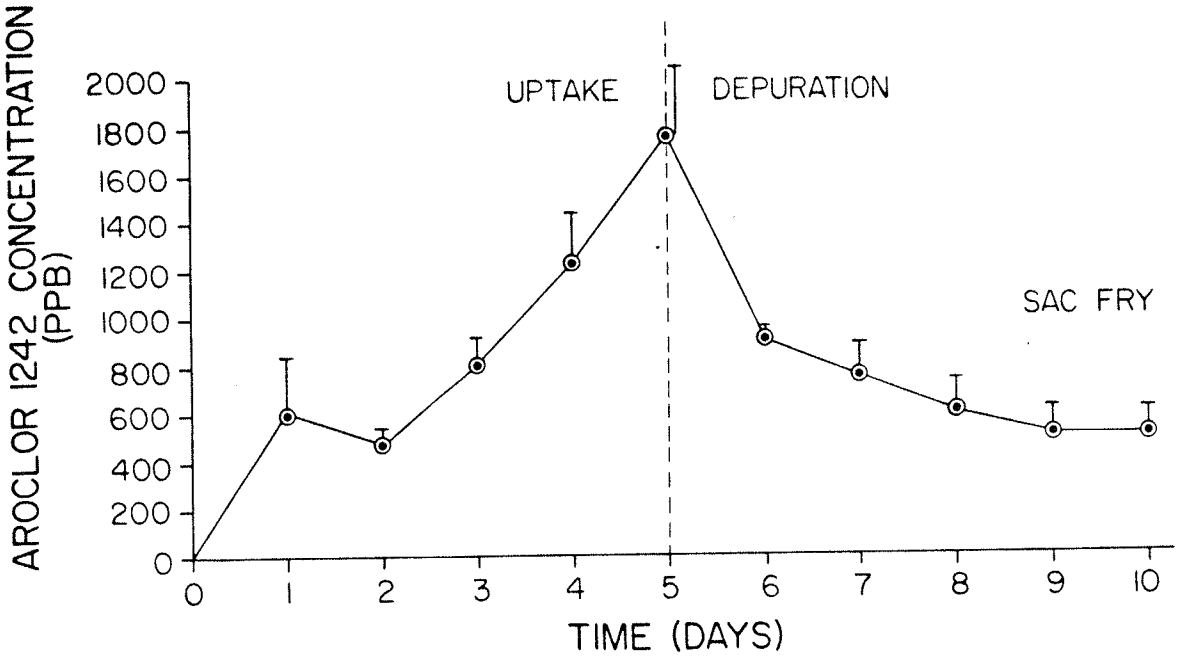

UPTAKE
DEPURATION
BENZO (a) PYRENE CONCENTRATION (PPB)
900
840
780
720
660
600
540
480
420
360
300
240
180
120
60
0
0 1 2 3 4 5 6 7 8 9 10
TIME (DAYS)
DAYS 27-37
DAYS 16-26
DAYS 5-15

Figure 6. Biological uptake and depuration of Aroclor 1242 at 1.0 PPB over three embryonic stages (5-15 , 16-26 , and 27-37 days post-fertilization) (Lower) and one post-hatch stage (50-60 days post-fertilization) (Upper) in rainbow trout. Vertical bars represent standard deviations.


AROCLOR 1242 CONCENTRATION (PPB)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
UPTAKE
DEPURATION
SAC FRY
0
1
2
3
4
5
6
7
8
9
10
TIME (DAYS)

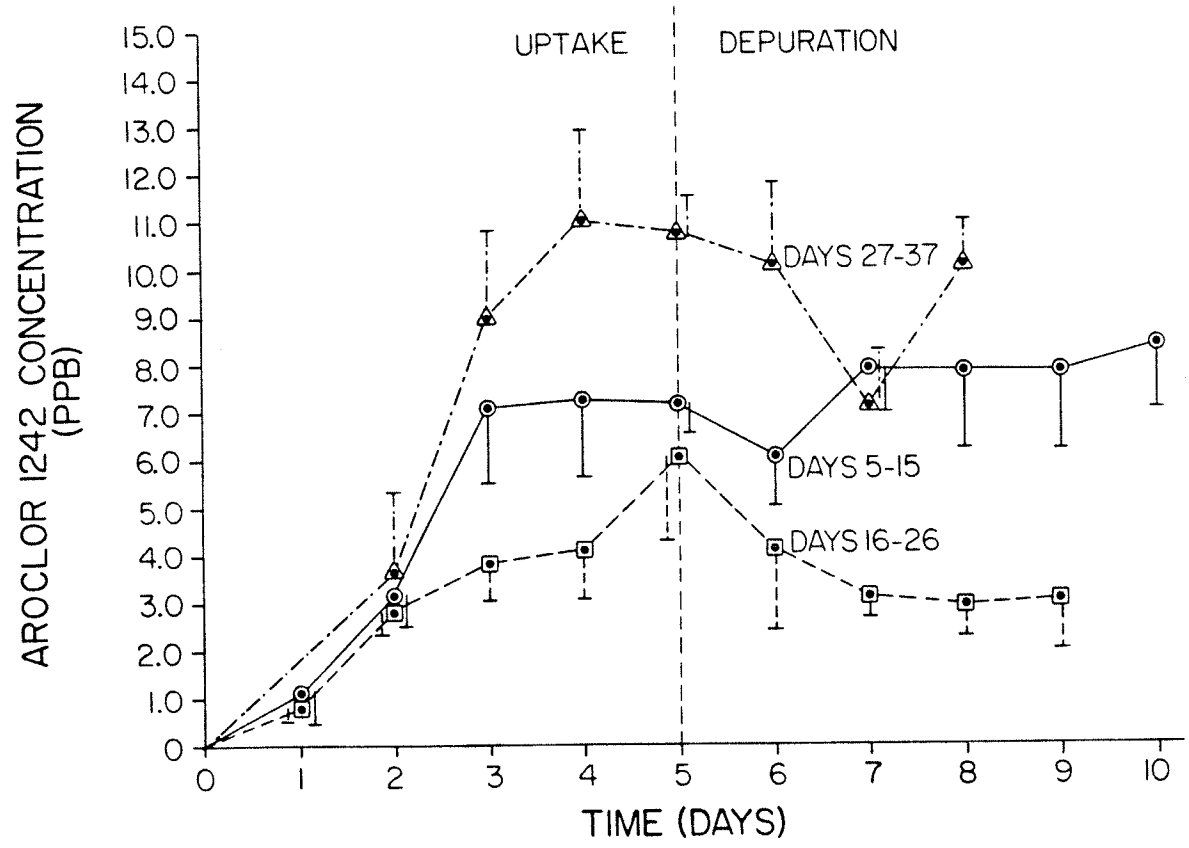
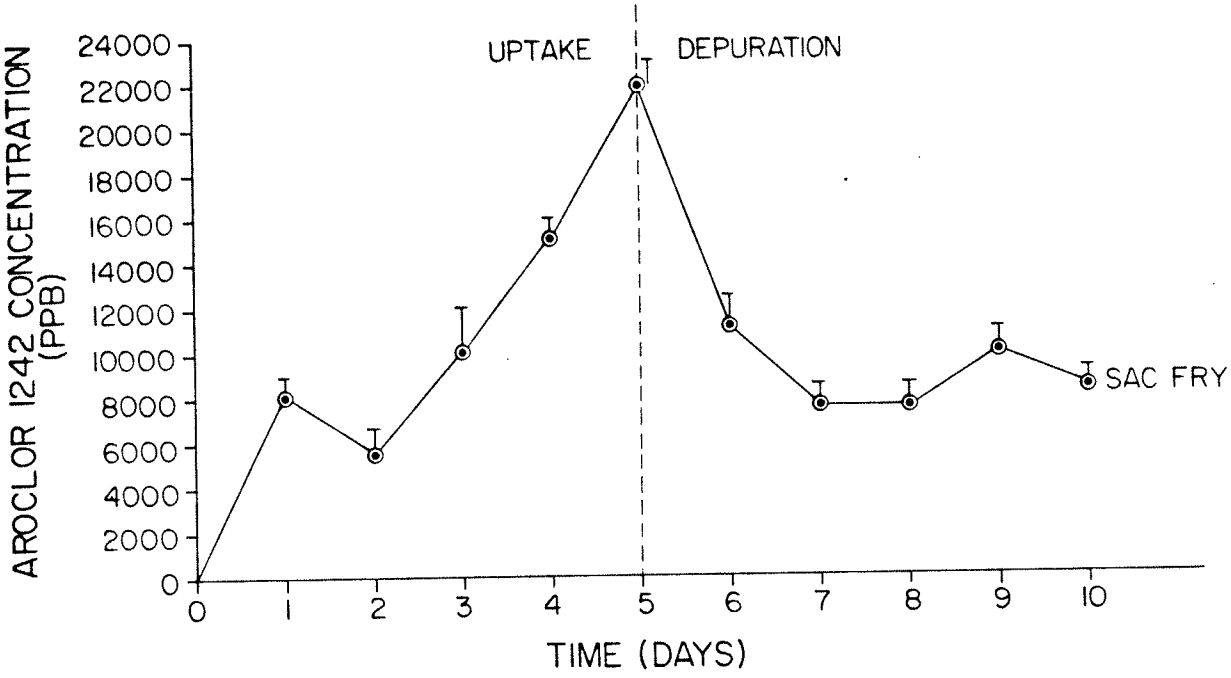

AROCLOR 1242 CONCENTRATION (PPB)
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
UPTAKE
DEPURATION
DAYS 27-37
DAYS 5-15
DAYS 16-26
0
1
2
3
4
5
6
7
8
9
10
TIME (DAYS)

Figure 7. Biological uptake and depuration of Aroclor 1242 at 10.0 PPB over three embryonic stages (5-15, 16-26, and 27-37 days post-fertilization) (Lower) and one post-hatch stage (50-60 days post-fertilization) (Upper) in rainbow trout. Vertical bars represent standard deviations.


AROCLOR 1242 CONCENTRATION (PPB)
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
UPTAKE
DEPURATION
SAC FRY
0
1
2
3
4
5
6
7
8
9
10
TIME (DAYS)

