AN ABSTRACT OF THE THESIS OF

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Title: EXPOSURE OF COHO SALMON (ONCORHYNCHUS KISUTCH) TO PETROLEUM HYDROCARBONS: EFFECT ON LIPID METABOLISM AND ARYL HYDROCARBON HYDROXYLASE ACTIVITY

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R. S. Caldwell

Coho salmon (Oncorhynchus kisutch) were exposed to sublethal levels of a water-soluble fraction (WSF) of Cook Inlet crude oil for periods ranging from 18 to 31 days, at four acclimation temperatures. The fatty acid composition of phospholipids extracted from muscle, gill, liver, and brain tissues, and, at three of the temperatures, the activity of the hepatic microsomal aryl hydrocarbon hydroxylase (AHH) system in the salmon, were determined.

Induction of the AHH system due to exposure of the fish to the toxicant was observed in the warm acclimated (20.0 C) fish only. With minor exceptions, the fatty acid composition of phospholipids in all of the tissues analyzed, including those from the warm acclimated fish, were unaltered by exposure of the fish to the crude oil WSF. In both control and exposed fish, AHH activity was greater in fish acclimated to 7.8 C than to 20.0 C. The phospholipids from both exposed and control cold acclimated fish contained a higher percentage of polyunsaturated fatty acids than the phospholipids from the corresponding warm acclimated fish.

The studies reported here indicate that exposure of coho salmon (O. kisutch) to sublethal levels of petroleum hydrocarbons does not appreciably alter the fatty acid composition of tissue phospholipids or affect the normal change in fatty acid composition associated with a change in acclimation temperature.

The data obtained are insufficient to permit any conclusion regarding the possibility of an interaction between the MFO system and the metabolsim of polyunsaturated fatty acids in oil exposed salmon.

Exposure of Coho Salmon (*Oncorhynchus kisutch*) to Petroleum Hydrocarbons: Effect on Lipid Metabolism and Aryl Hydrocarbon Hydroxylase Activity

By

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INTRODUCTION

In recent years, organisms have been increasingly subjected to organic pollutants which enter the ecosystem through a variety of pathways such as urban and industrial use, wastes, and accidents. Those foreign organic compounds (xenobiotics) which are taken up by the organisms must be excreted. Water soluble xenobiotics are easily excreted; however, the lipophilic compounds must first be converted to more polar forms (Ullrich and Kremers 1977). Mammals have a membrane bound, multicomponent enzyme system which is capable of catalyzing the transformation of both endogenous compounds and foreign organic compounds of widely differing chemical structures to more polar forms (Nebert et al. 1975). This system, called the monooxygenase or mixed function oxidase (MFO) system, is structurally and functionally very complex and has not yet been fully characterized (Nebert et al. 1975).

With most xenobiotics, the metabolic derivatives of this enzyme system are less toxic to the animal than the parent compound; however, some compounds become activated by the MFO system e.g. the desulfuration of malathion to malaoxon and the epoxidation of aldrin to dieldrin (Chambers and Yarbrough 1976, Krueger and O'Brien 1959). Some xenobiotics have been shown to inhibit or have no effect upon the MFO system while others can cause induction, as measured by an increase in the *in vitro* rate of transformation of the substrate (Nebert et al. 1975).

In mammals, the metabolism of foreign polycyclic aromatic hydro-

carbons is catalyzed by the mixed function oxidase enzyme system termed the aryl hydrocarbon hydroxylase (AHH) system (Sims and Grover 1974). There has been much concern with this enzyme system because it is thought that epoxides are the primary products of metabolism (Sims and Grover 1974). Some epoxides are chemically reactive and may interact with genetic material and pose a mutagenic or carcinogenic threat to the organism (Sims and Grover 1974). Many epoxides are capable of undergoing further metabolism to form compounds such as phenols, dihydrodiols, or to form conjugated derivatives which are more easily excreted (Figure 1) (Nebert et al. 1975).