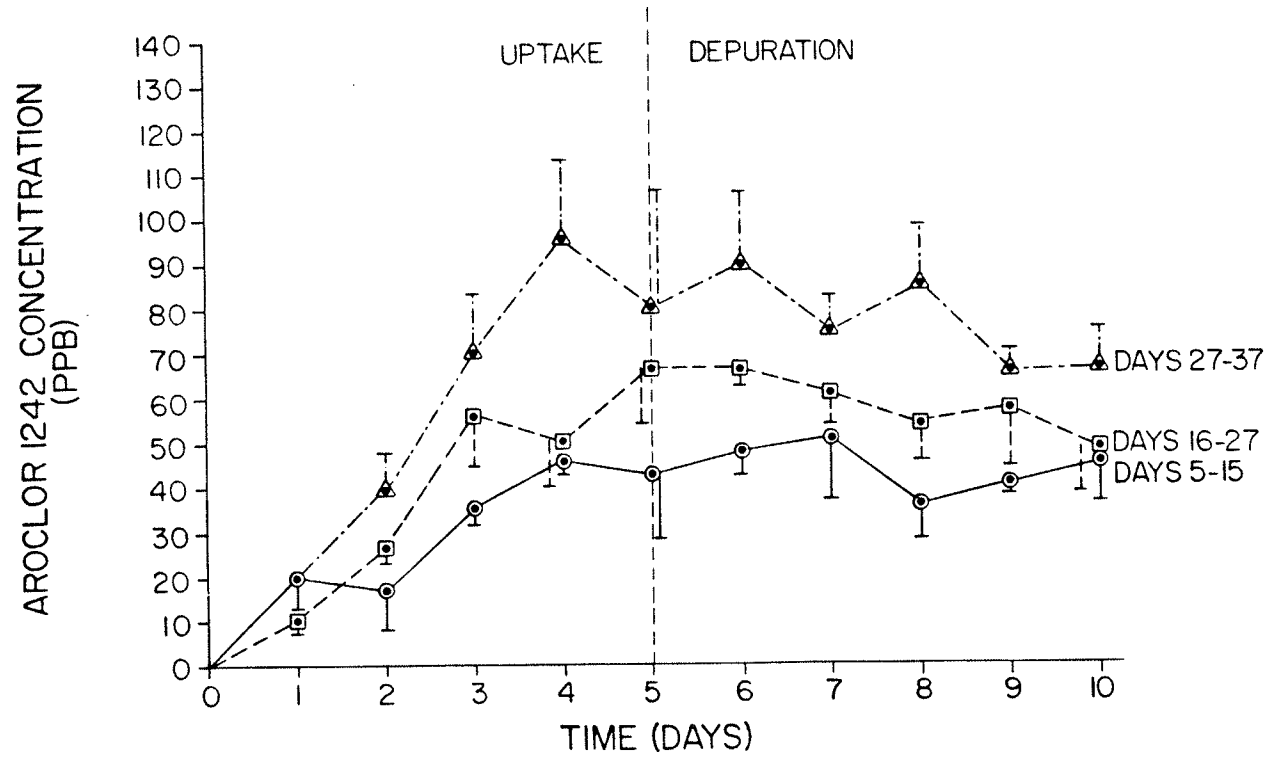
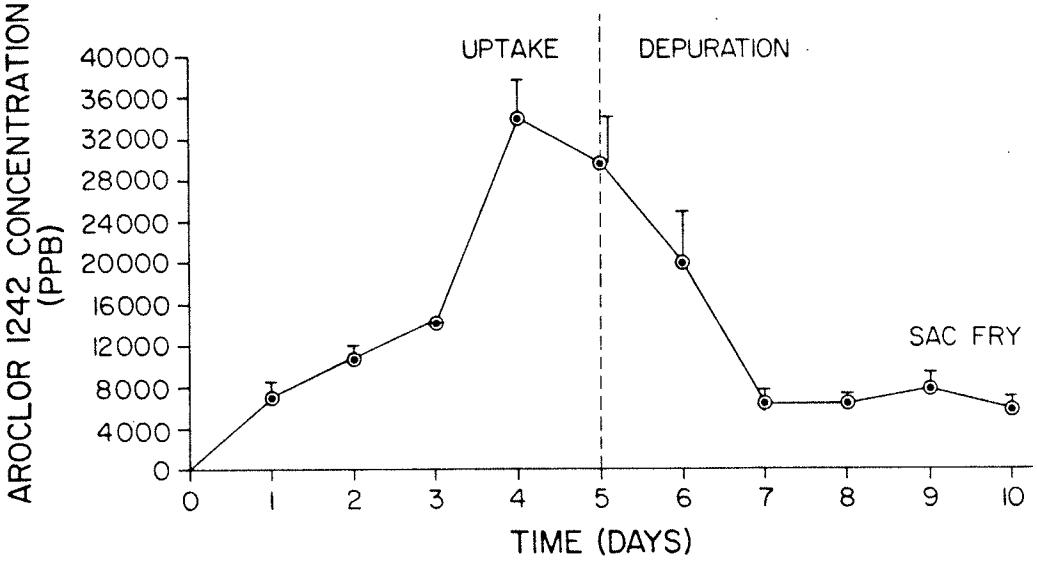
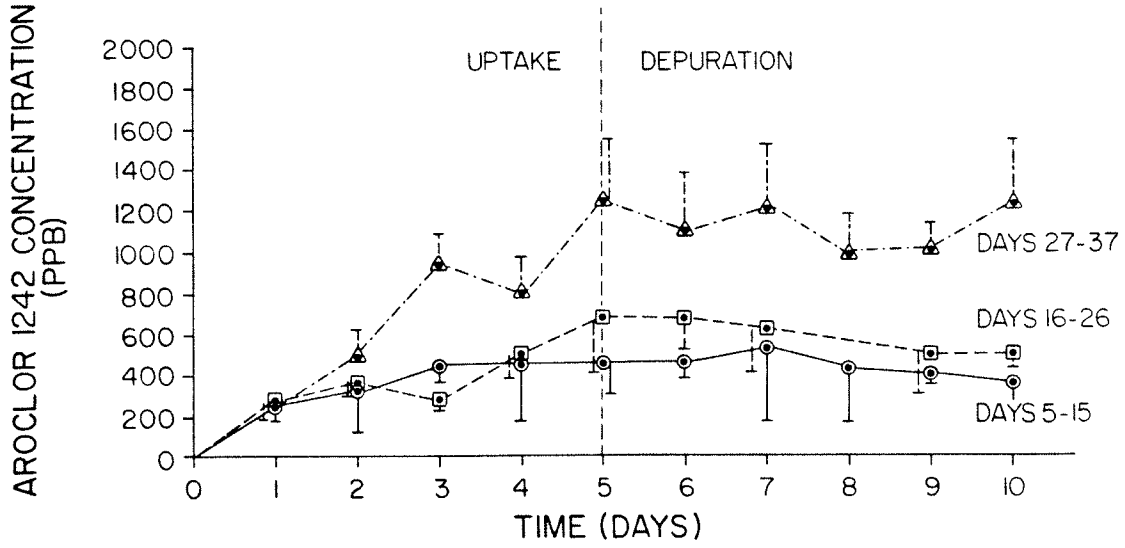

AROCLOR 1242 CONCENTRATION (PPB)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
UPTAKE
DEPURATION
DAYS 27-37
DAYS 16-27
DAYS 5-15
0
1
2
3
4
5
6
7
8
9
10
TIME (DAYS)

Figure 8. Biological uptake and depuration of Aroclor 1242 at 100.0 PPB over three embryonic stages (5-15 , 16-26 , and 27-37 days post-fertilization) (Lower) and one post-hatch stage (50-60 days post-fertilization) (Upper) in rainbow trout. Vertical bars represent standard deviations.


AROCLOR 1242 CONCENTRATION (PPB)
40000
36000
32000
28000
24000
20000
16000
12000
8000
4000
0
UPTAKE
DEPURATION
SAC FRY
0
1
2
3
4
5
6
7
8
9
10
TIME (DAYS)


AROCLOR 1242 CONCENTRATION (PPB)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
UPTAKE
DEPURATION
DAYS 27-37
DAYS 16-26
DAYS 5-15
0
1
2
3
4
5
6
7
8
9
10
TIME (DAYS)

as with benzo(a)pyrene data, eggs at the two earlier stages did not show the previously noted relationship between egg age and BCF. By day five, eggs at days 5-15 post-fertilization attained a BCF of 7.2 while eggs at days 16-26 post-fertilization recorded a BCF of 5.7. As with previous data the sac-fry bioconcentrated the Aroclor 1242 to a much greater extent than eggs. The sac-fry BCF was over 125 times greater than the maximum egg BCF while the uptake rate constant was more than 70 times larger.

Depuration rate constants observed in the eggs were low (Table 1). Eggs at the earliest developmental stage did not clear any Aroclor 1242 over the five day clearance period. Eggs at the two later stages showed some depuration with half-lives of 5.72 and 16.91 days calculated from eggs at 16-26 and 27-37 days post-fertilization respectively. This increase in half-life with egg age, for these two egg stages, was not observed in the previous data. Sac-fry demonstrated the most rapid depuration with a half-life of 3.14 days.

1.22 10.0 PPB exposure

Exposure of trout eggs and sac-fry to 10.0 PPB Aroclor 1242 in water over five days (Fig. 7A and B) resulted in uptake and depuration trends consistent with the benzo(a)pyrene data. Within the egg developmental stages both uptake rate constants (K1) and bioconcentration factors (BCF) increased with age (Table 1). This differed from the

data obtained with the exposure to 1.0 PPB Aroclor 1242 but was consistent with all other data. The K1 and BCF in the sac-fry were approximately 200 times greater than in the eggs. This was slightly greater than observed in data from the 1.0 PPB exposure to Aroclor 1242.

Depuration rate constants (K2) were low in all stages except sac-fry (Table 1). Clearance of Aroclor 1242 by the eggs yielded half-lives ranging from 33.0 to 10.8 days. Sac-fry eliminated these materials with a calculated half-life of 4.6 days.

1.23 100.0 PPB exposure

Exposure of trout eggs and sac-fry to 100.0 PPB Aroclor 1242 in water for five days resulted in biconcentration and depuration of the compound in a manner consistent with most data presented above (Fig. 8A and B). The previously noted relationship between increasing age and increasing K1 and BCF was found again in these exposures. Bioconcentration by sac-fry was approximately 30 times greater and K1 was about 15 time larger than observed in the eggs. These differences were considerably less than those observed in exposures to the two lower concentrations of Aroclor 1242. This decrease in K1 and BCF values relative to the eggs in the 100.0 PPB exposure was also found with the benzo(a)pyrene experiments.

Depuration rate constants (K2) were similar to those of the 10.0 PPB exposure. Again, sac-fry were the only

TABLE ONE: Uptake (K1) and depuration (K2) rate constants, observed bioconcentration factors (BCF) and depuration half lives ($t_{\frac{1}{2}}$) for Rainbow Trout exposed to either Benzo(a) pyrene or Aroclor 1242 at three different concentrations over four different periods of early development.

BENZO(a)PYRENE

1.0 PPB					10.0 PPB					100.0 PPB				
AGE*	K1	BCF(CW/CF)	K2	$t_{\frac{1}{2}}$(days)	AGE*	K1	BCF(CW/CF)	K2	$t_{\frac{1}{2}}$(days)	AGE*	K1	BCF(CW/CF)	K2	$t_{\frac{1}{2}}$(days)
5-15 (egg)	-----	7.0	+0.036	----	5-15 (egg)	0.52	2.6	-0.003	231.0	5-15 (egg)	0.31	1.9	-0.078	8.9
16-26 (egg)	-----	8.0	+0.018	----	16-26 (egg)	0.71	3.9	-0.033	21.0	16-26 (egg)	0.68	5.2	-0.160	4.3
27-37 (egg)	1.98	10.7	-0.030	23.1	27-37 (egg)	1.06	5.6	-0.023	30.1	27-37 (egg)	0.85	6.3	-0.150	4.6
50-60 (sacfry)	76.30	808.0	-0.270	2.5	50-60 (sacfry)	68.20	560.0	-0.183	3.8	50-60 (sacfry)	15.90	61.7	-0.257	2.7

AROCLOR 1242

1.0 PPB					10.0 PPB					100.0 PPB				
AGE*	K1	BCF(CW/CF)	K2	$t_{\frac{1}{2}}$(days)	AGE*	K1	BCF(CW/CF)	K2	$t_{\frac{1}{2}}$(days)	AGE*	K1	BCF(CW/CF)	K2	$t_{\frac{1}{2}}$(days)
5-15 (egg)	------	7.2	+0.055	----	5-15 (egg)	0.85	4.5	-0.021	33.0	5-15 (egg)	0.81	4.7	-0.060	11.6
16-26 (egg)	0.83	5.7	-0.121	5.7	16-26 (egg)	1.11	6.5	-0.061	11.4	16-26 (egg)	1.08	6.8	-0.090	7.7
27-37 (egg)	2.48	13.8	-0.041	16.9	27-37 (egg)	1.64	9.7	-0.064	10.8	27-37 (egg)	2.16	12.6	-0.060	11.6
50-60 (sacfry)	192.00	1760.0	-0.221	3.1	50-60 (sacfry)	293.00	2200.0	-0.152	4.6	50-60 (sacfry)	27.80	334.0	-0.311	2.2

*Age expressed as days from fertilization.

stage to show rapid depuration, with a calculated half-life of 2.22 days.

1.3 Localisation of benzo(a)pyrene and hexachlorobiphenyl within rainbow trout eggs

Analysis of radioactivity within dissected eggs exposed to BaP and HCBP over a period of six days revealed that 82 percent of benzo(a)pyrene and 88 percent of hexachlorobiphenyl was localised within the egg yolk (Table 2). Analysis was based on a sample size of ten for each treatment.

2.0 CHARACTERIZATION OF THE CYTOCHROME P-450 ISOZYME(S)

2.1 Spectral analysis of hepatic cytochrome P-450 in arctic charr

Intraperitoneal injection of benzo(a)pyrene (20 mg/kg fish) induced hepatic levels of cytochrome P-450. Analysis of hepatic microsomal suspensions by difference spectroscopy revealed an absorbance peak at 450 nm that was undetectable in control animals (Fig 9 and 10). This peak allowed calculation of the cytochrome P-450 content of the microsomal suspension at 0.16 nmol/mg microsomal protein.

TABLE TWO: Localization of benzo(a)pyrene and 2,2',4,4',5,5'-hexachlorobiphenyl within rainbow trout eggs exposed to 0.2 and 1.0 PPB concentrations respectively over six days.

	BENZO(A)PYRENE %	HEXACHLOROBIPHENYL %
CHORION	11.0 +/- 1.1	8.0 +/- 2.3
EMBRYO	7.0 +/- 1.5	4.0 +/- 1.2
YOLK	82.0 +/- 2.6	88.0 +/- 3.5
	TOTAL=100	TOTAL=100

Figure 9. Plotted difference spectra of cytochrome P-450 from Arctic Charr given interperitoneal injection of corn oil.

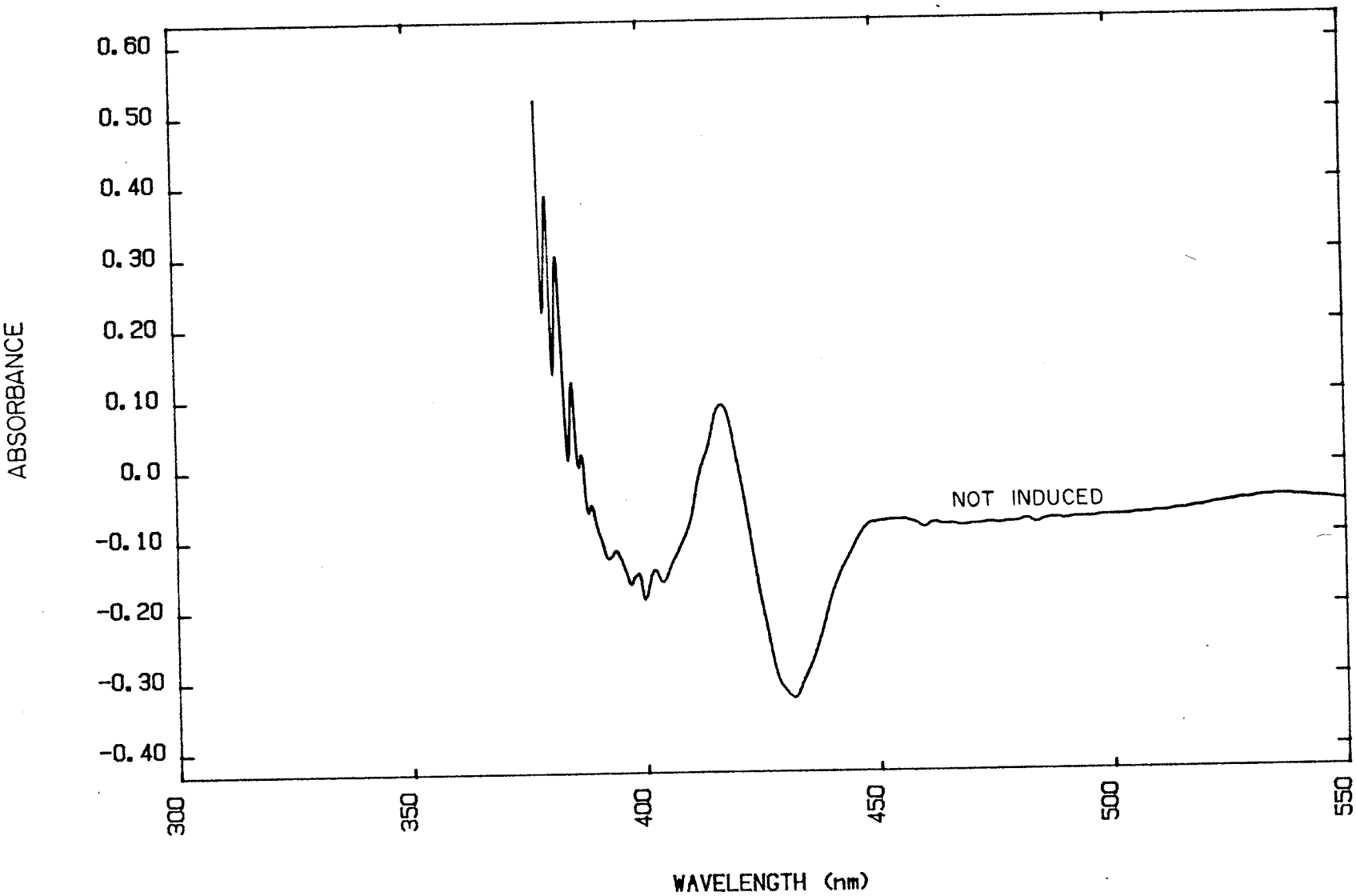
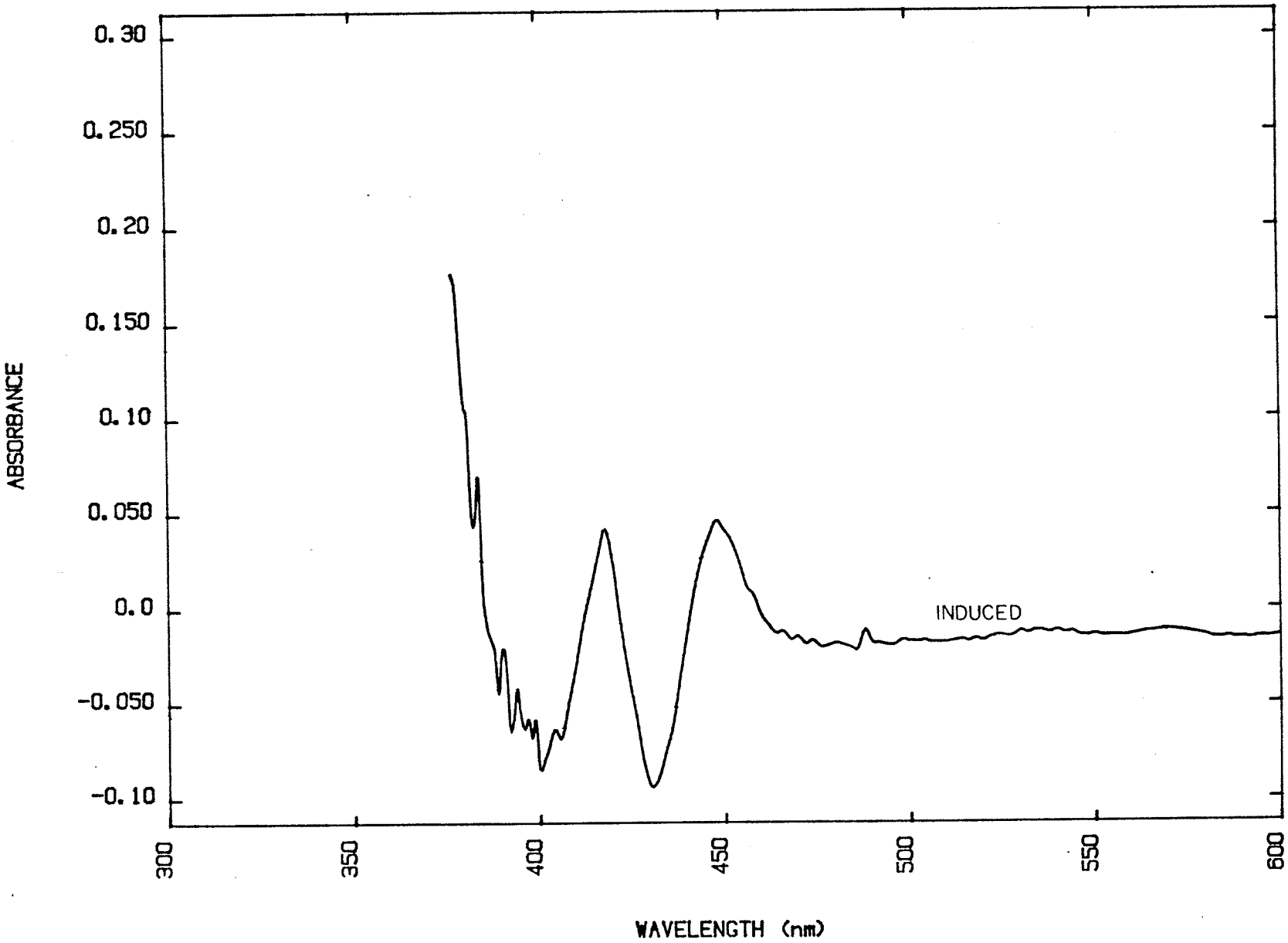

0.60
0.50
0.40
0.30
0.20
0.10
0.0
-0.10
-0.20
-0.30
-0.40
ABSORBANCE
300
350
400
450
500
550
WAVELENGTH (nm)
NOT INDUCED

Figure 10. Plotted difference spectra of cytochrome P-450 from Arctic Charr induced by interperitoneal injection of benzo(a)pyrene to a dose of 20mg BaP/kg fish.


0.30
0.250
0.20
0.150
0.10
0.050
0.0
-0.050
-0.10
ABSORBANCE
300
350
400
450
500
550
600
WAVELENGTH (nm)
INDUCED

2.2 Assay conditions for benzo(a)pyrene hydroxylase activity

2.21 Post-mitochondrial homogenate storage effect

Analysis of benzo(a)pyrene hydroxylase activity in post-mitochondrial homogenates, stored on ice, over 48 hours revealed a consistent decrease in BPHase activity with time (Fig. 11). Activity levels appeared be more dependant on time within the first 12 hours of storage, however this observation was based on only two assays. Analysis of the data as a whole allowed calculation of a BPHase activity half-life of 31.5 hours, assuming as a first approximation, first order decay kinetics.

2.22 Effect of incubation time on benzo(a)pyrene hydroxylase activity

The rate of metabolism of benzo(a)pyrene showed a strong inverse relationship with time over the first ten minutes of incubation (Fig. 12). After ten minutes there was a consistent but much slower decrease in activity. Measured BPHase activity levels over the first one minute of incubation were an order of magnitude greater than those observed over ten minutes (50.0 vs. 5.0 pg/min/mg protein). In contrast, the drop in BPHase activity over the next eighty minutes was only approximately 50 percent (5.0 vs. 2.2 pg/min/mg protein).

Figure 11. Effect of time on benzo(a)pyrene hydroxylase activity in post-mitochondrial homogenates stored on ice. Vertical bars represent standard deviations.