Fish have an MFO system which is comparable to that found in mammals (Ahokas et al. 1976, Bend et al. 1977a, Pohl et al. 1974). It is a multicomponent electron transport system that requires: cytochrome P450 which serves as the terminal oxidase; a reduced pyridine nucleotide; and molecular oxygen. This system is inhibited by carbon monoxide, and is capable of catalyzing reactions such as the hydroxylation of aniline, aldrin epoxidation, hydroxylation of benzo(a)pyrene, and N-demethylation of aminopyrine (Buhler and Rasmusson 1968, Burns 1976, Payne and Penrose 1975). There is also a liped requirement for the transfer of electrons to cytochrome P450. Using a reconstituted liver microsomal enzyme system, Strobel et al. (1970) determined phosphatidylcholine to be the essential lipid in mammals.

In fish, the liver microsomal fraction demonstrates the greatest MFO activity, although activity is found in other tissues as well (Pederson et al. 1974, Stegeman et al. 1979). It was originally thought that even under optimum conditions, fish had lower enzyme activities

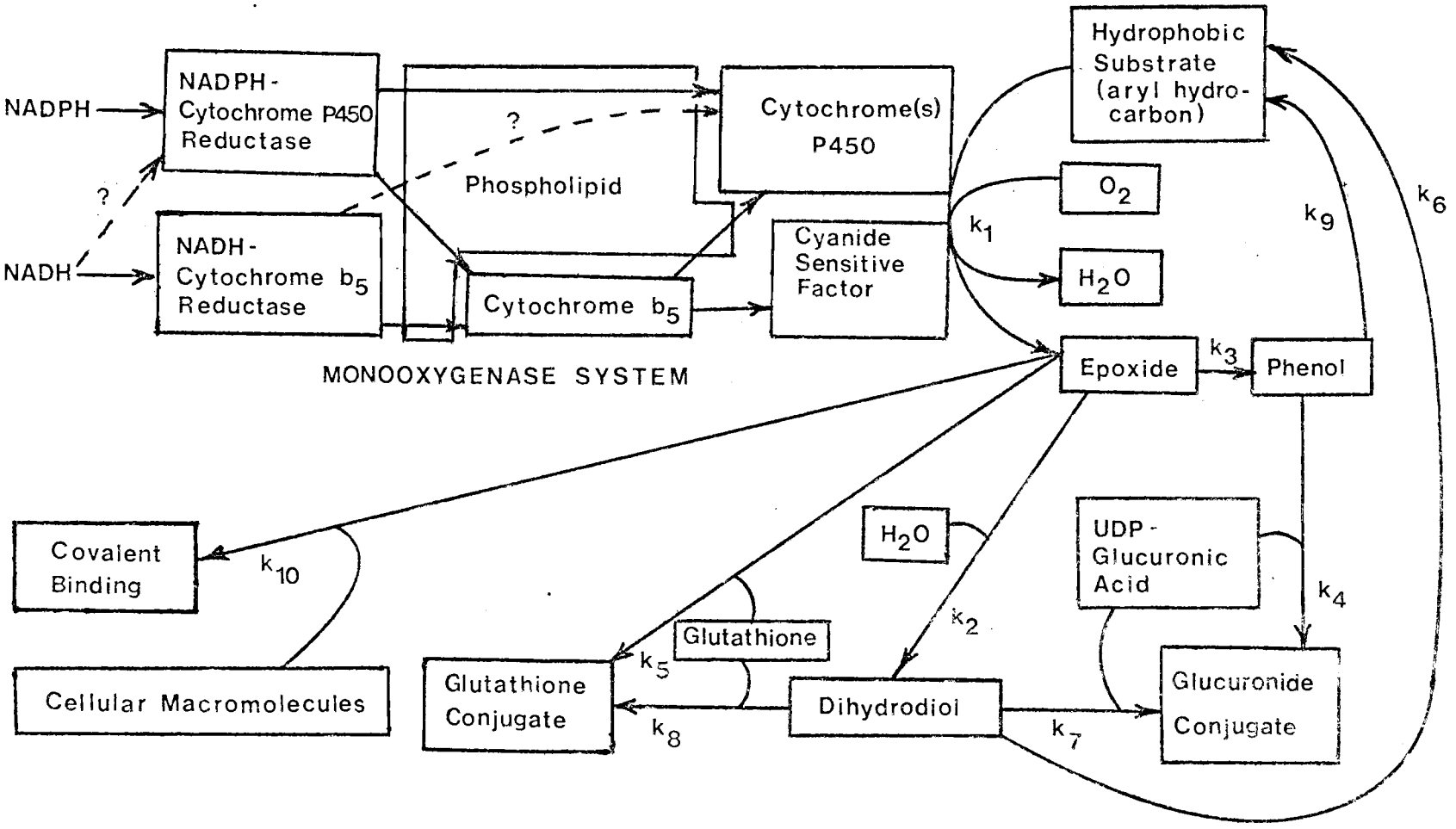

Figure 1. Possible pathways involved in the oxidative metabolism of aryl hydrocarbons (ref. Nebert et al. 1975). For any given substrate the relative rates of k_1 through K_{10} are not known.