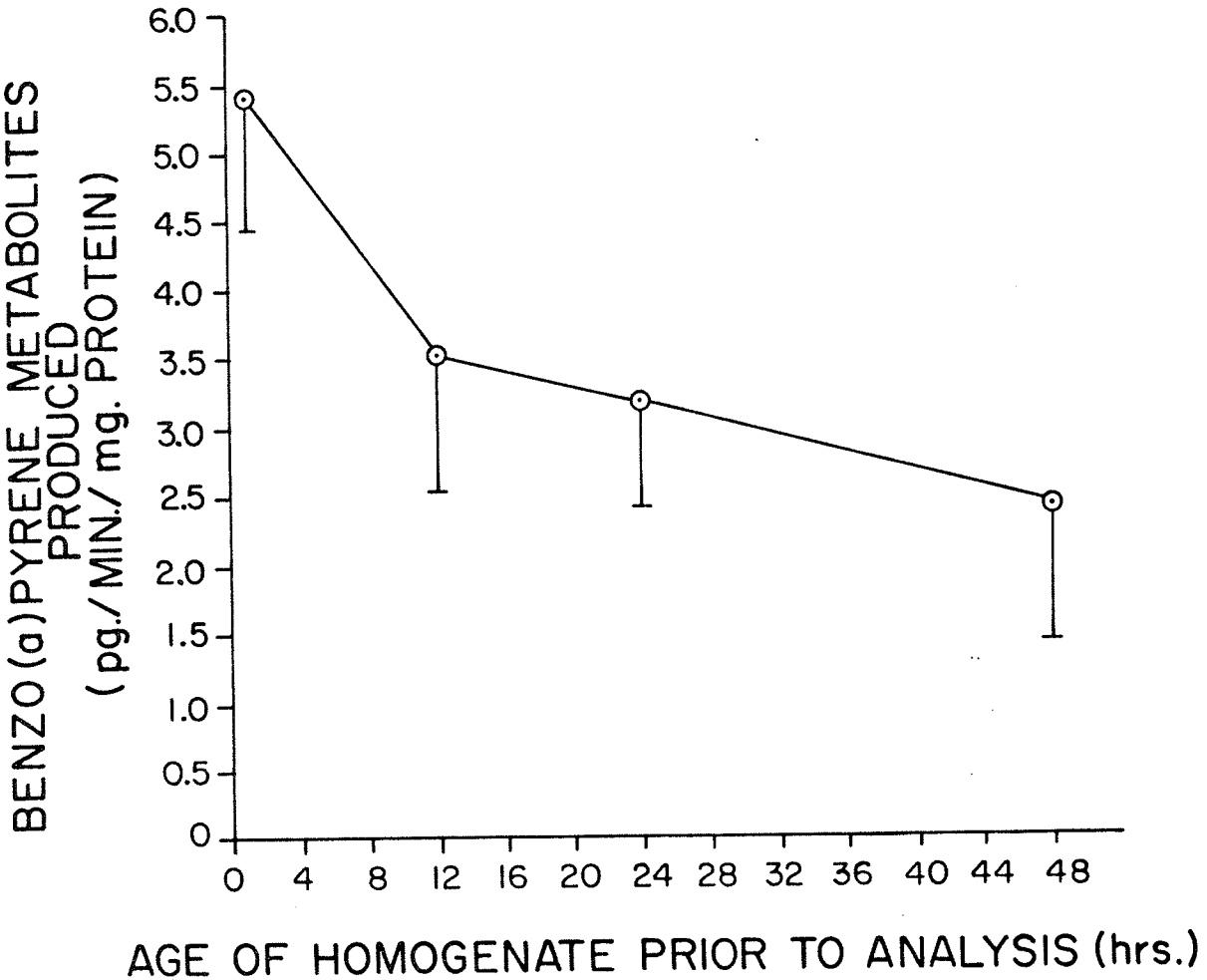
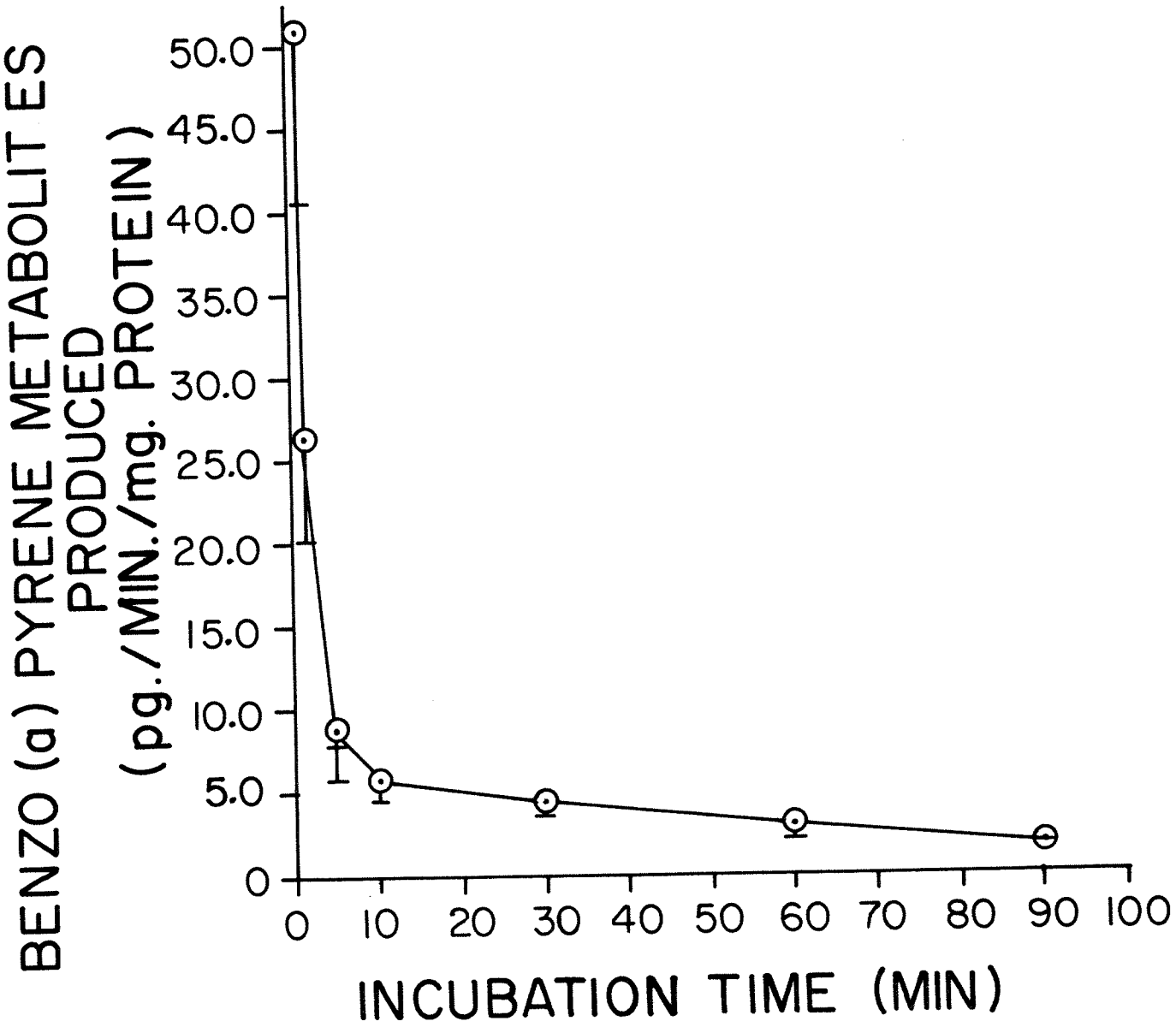

BENZO(a)PYRENE METABOLITES PRODUCED (pg./MIN./ mg. PROTEIN)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0 4 8 12 16 20 24 28 32 36 40 44 48
AGE OF HOMOGENATE PRIOR TO ANALYSIS (hrs.)

Figure 12. Effect of incubation time on benzo(a)pyrene hydroxylase activity. Vertical bars represent standard deviations.


BENZO (a) PYRENE METABOLIT ES PRODUCED (pg./MIN./mg. PROTEIN)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0
0
10
20
30
40
50
60
70
80
90
100
INCUBATION TIME (MIN)

2.23 Effect of post-mitochondrial homogenate protein concentration on benzo(a)pyrene hydroxylase activity

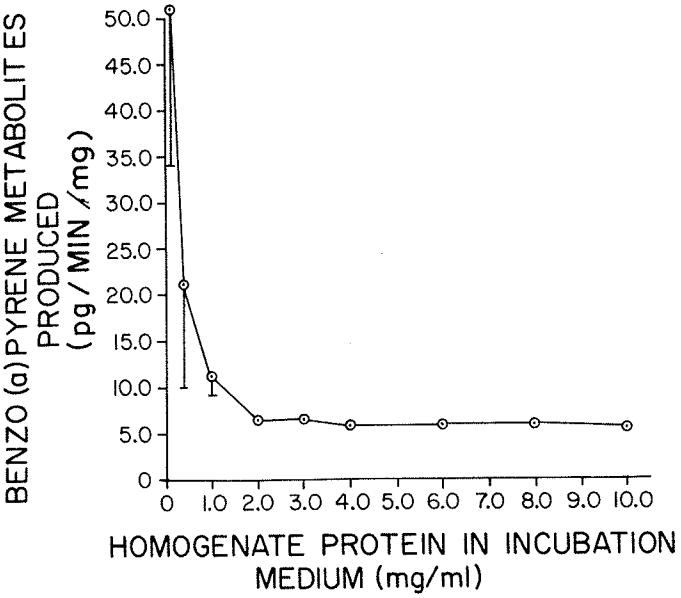
The rate of metabolism of benzo(a)pyrene was inversely related to homogenate protein concentrations between 0.2 and 2.0 mg/ml. Homogenate protein concentrations between 2.0 and 10.0 mg/ml had no effect on the rate of BPHase activity (Fig. 13A). Benzo(a)pyrene hydroxylase activity at the lowest protein concentration tested (0.2 mg/ml) was one order of magnitude greater than the corresponding activity at a protein concentration of 2.0 mg/ml.

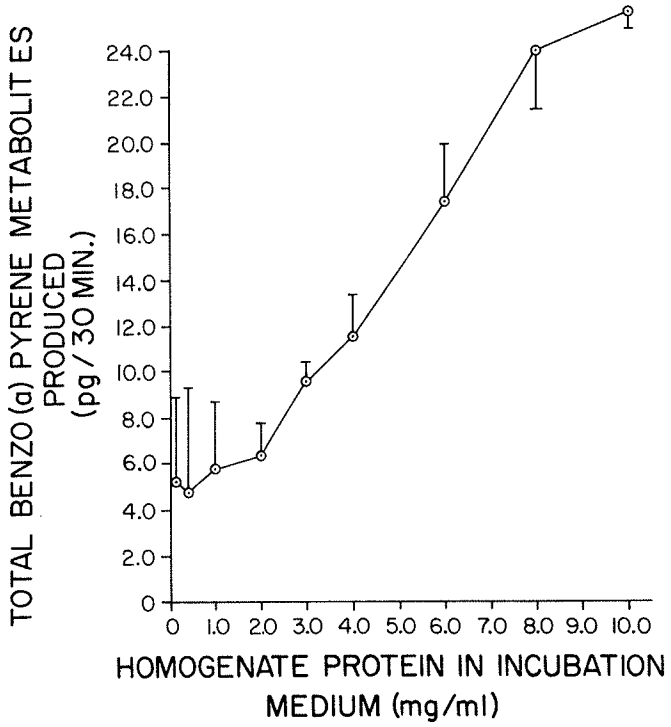
Analysis of the total production of benzo(a)pyrene metabolites as a function of homogenate protein concentration illustrated a direct relationship between the two at concentrations greater than 2.0 mg/ml, however, activity did not appear to be related to protein concentation below this value (Fig. 13B).

2.24 Effect of cofactor concentration on benzo(a)pyrene hydroxylase activity

Analysis of the effect of cofactor concentration on benzo(a)pyrene hydroxylase activity showed a significant difference between controls and incubations containing 50 percent of the suggested cofactor concentration. (DePierre et al. 1975) BPHase activity rates were lowered to 2.51 pg/min/mg protein in incubations containing 50 percent of normal cofactor concentrations from 5.41 pg/min/mg protein

Figure 13. Effect of varying concentrations of homogenate protein on benzo(a)pyrene hydroxylase assays. Lower:activity expressed per milligram of protein. Upper:activity expressed as the total activity for the protein present. Vertical bars represent standard deviations.


BENZO (a) PYRENE METABOLITES PRODUCED (pg / MIN / mg)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0
0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0
HOMOGENATE PROTEIN IN INCUBATION MEDIUM (mg/ml)


TOTAL BENZO (a) PYRENE METABOLITES PRODUCED (pg / 30 MIN.)
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0
HOMOGENATE PROTEIN IN INCUBATION MEDIUM (mg/ml)

in controls. Analysis by t-test indicated a significance greater than alpha=0.005.

Addition of twice the normal cofactor concentration had no significant effect on BPHase activity when compared to controls (5.41 vs. 5.48 pg/min/mg protein respectively).

2.25 The effect of buffers on benzo(a)pyrene hydroxylase activity

Benzo(a)pyrene hydroxylase activity in assays containing Tris/HCl (ph 7.5) or Hepes/KCl (ph 7.4) as the only buffer did not differ significantly from each other or from assay mixtures in which both buffers were mixed. (7.06 versus 6.9 versus 6.8 pg/min/mg protein respectively).

2.26 Effect of substrate carrier solvent on benzo(a)pyrene hydroxylase activity

2.261 Dimethylsulphoxide versus acetone

The use of acetone as a substrate carrier solvent yielded BPHase activity levels that were approximately 2 to 3 times higher than identical assays in which DMSO was employed as a carrier. When DMSO was used as a carrier BPHase rates of 2.71 pg/min/mg protein were observed as compared to acetone assays with rates of 7.8 pg/min/mg protein.

2.262 Effect of the concentration of the substrate carrier acetone on benzo(a)pyrene hydroxylase activity

It was found that benzo(a)pyrene hydroxylase activity was strongly dependant on the amount of acetone used to deliver the benzo(a)pyrene substrate to the incubation medium (Fig. 14). A peak in BPHase activity could be seen when the acetone concentration, expressed as percent by volume, reached four percent. The peak in activity was sharp, with BPHase activity at three percent and five percent (of test 2 (Fig.14)) showing activity levels 30 and 50 percent lower respectively. As acetone concentrations reached eight percent and higher observable inhibition of BPHase activity occurred.

3.0 BENZO(a)PYRENE HYDROXYLASE IN EMBRYONIC RAINBOW TROUT

Assays of three populations of rainbow trout eggs (A, control; B, those exposed to 100.0 PPB Aroclor 1254; C, those exposed to 200.0 PPB Aroclor 1254) for benzo(a)pyrene hydroxylase activity resulted in the first measurable activities on day 29 post-fertilization (Fig. 15). Over the next three day period levels of BPHase increased such that all three treatments had measureable activities. Upon hatch, which occurred over days 32 and 33, the activity of BPHase increased significantly to approximately four

Figure 14. Effect of varying volume of acetone substrate carrier on benzo(a)pyrene hydroxylase activity. Vertical bars represent standard deviations.

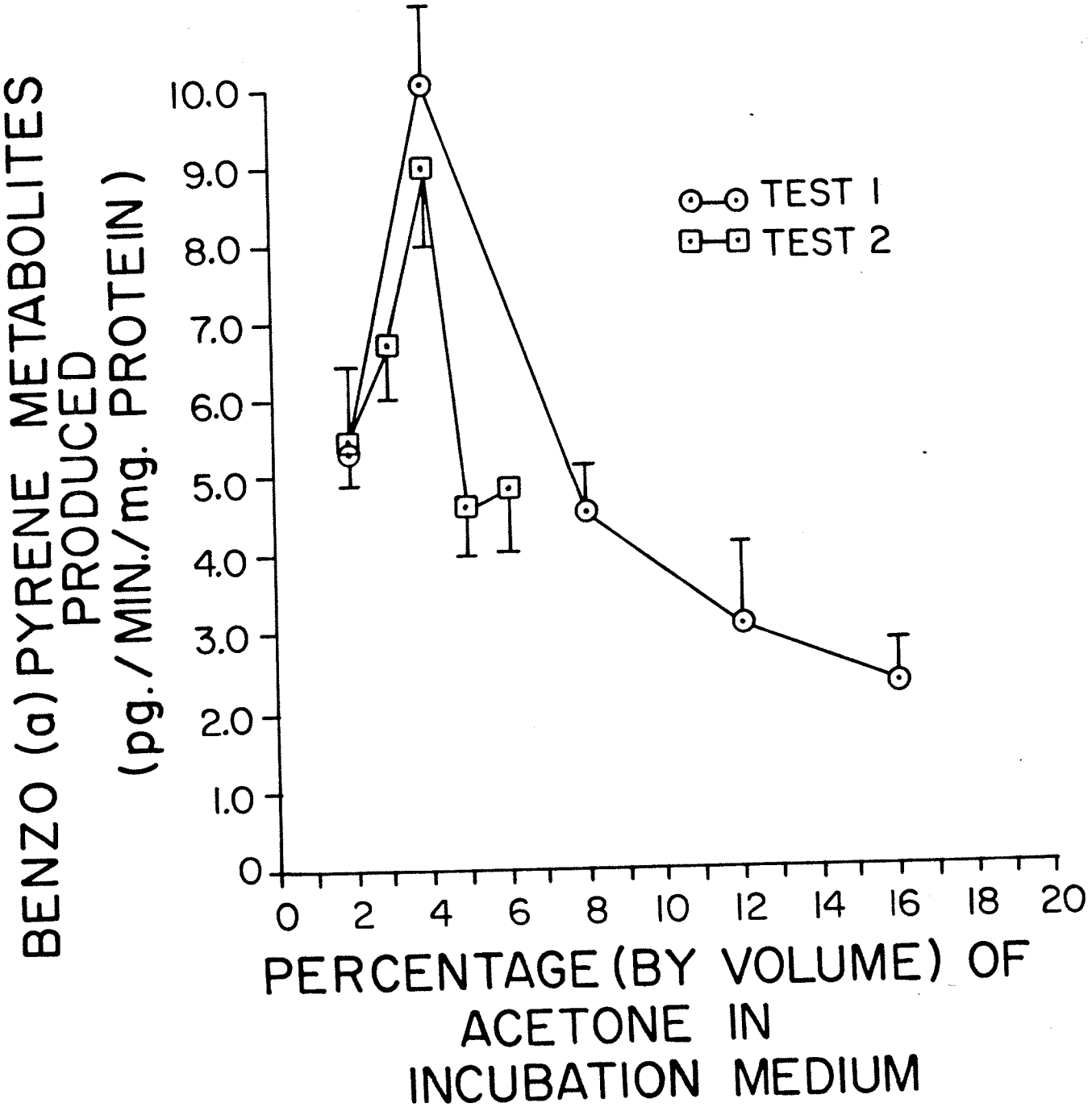
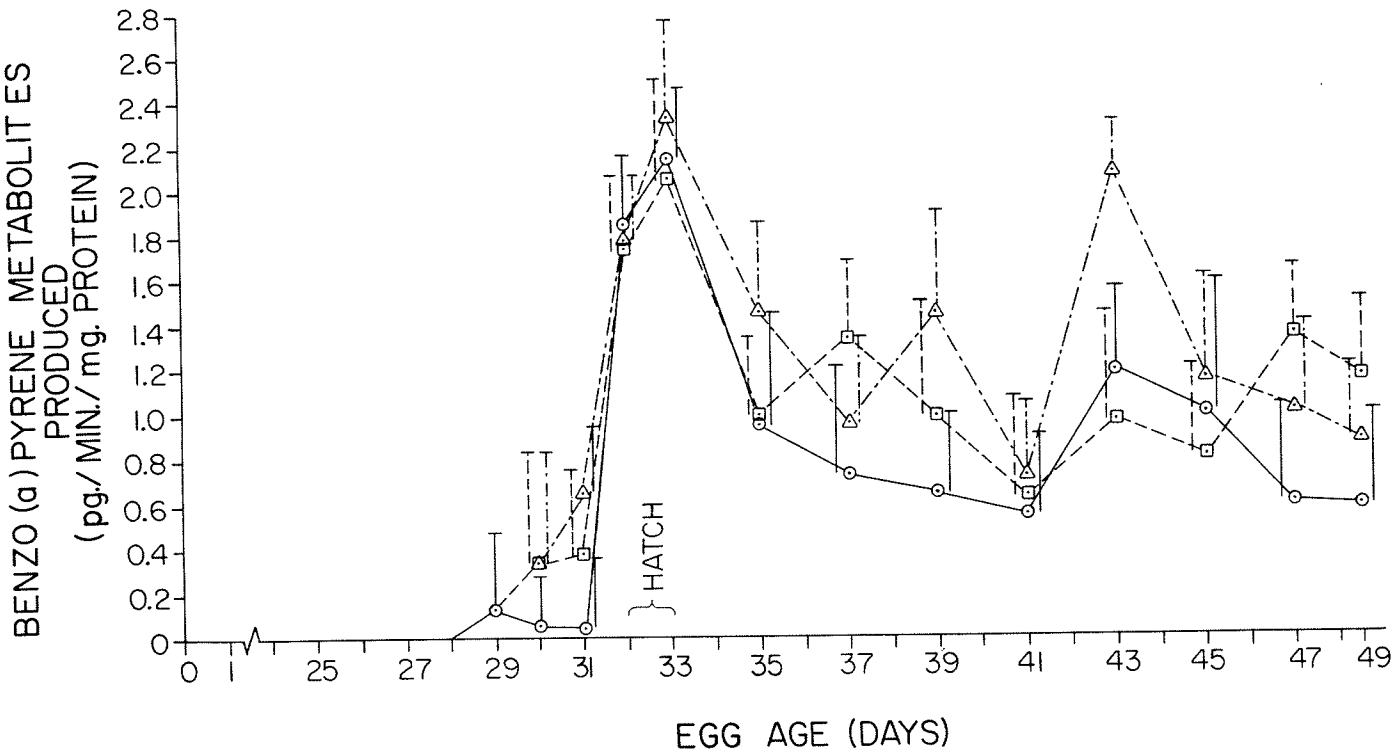

BENZO (a) PYRENE METABOLITES PRODUCED (pg./MIN./mg. PROTEIN)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0 2 4 6 8 10 12 14 16 18 20
PERCENTAGE (BY VOLUME) OF ACETONE IN INCUBATION MEDIUM
TEST 1
TEST 2

Figure 15. Induction of benzo(a)pyrene hydroxylase by Aroclor 1254 during the early life history of rainbow trout. Controls represented by circles, 100 PPB exposure to Aroclor 1242 represented by squares, 200 PPB exposure to Aroclor 1242 represented by triangles. Vertical bars represent standard deviations.


BENZO (a) PYRENE METABOLITES PRODUCED (pg./MIN./mg. PROTEIN)
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
HATCH
0
1
25
27
29
31
33
35
37
39
41
43
45
47
49
EGG AGE (DAYS)

times the levels found in the pre-hatch embryos. Following the hatch period BPHase activity dropped off to approximately 50 percent of the hatch peak. Throughout the remainder of the study period BPHase rates remained relatively constant, with one anomalous high value on day 43 post-fertilization. Analysis of the data obtained after day 35 by t-test indicated that BPHase rates were significantly (alpha=0.005) higher in the eggs exposed to 100.0 and 200.0 PPB Aroclor 1254 than in the control eggs. BPHase activities between 100.0 and 200.0 PPB Aroclor 1254 could not be resolved statistically. In addition, pooled BPHase activities prior to hatch differed significantly (alpha=0.005) from the activities in larvae.