than mammals; however, more recent studies have found untreated brown trout (Salmo trutta) livers to contain five to ten times the AHH activity found in male rat liver (Ahokas et al. 1975) and untreated scup (Stenotomus versicolor) to contain ten times more benzo(a)pyrene hydroxylase activity than mice (Stegeman and Binder 1979). The optimum in vitro incubation temperature for fish MFO enzymes is lower than for mammalian MFO systems (Buhler and Rasmusson 1968; Clark and Diamond 1977, Dewaide 1970).

In recent years, there has been a growing interest in the effects of petroleum hydrocarbons on organisms and ecosystems due to the increasing amount of petroleum entering the aquatic environment. Researchers have found that many fish demonstrate induction of the AHH system upon exposure to oil. This has been shown in brown trout (S. trutta) from a petroleum contaminated lake (Payne and Penrose 1975), in brown trout and capelin (Mallotus villosus) after exposure to a crude oil emulsion in the laboratory for 17 days (Payne and Penrose 1975), in young coho salmon (Oncorhynchus kisutch) after six days of exposure to a 150 ppb water-soluble fraction of crude oil (Grueger et al. 1977), and in fish taken from a natural oil spill site (Kurelec et al. 1977).

It is thought that induction of the fish AHH system during hydrocarbon exposure results in an increase in the in vivo metabolism of petroleum hydrocarbons. Statham et al. (1978) determined that pretreatment of rainbow trout (Salmo gairdneri) with an AHH inducer resulted in an increase in the metabolism and biliary excretion of 2-methylnaphthalene in vivo. However, the significance to the fish of this increase in metabolism of the petroleum hydrocarbons has not been determined. If

the epoxide metabolizing enzymatic activities are not also increased, there might actually be a greater risk of carcinogenicity or mutagenicity to the fish. Bend et al. (1977b) determined that several marine teleosts contained microsomal epoxide hydrase activity and glutathione-S-transferase activity with different oxide substrates. These activities are generally a detoxication reaction in mammals. However, after induction of AHH activity in the marine fish Archosargus probatocephalus by 3-methylcholanthrene (3MC) they did not find a concomitant induction of these detoxication reactions.

Due to the increasing frequency and severity of oil spills in the oceans, it has become important to determine the effect of petroleum hydrocarbons which can be dissolved in seawater on marine organisms. The water-soluble fraction (WSF) of petroleum is a very complex mixture of hydrocarbons; however, it is generally agreed that the soluble aromatic hydrocarbons are the most toxic components (Anderson et al. 1974b, Craddock 1977, Rice et al. 1979). The concentration of specific aromatic hydrocarbons in a WSF is a function of their concentration in the parent crude oil and their solubility in water (Anderson et al. 1974b).

Most of the AHH activity measurements in marine fish exposed to oil have been based on the in vitro metabolism of a single substrate, such as benzo(a)pyrene, thus the specific compounds in the WSF responsible for induction of the AHH system have not been identified nor has the capability of the fish to metabolize the complex mixture of hydrocarbons been determined. However, researchers have determined that marine fish are capable of metabolizing and excreting at least some of the hydrocarbons usually found in a WSF of crude oil. Lee et al. (1972)

traced the uptake of ^{14}C-naphthalene and ^{3}H-benzo(a)pyrene in three species of marine fish. He found an initial rapid increase in radioactivity in the liver, gut, gill, and brain tissues, and later in the gall bladder. This was followed by an increase in hydroxylated or conjugated derivatives of the parent compounds in the liver, gall bladder, and urine, Roubal et al. (1977) found similar results after administering ^{14}C labeled benzene, naphthalene and anthracene to young coho salmon (O. kisutch) in the food and by intraperitoneal injection. Discontinuing the administration of hydrocarbons usually results in a rapid depuration of the parent compounds and their derivatives from the fish tissues (Anderson et al. 1974a, Lee et al. 1972). Roubal et al. (1977, 1978) found that some aromatic hydrocarbons can be stored in coho salmon tissue for extended periods of time, with the retention of the hydrocarbons increasing as the number of aromatic rings increases.