DISCUSSION

1.0 BIOLOGICAL UPTAKE AND DEPURATION

Applied toxicologists have long been interested in the embryonic and larval stages of diverse species of fish for use in toxicological assays (Schimmel et al., 1974; Birge et al., 1979). These early life stages have been valued on the one hand for the practical benefits of their small size in exposure experiments and on the other hand for the environmentally significant finding that they are the most susceptible to poisons (Mauck et al., 1978; Weiss and Weiss, 1978; Passino and Kramer, 1980). This latter finding enabled toxicologists to use embryo-larval toxicity tests to quickly estimate a parameter known as the Maximal Acceptable Toxicant Concentration (MATC) thus avoiding lengthy and expensive chronic exposure experiments (McKim et al., 1977; Woltering, 1984). A variety of toxicity criteria have been developed for use in these tests, such as egg hatchability and hatching time (Mauck et al., 1978), heart rate (Linde, 1974), respiratory rate (Maki, 1979), abnormal pigmentation (Wilson, 1970), gross abnormalities (Smith and Cameron, 1979; Stross and Haines, 1979), or lethality (Halter and Johnson, 1974; DeFoe, 1978). Unfortunately, despite a wealth of data pertaining to embryo-larval toxicology a

paucity of research has been directed into investigating the kinetics of uptake, depuration, and metabolism of toxins at these early life stages. Of the limited work assessing uptake and depuration rates in fish eggs this work is the first, to the knowledge of the author, to determine these rates at different stages of embryonic development and attempt a correlation to the known biochemical factors which govern the elimination of foreign organic compounds in adults.

It would not be reasonable to infer that the uptake of benzo(a)pyrene and Aroclor 1242 by eggs or sac-fry reached steady-state equilibrium over the course of the five day exposures in this study (Fig. 3-8). This would be consistent with the findings of Korn and Rice (1981) with coho salmon eggs which were exposed to toulene and naphthalene (1.8 PPM and 108 PPB respectively) and required 10 days to reach steady-state equilibrium. In contrast to the sac-fry data however, Korn and Rice (1981) reported that sac-fry reached steady-state equilibrium with toulene and naphthalene in three and ten hours respectively. The much longer time required in this present study (it was not reached over five days) may be correlated to the much lower water solubility of the benzo(a)pyrene and Aroclor 1242. This would be consistent with the uptake rate differences between toulene and naphthalene reported by Korn and Rice (1981) and the work of Kuhnhold and Busch (1978) who also reported that naphthalene was accumulated faster than

benzo(a)pyrene in salmon (Salmo salar) eggs.

Uptake rate constants (K1) and bioconcentration factors (BCF) determined at four different stages of trout development showed a clear increase (Table 1) in K1 and BCF with increasing age (over the periods tested). Only the 1.0 PPB Aroclor 1242 uptake patterns showed some deviation. As far as the author is aware, these increases in K1 and BCF within egg stages were the first reported. This increase was not originally anticipated. The conversion of lipid to more water soluble body components (with no net weight gain) and the presumed appearance of metabolising enzymes (Binder and Stegeman, 1980; 1982; 1983; 1984) with age would suggest a decrease in bioconcentration. This increase in bioconcentration and uptake may be explained by limited penetration of the egg by foreign organic compounds prior to development of the vascular system to the egg periphery. The extreme lipophilicity of benzo(a)pyrene and Aroclor 1242 would presumably cause adsorption in the lipid of the yolk. Saturation of binding sites at the egg periphery at the earliest stages (days 5-15 post-fertilization) with no mechanism for movement into the egg core may have resulted in lower BCF compared to older eggs with a developing vascular system. The vascular system would provide a mechanism to increase penetration of foreign organics thus increasing the overall burden to the egg. This hypothesis could be tested by autoradiography of exposed eggs.

Bioconcentration factors were observed within each egg

stage for benzo(a)pyrene to be an inverse function of the concentration in the water. The bioconcentration decreased between the 1.0 PPB exposure and the 10.0 and 100.0 PPB exposures (Table 1). It could be suggested that the high bioconcentration factor observed in the 1.0 PPB exposure may be due to non-saturation of binding sites within the egg, thus making BCF only a function of the rate of transport (passive) across the chorion. The near equal BCF observed between 10.0 and 100.0 PPB exposures could be related to both concentration in the water and competition for unsaturated binding sites, moderated by passive transport across the chorion. Mayer (1976) observed a constant decrease in bioconcentration with increasing concentration of exposure to di-(2-ethylhexyl)-phthalate in adult fathead minnows. Mayer hypothesized that this might have been due to the metabolism of the pollutant. The low cytochrome P-450 activity observed in this work (discussed later), especially at early egg stages, and those levels reported by Binder and Stegeman (1980; 1982; 1983; 1984) would argue against this hypothesis in eggs. More likely this process is passive, as suggested, and not active.

It is not possible to compare analagous egg data in the Aroclor 1242 exposures due to anamolous values recorded at 1.0 PPB uptake (Table 1). However, as noted in the benzo(a)pyrene data the 10.0 and 100.0 PPB exposures were similar. Thus it is possible that the same effect of uptake occurred with exposure to Aroclor 1242, but this is not

evident from the data. Observation of bioconcentration factors within sac-fry at different exposure concentrations, for benzo(a)pyrene, show a more constant decrease in BCF with increasing exposure concentration. These data are more analogous to those of Mayer (1976). However, caution should be excersised in interpeting the low BCF as the result of metabolism by embryos at this later stage of development. Observation of the analogous Aroclor 1242 data (sac-fry BCF against concentration) (Table 1) show a similar significant decrease in BCF at 100.0 PPB compared to 10.0 or 1.0 PPB exposures. The lack of metabolism of PCBs by trout (Hutzinger, 1972; Frederick, 1975) imply that metabolism is inconsequential and the decrease in BCF is not dependant upon it. Again, a passive mechanism may be suggested. The much greater uptake rates (K1) observed in sac-fry (Table 1) would greater accessability of the compounds (benzo(a)pyrene and Aroclor 1242) to binding sites. Thus the loss of available binding sites could be playing an important role in BCF at higher concentrations, as suggested by Zitko in 1979.

The largest and most consistent differences in K1, BCF, K2 and t1/2 in the data exist between egg and sac-fry stages for both benzo(a)pyrene and Aroclor 1242 (Table 1). Increases ranging from one-half to two orders of magnitude were observed in sac-fry for all kinetic parameters. This large increase is presumably due to the more active and efficient penetration of the compounds through the sac-fry

gills as compared to the egg chorion. This hatch effect has been observed by other researchers (Broyles and Noveck, 1979; Korn and Rice, 1981; Lockhart, unpublished). It should be noted however that for a few days after hatch the sac-fry obtains oxygen primarily through the highly vascularized sac (Knight, 1963). To avoid this complication in this study exposures of sac-fry were performed at least 15 days after hatch.

Of early life stages, sac-fry have generally been shown to be the most sensitive to toxic compounds in the water (McKim, 1977; Birge, et al. 1979). Their small size and presence of a lipid sac to act as an organic repository help to explain the rapid uptake of foreign compounds and theobserved poison sensitivity.

The distribution of accumulated organic compounds is reported to differ between sac-fry and eggs. Data obtained in this study indicate that greater than 80 percent of benzo(a)pyrene and 2,2',4,4',5,5' hexachlorobiphenyl (Table 2) accumulated in the yolk. This is consistent with the work of Broyles and Noveck (1977) who investigated partitioning of 2,2',5,5'-tetrachlorobiphenyl (TCB) in lake trout eggs at a 500 PPB exposure concentration. They found that 87-88 percent of the TCB was localised in the yolk. In contrast localisation in sac-fry similarily exposed, revealed that 70 percent of TCB was found in tissues other than yolk. This latter experiment was not repeated in this study.

Depuration rates in eggs observed in exposures to benzo(a)pyrene and Aroclor 1242 over all concentrations tested were low in comparison to sac-fry. The presence of the chorion and the partitioning of lipophilic organics to non-metabolic yolk suggest the slow depuration observed. This has been observed by other researchers (Broyles and Noveck, 1979; Korn and Rice, 1981; Solbakken et al. , 1984; Lockhart, unpublished). The increase in K2 as eggs develop may be due to loss by the vascular system of the foreign organic compound, and to a lesser extent to the loss of compound adsorbed to non-metabolic yolk. This is almost certainly the case in sac-fry and explains the biphasic nature of their depuration rates.

As noted previously, work of Broyles and Noveck (1979) show that 70 percent of TCB taken into sac-fry is present in the non-yolk tissues. Sac-fry depuration over time (Figs. 3-8 B) may be a function of this partitioning. It can be observed that the decrease in body burden is rapid over the first 48 hours, releasing 50-75 percent of the total accumulation. Following this period there is a slow rate of depuration. This may represent the rapid loss of accumulated organics from the low-lipid, metabolic organs of the sac-fry followed by the slow release of the foreign organic from the high-lipid yolk sac.

2.0 CHARACTERISATION OF THE CYTOCHROME P-450 ISOZYME(S)

2.1 Induction of cytochrome P-450 by benzo(a)pyrene: spectral analysis

Exposure of most species (vertebrate and invertebrate) to a wide variety of foreign organic compounds results the induction of the cytochrome P-450 mediated hydroxylation system (Gelboin, 1980; Stegeman et al., 1981; Addison et al., 1982). Quantitation of cytochrome P-450 levels in microsomes of many tissues (especially hepatic) can be accomplished through spectral analysis (Omura and Sato, 1964a & b). Induction of cytochrome P-450 in fish has been reported upon exposure to a variety of compounds such as DDT, Clophen A50 (a PCB mixture), and benzo(a)pyrene (Lidman et al., 1976; Ahokas et al., 1976; Gerhart and Carlson, 1978; Elcombe and Lech, 1979). Injection of arctic charr in this study (used here instead of rainbow trout because of optimal size availablility) with benzo(a)pyrene resulted in induction of cytochrome P-450 to levels detectable by spectral analysis (Figs. 9 and 10). This supports the premise underlying this overall work that cytochrome P-450 is present in fish microsomes, and is inducible.

2.2 The benzo(a)pyrene hydroxylase assay

Benzo(a)pyrene hydroxylase is a type of Aryl Hydrocarbon Hydroxylase (AHH) that belongs to a class of isozymes mediated by cytochrome P-450 (Gelboin, 1980). Determination of BPHase activity is usually performed by one of two general methods. The first technique utilizes the production of a highly flourescent metabolite 3-hydroxybenzo(a)pyrene. The activity of BPHase is determined as a function of the degree of flourescence recorded after incubations. This technique is generally sensitive to one pmol of product and was orginally described by Kuntzman et al. in 1966. The other technique (used in this study) is radiometric. Initially described by DePierre et al. (1975), this assay allows a measurement of all polar metabolites produced by BPHase. As the radiometric assay measures all metabolites produced, its sensitivity is dependant upon the specific activity of the substrate utilized. This can vary, with sensitivity usually between 40 and 100 pmol (Gelboin, 1980) but can be as sensitive as 1 pmol (Binder and Stegeman, 1984). In this study sensitivity reached 32 pmol.

The presumed low activity of BPHase in rainbow trout eggs stimulated the need to define the essential parameters of the assay to maximize observed activity. Several components of the assay were analysed to reveal their effect on the overall assay.

It was found that the storage of post-mitochondrial

homogenates on ice had an effect on BPHase activity with time (Fig. 11). A half-life of 31.5 hours was calculated from the decrease in activity, assuming first order kinetics. Therefore, to avoid any appreciable decrease in BPHase activity all homogenates prepared were utilized within 1-1.5 hours of preparation.

The effect of cofactor concentration on BPHase activity showed that an optimal concentration was being added to incubation media. A decrease in BPHase activity was only observed when the concentration was reduced. The concentration employed in this study was that suggested by DePierre et al. in 1975.

Laboratory procedures utilized Hepes/KCl buffer when preparing sub-cellular homogenates because of the low osmotic pressure which aided in disruption of cells. Tris/HCl buffer was routinely used in incubation media. As any incubation would require some degree of mixing these buffers, the effect that they exert on BPHase was determined. The finding that both buffers used together or separately had no effect on BPHase activity eliminated mixing as a possible source of error in the BPHase assay.