Research is continuing on the AHH system to better characterize its components and electron pathways. There is evidence indicating that multiple forms of cytochrome P450 exist and each may have a different substrate specificity (Nebert et al. 1975, Ullrich and Kremers 1977). In mammals, factors such as sex, age, diet, and environmental temperature, can effect MFO induction (Conney 1967). Unfortunately, the effect of these parameters on fish has not been studied.

Another hepatic microsomal electron transport system is the fatty acid desaturase system. In mammals, this system has been directly linked to the $\Delta 9$ fatty acyl CoA desaturase pathway and possibly the $\Delta 6$ pathway (Schenkman et al. 1976). This multi-component system contains a hemoprotein (cytochrome b_5) and requires a reduced pyridine nucleotide

and oxygen as co-factors. A non-heme iron protein, called the cyanide sensitive factor, is the terminal oxidase in the pathway and is probably the desaturase (Schenkman et al. 1976). There is also a lipid requirement for electron transfer from NADH-cytochrome b_5 reductase to cytochrome b_5 (Schenkman et al. 1976). This system has not been fully characterized in mammals and even less is known about its components in fish; however, it is believed that the pathway of synthesis of polyunsaturated fatty acids (PUFA) by a series of elongation and desaturation reactions is very similar in both groups (Brenner et al. 1963, Reiser et al. 1963).

It is not clear how the fatty acid desaturase and MFO system interact with each other; however, it appears that interactions between the two systems do exist. Jansson and Schenkman (1975) determined that feeding rats a high carbohydrate diet caused a large increase in stearyl CoA desaturation ($\Delta 9$) and a decrease in MFO activity as measured by the in vitro rate of aminopyrine demethylation. Injection of the rats with phenobarbital induced MFO activity but caused a 50% decrease in stearyl-CoA desaturase activity. Fasting of the rats for 48 hours decreased the desaturase activity by 60-75% but did not affect the rate of aminopyrine demethylation. Oshino and Sato (1972) have found similar results. Upon refeeding of starved rats, the expected induction of stearyl-CoA desaturase was accompanied by a decrease in the level of cytochrome P450 by as much as 40% and the hydroxylation of aniline (an MFO reaction) was decreased to an even greater extent. Starved rats injected daily for four days with an MFO inducer (phenobarbital) exhibited a four-fold increase in cytochrome P450, a two-fold increase in cytochrome b_5, but

less than half the ability to induce stearyl-CoA activity upon refeeding (Oshino 1972). One explanation for these observations is that the synthesis of the cyanide sensitive factor and cytochrome P450 may be competitive.

Ninno et al. (1974) found that the administration of 150 ppm dieldrin (an MFO inducer) in the diet of the rats resulted in a substantial and persistent increase in the activity of the hepatic microsomal Δ9 desaturation of palmitic acid (16:0). The same treatment resulted in a transient 50% increase in the Δ5 desaturation of eicosa-8,11-dienoic acid (20:2ω9) and eicosa-8,11,14-trienoic acid (20:3ω6), followed by a slight inhibition by the seventh day of the experiment. The rate of Δ6 desaturation of linoleic acid (18:2ω6) and linolenic acid (18:3ω3) was relatively unaffected. In contrast to the studies by Oshino (1972) and Jansson and Schenkman (1975), these results suggest that the Δ9 desaturation system and MFO system were co-induced.

It has been repeatedly shown that the fatty acid composition of fish lipids is altered in a predictable way as a result of changes in the environmental temperature; cold adapted organisms have a higher degree of lipid unsaturation than those that are warm adapted. Caldwell and Vernberg (1970) found an increase in the level of polyunsaturated fatty acids in gill mitochondria from cold acclimated goldfish (Carassius auratus) and yellow bullheads (Ictalurus natalis) and a decrease in the levels of saturated and monounsaturated fatty acids when compared to warm acclimated fish. Similar changes were noted in the fatty acid pattern of lipids from cold and warm acclimated rainbow trout (S. gairdneri) (Knipprath and Mead 1965), mosquito fish (Gambusia affinis) and

guppies (Lebistes reticulatus) (Knipprath and Mead 1966) and goldfish brain (Johnston and Roots 1964) and intestines (Kemp and Smith 1970).

Not only do total lipids show this pattern of change with temperature but also the phospholipids, which are one of the main components of membranes. Caldwell et al. (1979) found a significant increase in the level of phospholipid PUFA in sculpins (Leptocottus armatus) acclimated to 7.0 C when compared to fish acclimted to 20.0 C. It is now generally accepted that these changes in lipid composition perform an essential role in preserving specific biophysical properties of biological membranes during periods of temperature change, and that the control of these properties is essential for maintaining optimal membrane function (Hazel 1973, Prosser 1973).

The mechanisms responsible for the change in the fatty acid composition of phospholipids from thermally acclimating fish has not been determined. The possibilities include: selection of the kinds of phospholipids incorporated into biomembranes; selection of specific fatty acids incorporated into membrane phospholipids; or a change in the composition of the free fatty acid pool available for incorporation into membrane lipids i.e. an increase in the rate of desaturation of the available fatty acids.

Recent experiments indicate that the latter mechanism may be involved. Kayama et al. (1963) injected methyl (1-C^{14}) linolenate into kelp bass acclimated to 18 C, then transfered the fish to 9.6 C for 5.5 hours. He found that the radiolabled linolenic acid was converted to eicosapentaenoic acid (20:5ω3), which was then incorporated in 22:6ω3. Ninno et al. (1974) found a two-fold increase in the rates of Δ6, Δ9,

and Δ5 desaturations in liver microsomes of the fish Pimelodus maculatus acclimated to 15.6 C for three weeks when compared to fish acclimated to 29.2 C. Reiser et al. (1963) determined that when fish containing a high initial level of 18:2ω6 and 18:3ω3 were placed on a 20% ethyl myristate-ethyl laurate diet there was a significant conversion of 18:2ω6 and 18:3ω3 to more highly unsaturated fatty acids at 13 C. However, when these fish were acclimated to 23 C the lauric and myristic fatty acids were deposited and there was little change in the levels of 18:2ω6 and 18:3ω3. DeTorrengo and Brenner (1976) found significant increases in rates of desaturation and chain elongation activities in liver microsomes of the fish P. maculatus acclimated to 14 C for three weeks when compared to fish acclimated to 29C. They felt that the increase in the rate of Δ6 desaturation of oleic, linoleic, and linolenic acids at the lower acclimation temperature was due to an increase in the amount of enzyme; however, they also found a decrease in the specific reaction rates of the desaturases at that temperature, making the overall effect on the fatty acid composition questionable.

Caldwell et al. (1979) determined that staghorn sculpin (L. armatus) acclimated to both 7.0 C and 19.2 C demonstrated altered whole body phospholipid fatty acid patterns 12 days after injection with Aroclor 1254, a potent MFO inducer in animals. The authors felt that the changes in the fatty acid composition of the fish after treatment with the Aroclor 1254 may have been a result of interactions between the MFO and fatty acid desaturase systems, though the paper did not present any direct evidence for induction of the MFO system or changes in fatty acid desaturation rates. What they observed was a decrease in levels of long

chain PUFA and a compensatory increase in short chain saturated and mono-unsaturated fatty acids in the PCB treated fish at both acclimation temperatures. The authors felt that these fatty acid changes may adapt the fish, with respect to the biophysical properties of the cell membranes, to a temperature approximately 13 C above their actual acclimation temperature.

The working hypothesis in the present study was that the fatty acid composition of phospholipids from coho salmon (O. kisutch) would be affected by treatment of the fish with an AHH inducing chemical as a result of interactions between the fatty acid desaturase and AHH systems.

The specific objectives were:

1) to determine whether exposure of coho salmon to a crude oil WSF (a potential AHH inducer) affected the fatty acid composition of tissue phospholipids
2) to determine whether exposure of coho salmon to a crude oil WSF affected the normal change in fatty acid composition of tissue phospholipids associated with a change in acclimation temperature; and
3) to determine whether any observed changes in the fatty acid composition of tissue phospholipids could be related to changes in AHH activity.