The time of incubation exerted a strong effect on observed BPHase activity (Fig. 12), and illustrated the major limitation of this assay. It can be seen (Fig. 12) that activity rates expressed per minute are an order of magnitude higher after one minute of incubation as compared to ten minutes. This change in activity is presumably due

to the extreme water insolubility of benzo(a)pyrene. This has also been reported by Hansen and Fouts (1972). The addition of benzo(a)pyrene to incubation media results in the majority of the compound immediately adsorbing to the glass or to non-hydroxylating sites on protein. Thus at a specific homogenate protein concentration available substrate is used within one minute of addition and little activity occurs thereafter. This theory is supported by additional work (not presented here) performed by the author in which substrate concentration was varied. No change in BPHase activity was observed between 10-150 uM BaP concentrations tested, at incubation times of 30 minutes.

As with the time of incubation the concentration of protein exerted an effect on BPHase activity (Fig. 13 a and b). Activity expressed per mg of protein (Fig. 13a) shows much higher activity (one order of magnitude) at 0.2 mg/ml as observed at 2.0 mg/ml. Presumably this was caused by the same factor as that thought to be operating on the incubation time, namely the solubility of the BaP substrate. It should be noted that over this range (0.2-2.0 mg/ml) the total amount of metabolite produced was the same (Fig. 13b). Thus, as the amount of protein increased over this range there was less substrate available for it to utilize causing a decrease in activity per unit of protein. This effect was not observed beyond 2.0 mg/ml where the total amount of metabolite produced were proportional to the protein concentration (Fig. 14b). This has also been observed by

Alvores et al. (1970), Hanson and Fouts (1972), and Hayakowa and Udenfriend (1973). The presumed increase in available substrate beyond 2.0 mg/ml was presumed due to the effect of the protein in solubilizing the substrate (Alvores et al. ,1970). Hansen and Fouts (1972) suggested that the substrate was not solubilised but rather bound to protein in such a manner that appreciable dissociation occured. In this study all assays were performed at 3.0 to 3.5 mg protein per ml of homogenate.

During the course of the study it was found that BPHase rates obtained after addition of the substrate dissolved in DMSO were consistently 30-35 percent of those obtained when acetone was used as a substrate carrier. This suggests that the substrate carrier used was exerting an effect on either the availability of substrate or on the activity of the isozyme(s). As benzo(a)pyrene has been shown to be the rate-limiting factor, the substrate carrier would appear to be affecting the availability of the substrate. It could be suggested that the acetone forms small micelles in the incubation medium as a function of its lack of polarity and in doing so solubilizes the benzo(a)pyrene within the micelle. DMSO however is quite polar and would dissolve completely in the medium providing no protection for the hydrophobic substrate. Additional evidence for the interaction of acetone and benzo(a)pyrene was obtained by examining the elution profile of aqueous solutions of benzo(a)pyrene, added to water by acetone or

DMSO (work by the author not presented here), from reverse phase SEP-PAKs (Waters and Associates). The SEP-PAK has a non-polar packing (octadecyl-silane) which would be expected to adsorb benzo(a)pyrene readily. This was in fact observed when aqueous solutions of benzo(a)pyrene are passed through it in which the BaP was added by DMSO. In contrast when aqueous solutions of BaP (added to solution by acetone) were passed through the SEP-PAK almost all of the BaP passed through the SEP-PAK without interacting with it.

As acetone did seem to exert an effect on BaP solubility the amount of acetone used was tested to see if it could be optimized. An increase in BPHase activity was observed at four percent (by volume, to incubation medium) concentration in which BPHase activity almost doubled (Fig. 15). Prior to this finding acetone was typically added to assays at a two percent concentration. All work reported in this study was carried out with a four percent acetone concentration.

To the best of the author's knowledge this effect has not been reported in the literature. Considering the limitation of this assay caused by the insolubility of the substrate it would be of benefit to utilize this acetone effect. The effect that acetone exerts in the BPHase assay may also be realized in other enzymatic assays limited by substrate solubility. In addition, this may have important implications in experiments determining the rate of uptake or lethality of a drug or organic pollutant. If acetone

makes the compound more water soluble the rate of uptake may be observed to increase. This effect was not tested in this study.

3.0 INDUCTION OF BENZO(a)PYRENE HYDROXYLASE IN EMBRYONIC RAINBOW TROUT BY AROCHLOR 1254

Direct analysis of cytochrome P-450 mediated hydroxylations during the early life stages in fish are almost completely lacking in the literature. To date, BPHase and its induction by Aroclor 1254 in the embryonic killifish (Fundulus heteroclitus) (Binder and Stegeman 1980; 1982; 1983) and in the brook trout (Salvelinus fontinalis) (Binder and Stegeman, 1983) have been described. The work presented in this study (Fig. 14) corresponded in overall nature with this previous work. BPHase activity levels observed during this study were an order of magnitude lower than those observed by Binder and Stegeman (1980; 1983; 1984). This would be expected as post-mitochondrial homogenates were used in this study while Binder and Stegeman were able to collect microsomal fractions.

Benzo(a)pyrene hydroxylase activity was observed to increase by nine fold and three fold in killifish and brook trout respectively at hatch (Binder and Stegeman, 1980; 1982). This is consistent with the findings of this study in which BPHase levels rose three to four fold at hatch. It

should be noted however that BPHase activity in pre-hatch rainbow trout was only observed two days prior to hatch, thus making the determination of pre-hatch activities tenuous. In contrast to the work of Binder and Stegeman (1982) with brook trout, a decrease in BPHase activity was observed after the hatch period in this study, however the BPHase rates did not return to pre-hatch levels. Binder and Stegeman (1982) did not report a decrease in activity after hatch. It should be noted however that they tested only two periods during post-hatch.

Induction of BPHase by Aroclor 1254 (Fig.14) is consistent with the work of Binder and Stegeman (1980 and 1983). The inability to distinquish between BPHase rates in eggs exposed to 100 and 200 PPB Aroclor 1254 was also observed by Binder and Stegeman (1982) in brook trout. It contrasts however with earlier work of Binder and Stegeman (1980) where killifish did show dose-dependant induction.

Indirect determination of cytochrome P-450 mediated hydroxylations in brook trout eggs and fry were reported by Atchison and Johnson in 1975. Adult brook trout were fed p,p'-DDT over a period of time and the eggs obtained from these animals were fertilized. Of the amount of DDT present in the eggs at fertilization they found that 35 percent had been converted to DDE and DDD over a 72 day period. The work presented in this study, and that of Binder and Stegeman (1980; 1983) support the hypothesis that the DDT was metabolised enzymatically.

Although BPHase activity in the embryo is high during hatch (about 50 percent of the level found in adult liver) this study gave little evidence to conclude that it plays a significant role in depuration of the foreign organic compounds. The similarity of the elimination rate constants between benzo(a)pyrene and Aroclor 1242 (which is not metabolised) suggest that some other mechanism is primarily responsible for depuration during early life stages. Metabolites of benzo(a)pyrene may not have been in sufficient quantity during the exposure experiments to cause a significant change in exposure kinetics or they may have been bound within the animal and not excreted. It should be noted that measurement of 14-C on oxidised samples does not discriminate parent compound from possible metabolites. No information has been published with regard to the development of Phase Two elimination enzymes (those that work by conjugation after Phase One hydroxylation) in embryonic fish. Thus it is currently impossible to assess the role that these play in the metabolic elimination at the early life stages. From the present work, however, it appears that the enzymatic hydroxylation represented by BPHase are not the rate-limiting component in clearance of a compound such as benzo(a)pyrene.

LITERATURE CITED

Addison.R.F., M.E. Zinck, D.E. Willis, and J.J. Wrench. 1982. Induction of hepatic mixed function oxidase activity in trout(Salvelinus fontinalis) by Aroclor 1254 and some aromatic hydrocarbon PCB replacements. Toxicol. Appl. Pharmacol. 63: 166-172.

Alvares, A.P., G. Schilling, A. Garbut, and R. Kuntzman. 1970. Studies on the hydroxylation of 3,4-Benzpyrene by hepatic microsomes: Effect of albumin on the rate of hydroxylation of 3,4-benzpyrene. Biochem. Pharmacol. 19: 1449-1455.

Atchison, G.L., and H.E. Johnson. 1975. The degradation of DDT in brook trout eggs and fry. Trans. Am. Fish. Soc. 4: 782-784.

Binder, R.L., and J.J. Stegeman. 1980. Induction of aryl hydrocarbon hydroxylase activity in embryos of an estuarine fish. Biochem. Pharmacol. 29: 949-951.

Binder, R.L., and J.J. Stegeman. 1982. The ontogeny of aryl hydrocarbon hydroxylase activity and associated electron transport activities during embryonic development in an estuarine fish. Fed. Proc. Fed. Amer. Soc. Exp. Biol. 41: 1560.

Binder, R.L., and J.J. Stegeman. 1983. Basal levels and induction of hepatic aryl hydrocarbon hydroxylase activity during the embryonic period of development in brook trout. Biochem. Pharmacol. 32(7): 1324-1377.

Binder, R.L., and J.J. Stegeman. 1984. Microsomal electron transport and xenobiotic monoxygenase activities during the embryonic development in the killifish, Fundulus heteroclitus. Toxicol. Appl. Pharmacol. 73: 432-443.

Birge, W.J., J.A. Black, J.E. Hudson, and D.M. Bruser. 1979. Embryo-larval toxicity tests with organic compounds. In Aquatic Toxicology, ASTM STP 667, L.L. Marking and R.A. Kimerle, Eds., American Society for Testing and Materials. pp. 131-147.

Branson, D.R., G.E. Blau, H.C. Alexander, and W.B. Neely. 1975. Bioconcentration of 2,2',4,4'-tetrachlorobiphenyl in rainbow trout as measured by an accelerated test. Trans. Am. Fish. Soc. 4: 785-792.

Broyles, R.H., and M.I. Noveck. 1979. Uptake and distribution of 2,5,2',5'-tetrachlorobiphenyl in developing lake trout. Toxicol. Appl. Pharmacol. 50: 291-298.

Creaven, P.J., P.V. Parke, and R.T. Williams. 1965. A flourimetric study of the hydroxylation of biphenyl in vitro by liver preparations of various species. Biochem. J. 96: 870.

DeFoe, D.L., G.D. Veith, and R.W. Carlson. 1978. Effects of Aroclor 1248 and 1260 on the fathead minnow (Pimephales promelas). J. Fish. Res. Board Can. 35: 997-1002.

De Pierre, J.W., M.S. Moron, K.A.M. Johannesen, and L. Ernster. 1975. A reliable, sensitive, and convenient radioactive assay for Benzo(a)pyrene monooxygenase. Anal. Biochem. 63: 470-484.

Elcombe, C.R., and J.J. Lech. 1979. Induction and characterization of hemoprotein(s) P-450 and monooxygenation in rainbow trout(Salmo gairdneri). Toxicol. Appl. Pharmacol. 49: 437-450.

Ernst, W. 1977. Determination of the bioconcentration potential of marine organisms: A steady state approach. I. Bioconcentration data for seven chlorinated pesticides in mussels (Mytilus edulis) and their relation to solubility data. Chemosphere 11: 731-740.

Essner, H.O., and P. Moser. 1982. An appraisal of the measurement and evaluation of bioaccumulation. Ecotoxicol. Environ. Safety 6: 131-148.

Frederick, L.L. 1975. Comparative uptake of a polychlorinated biphenyl and dieldrin by the white sucker (Catastomus commersoni). J. Fish. Res Board Can. 32: 1705-1709.

Forlin, L., and U. Lindman. 1978. Effects of Clophen A50, 4-2,5,2',5'-tetra- and 2,4,5,2',4',5'-hexa-chlorobiphenyl on the mixed function oxidase system of rainbow trout (Salmo gairdneri Richardson) liver. Comp. Biochem. Physiol. 60C: 193-197.

Gelboin, H.V. 1980. Benzo(a)pyrene metabolism, activation, and carcinogenesis: The role and regulation of mixed-function oxidases and related enzymes. Physiological Reviews 60: 1107-1166.

Gerhart, E.H., and R.M. Carlson. 1978. Hepatic mixed-function oxidase in rainbow trout exposed to several polycyclic aromatic compounds. Environ. Res. 17: 284-295.

Gooch, J.A. and M.K. Hamdy. 1982. Depuration and biological half-life of 14C-PCB in aquatic organisms. Bull. Environm. Contam. Toxicol. 28: 305-312.

Halter, M.T. and H.E. Johnson. 1974. Acute toxicities of a polychlorinated biphenyl (PCB) and DDT alone and in combination to early life stages of coho salmon (Oncorhynchus kisutch). J. Fish. Res. Board Can. 31: 1543-1547.

Hamelink, J.L. 1977. Current bioconcentration test methods and theory. Aquatic toxicology and hazard evaluation, ASTM STP 634, F.L. Mayer and J.L. Hamelink, Eds., ASTM. pp. 149-161.

Hanson, A.R., and J.R. Fouts. 1972. Some problems in Michaelis-Menton kinetic anaylsis of benzpyrene hydroxylase in hepatic microsome from polycyclic hydrocarbon pretreated animals. Chem.-Biol. Interactions 5: 167-182.

Hayakana, T., and S. Udenfriend. 1973. A simple radioisotope assay for microsomal aryl hydrocarbon hydroxylase. Anal. Biochem. 51: 501-509.

Hinz, R., and F. Matsumura. 1977. Comparative metabolism of PCB isomers by three species of fish and the rat. Bull. Environ. Contam. Toxicol. 18: 631-639.

Hodgson, E., and F. Guthrie. 1980. Introduction to biochemical toxicology. Elsevier North Holland, Inc., New York. pp. 437.

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

Hutzinger, O., D.M. Nash, S. Safe, A.S.W. DeFreitas, R.S. Norstrom, D.J. Wildish, and V. Zitko. 1972. Polychlorinated biphenyls: metabolic behaviour of pure isomers in pigeons, rats, and brook trout. Science 178: 312-314.

Kang, Y.H., M.T. Bui-Tran, W.L. West. 1982. Benzo(a)pyrene induced cytopathologic changes and peroxidase activity in the endometrium of ovariectomized rats. Toxicol. Appl. Pharmacol. 63: 242-256.

Kleinbaum, D.G. and L.L. Kupper. 1978. Applied regression analysis and other multivariate methods. Duxbury Press, North Scituate, Massachusettes. pp. 556.

Klingenberg, M. 1958. Pigments of rat liver microsomes. Arch. Biochem. Biophys. 75: 376-386.

Knight, A.E. 1963. The embryonic and larval development of the rainbow trout. Trans. Am. Fish. Soc. 92: 344-356.

Korn, S. and S. Rice. 1981. Sensitivity to, and accumulation and depuration of, aromatic petroleum components by early life stages of coho salmon (Oncorhynchus kisutch). Rapp. P. U. Reun. Cons. Int. Explor. Mer., 1978: 87-92.

Kuntzman, R., L.C. Mark, L. Brand, M. Jacobson, W. Levin, and A.H. Conney. 1966. Metabolism of drugs and carcinogens by human liver enzymes. J. Pharmacol. Exp. Ther. 152: 151-156.

Linden, O. 1974. Effects of oil spill dispersants on the early development of Baltic herring. Ann. Zool. Fennici. 14: 141-148.

Lidman, U., L. Forlin, O. Molander, and G. Axelson. 1976. Induction of the drug metaboliaing system in rainbow trout(Salmo gaidneri) liver by polychlorinated biphenyls (PCBs). Acta. Pharmacol. et Toxicol. 39: 262-272.

Loomis, T.A. 1974. Essentials of toxicology. 2nd ed. Lea and Febiger, Philadelphia. pp. 223.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

MacKay, D. 1982. Correlation of bioconcentration factors. Environ. Sci. Technol. 16: 274-278.

Maki, A.W. 1979. Respiratory activity of fish as a predictor of chronic fish toxicity values for surfactants. In Aquatic Toxicology ASTM ATP 667, L.L. Marking and R.A. Kimerle, Eds., American Society for Testing and Materials. pp. 77-95.

Mallon, B.J. and F.L. Harrison. 1984. Octanol-water partition coefficient of benzo(a)pyrene: measurement, calculations, and environmental implications. Bull. Environ. Contam. Toxicol. 32: 316-323.

Mauck, W.L., P.M. Mehrle, and F.L. Mayer. 1978. Effects of the polychlorinated biphenyl Aroclor 1254 on growth, survival, and bone development in brook trout. J. Fish. Res. Board Can. 35: 1084-1088.

Mayer, F.L. 1976. Residue dynamics of di-2-ethylhexyl phthaliate in fathead minnnows (Pimephales promelas). J. Fish. Res. Board Can. 33: 2610-2613.

McKim, J.M. 1977. Evaluation of tests with early life stages of fish for predicting long-term toxicity. J. Fish. Res. Board Can. 34: 1148-1154.

Melancon, M.J., and J.J. Lech. 1976. Isolation and identification of a polar metabolite of tetrachlorobiphenyl from bile of a rainbow trout exposed to 14C-tetrachlorobiphenyl. Bull. Environ. Contam. Toxicol. 15: 181-188.

Neely, W.B., D.R. Branson, and G.E. Blaw. 1974. Partition coefficient to measure bioconcentration potential of organic chemicals in fish. Environ. Sci. Technol. 8(13): 1113-1115.

Neff, J.M., B.A. Cox, D. Dixit, and J.W. Anderson. 1976. Accumulation and release of petroleum-derived aromatic hydrocarbons by four species of marine animals. Mar. Biol. 38: 279-289.

Omura, T. and R. Sato. 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239: 2370-2378.

Omura, T. and R. Sato. 1964. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. J. Biol. Chem. 239: 2379-2385.

Passino, D.M. and J.M. Kramer. 1980. Toxicity of arsenic and PCBs to fry of deepwater ciscoes (Coregonus sp.). Bull. Environ. Contam. Toxicol. 24: 527-534.

Roubal, W.T., T.K. Collier, and D.C. Malins. 1977. Accumulation and metabolism of carbon-14 labeled benzene, naphthalene, and anthracene by young coho salmon. Arch. Environm. Contam. Toxicol. 5: 513-529.

Safe, S. 1984. Polychlorinated biphenyls (PCBs) and polybrominated bihenyls (PBBs): Biochemistry, toxicology, and mechanism of action. CRC Critical Reviews in Toxicology. 13(4): 319-395.

Schimmel, S.C., D.J. Hanson, and J. Forester. 1974. Effects of Aroclor 1254 on laboratory-reared embryos and fry of sheepshead minnows (Cyprinodon variegatus). Trans. Amer. Fish. Soc. 3: 582-586.

Smith, R.L. and J.A. Cameron. 1979. Effect of the water soluble fraction of Prudhoe Bay crude oil on embryonic development of Pacific herring. Trans. Am. Fish. Soc. 108: 70-75.

Solbakken, J.E., S. Tilseth, and K.H. Palmork. 1984. Uptake and elimination of aromatic hydrocarbons and a chlorinated biphenyl in eggs and larvae of cod (Gadus morhua). Mar. Eco. 16: 297-302.

Stegeman, J.J., A.V. Klotz, B.R. Woodin, and A.M. Pajor. 1981. Induction of hepatic cytochrome P-450 in fish and the indication of environmental induction in scup (Stenotomus chrysops). Aquatic Toxicol. 1: 197-212.

Stross, 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 toxicant concentration. Environ. Pollut. 20: 139-148.

Ueda, K. 1976. Toxicolgical aspects I: The toxicity of PCBs. In: PCB poisoning and pollution. K. Huguchi ed. Academic Press, New York. pp. 25-40.

Van Cantfort, J., J. DeGraeve, and J.E. Gielen. 1977. Radioactive assay for aryl hydrocarbon hydroxylase, improved method and biological importance. Biochem. Biophys. Res. Commun. 79: 505-512.

Vandermeulen, J.H. and W.R. Penrose. 1978. Absence of aryl hydrocarbon hydroxylase (AHH) in three marine bivalves. J. Fish. Res. Board Can. 35: 643-647.

Weiss, P. and J.J. Weis. 1982. Toxicity of the PCBs Aroclor 1254 and 1242 to embryos and larvae of the mummichog, Fundulus heteroclitus. Bull. Environ. Contam. Toxicol. 28: 298-304.

Wilson, K.W. 1976. Effects of oil dispersants on the developing embryos of marine fish. Marine Biology 36: 259-268.

Woltering, D.M. 1984. The growth response in fish chronic and early life stage toxicity tests: A critical review. Aquatic Toxicology. 5: 1-21.

Yoshimura, H. and S. Yamamoto. 1976. Toxicological Aspects. II. The metabolic fate of PCBs and their toxicological evaluation. In PCB Poisoning and Pollution, K. Higuchi, Ed., Kodansha Ltd., Tokyo/Academic Press, New York. pp. 41-67.

Zitko, V. 1979. Relationships governing the behaviour of pollutants in aquatic ecosystems and their use in risk assessment. Can. Tech. Rep. Fish. Aquat. Sci. 975: 243-265.

Zitko, V. and R.L. Saunders. 1979. Effect of PCBs and other organochlorine compounds on the hatchability of Atlantic salmon (Salmo salar) eggs. Bull. Environm. Contam. Toxicol. 21: 125-130.

Appendix 1a: Water concentrations (in PPB) of benzo(a)pyrene during five day uptake periods over four stages of development in rainbow trout.

BENZO(a)PYRENE

Day 5-15 (egg)				Day 16-26 (egg)			
Time (days)	1 PPB	10 PPB	100 PPB	Time (days)	1 PPB	10 PPB	100 PPB
0	0	0	0	0	0	0	0
1	0.5	5.9	46.8	1	0.5	5.1	52.6
2	0.5	5.8	46.6	2	0.6	5.1	52.0
3	1.0	5.1	80.1	3	0.6	4.0	54.0
4	0.9	6.9	65.0	4	0.6	6.3	55.9
5	0.5	6.1	70.7	5	0.6	6.5	52.7

Day 27-37 (egg)				Day 50-60 (sac-fry)			
Time (days)	1 PPB	10 PPB	100 PPB	Time (days)	1 PPB	10 PPB	100 PPB
0	0	0	0	0	0	0	0
1	0.4	5.5	59.1	1	0.6	7.5	97.8
2	0.7	6.6	72.9	2	0.8	8.3	131.1
3	0.6	5.1	123.9	3	0.9	8.2	99.5
4	0.7	6.3	75.8	4	0.8	10.0	98.7
5	0.7	7.6	75.8	5	0.8	8.2	99.4

Appendix 1b: Water concentrations (in PPB) of Aroclor 1242 during five day uptake periods over four stages of development in rainbow trout.

AROCLOR 1242

Day 5-15 (egg)				Day 16-26 (egg)			
Time (days)	1 PPB	10 PPB	100 PPB	Time (days)	1 PPB	10 PPB	100 PPB
0	0	0	0	0	0	0	0
1	0.4	5.2	51.5	1	0.7	5.7	53.6
2	0.6	4.8	52.8	2	0.4	4.1	56.1
3	0.6	6.7	55.1	3	0.5	4.3	53.9
4	0.5	5.1	57.2	4	0.6	6.7	26.2
5	0.5	5.3	48.2	5	0.6	5.9	52.2

Day 27-37 (egg)				Day 50-60 (sac-fry)			
Time (days)	1 PPB	10 PPB	100 PPB	Time (days)	1 PPB	10 PPB	100 PPB
0	0	0	0	0	0	0	0
1	0.4	7.4	57.7	1	0.5	5.2	58.4
2	0.5	6.2	83.0	2	0.5	5.8	53.8
3	0.6	5.8	64.7	3	0.5	5.7	53.1
4	0.6	5.2	61.1	4	0.6	6.7	57.3
5	0.6	8.8	50.6	5	0.5	5.7	53.1