MATERIALS AND METHODS

Animals

Coho salmon (Oncorhynchus kisutch) were obtained from the Oregon State University Fish Disease Laboratory in Corvallis, Oregon. These fish had been spawned at the Bonneville (Columbia River) Hatchery in the Fall of 1976, and raised from eggs in well water at the Fish Disease Laboratory. Two weeks before the start of the first experiment, the salmon (1½-2 year olds) were transported to the Oregon State University Marine Science Center, Newport, Oregon and held in large flowing seawater tanks.

During the holding period, the fish were fed a 1/8" standard pelleted fish diet (Oregon Moist Pellet, Bioproducts, Inc., Warrenton, Oregon) (OMP) daily at a rate of 1% of their body weight. One group of thirty fish was fed a semi-purified diet at a rate of 1% of their body weight daily for three weeks prior to its use in the second experiment. This diet (Oregon Test Diet) was a modification of a rainbow trout diet developed by Sinnhuber et al. (1977) for use in nutrition studies with lipids (Table 1). With this diet, which used linseed oil as the lipid source, any fatty acid containing more than three double bonds would have to be synthesized by the fish, rather than being obtained through the food, since linseed oil contains essentially no ω3 polyunsaturated fatty acids other than linolenate. A new batch of the Oregon Test Diet (OTD) was prepared every two weeks and stored at -5 C until just before feeding. The final moisture content of the diet was 65%.

Table 1. Modified Oregon Test Diet.

Ingredients (%)	
Premix	
Casein	49.5
Gelatin	8.7
Dextrin[a]	15.6
Mineral mix[b]	4.0
Carboxymethyl cellulose[c]	1.0
α-Cellulose	8.2
Choline chloride (70%)	1.0
Vitamin mix no. 3[d]	2.0
Linseed oil	10.0

Modified from the Oregon Test Diet of Sinnhuber et al. (1977) by the substitution of linseed oil for whole fish oils.

[a]American Maize Products Co., Seattle, Washington.

[b]Bernhart-Tomerelli Salt Mix (1966) modified by the addition of NaF and $CoCl_2$ at 0.002 and 1.02%, respectively.

[c]Hercules Powder Co., San Francisco, California.

[d]Thiamine hydrochloride (0.3200%); riboflavin (0.7200%); niacinamide (2.5600%); biotin (0.0080%); calcium-pantothenate (D, 1.4400%); pyridoxine hydrochloride (0.2400%); folic acid (0.0960%); menadione (0.0800%); vitamin B_{12} (cobalamine, 3000 ug/g, 0.2667%); i-inosital (meso) (12.5000%); ascorbic acid (6.0000%); p-aminobenzoic acid (2.0000%); vitamin D (500,000 units/g, 0.0400%); vitamin A (250,000 IU/g, 0.5000%); dl-α-tocopherol acetate (2.5000%); and Celite (70.7293%).

Determination of Incipient Lethal Concentration of Total Aromatic Hydrocarbons in Seawater

Preliminary bioassays were conducted in order to determine the incipient lethal level of Cook Inlet crude oil water-soluble fraction. The incipient lethal level is defined as the highest concentration of total measured aromatic hydrocarbons dissolved in seawater which could be tolerated indefinately by 50% of the salmon at the assay temperature. Once the incipient lethal concentration was known, appropriate sublethal WSF concentrations for the experiments could be chosen.

The stock crude oil WSF for these assays was prepared using two continuous-flow water solublilizers (Nunes and Benville 1978) arranged in series. The solubilizers were made by General Glassblowing, Richmond California. Each solubilizer consisted of a modified glass bottle containing 1.5 L of Cook Inlet crude oil layered on top of 6.0 L of seawater. The solubilizers were fitted with an inlet and outlet for oil at the level of the oil layer. Oil in the solubilizer was continuously replenished by pumping oil from a reservoir through the bottle at a rate of 1.0 ml/min; the waste oil was collected at the outlet. Filtered ultraviolet light (UV) sterilized seawater flowed at a rate of 1,080 ml/min through a diffuser plate in the top of the first bottle and broke up into small droplets. As the droplets passed through the oil layer hydrocarbons became dissolved in the water and this effluent flowed out of a side arm into the second solubilizer. The effluent from the second solubilizer was supplied to a proportional flow diluter which delivered 400 ml/min of each of four concentrations of WSF to aquaria.

A fifth aquarium received seawater only.

Five groups of twelve OMP fed coho salmon (initial group mean weights ranging from 16.0-21.0 g) were placed in the individual 60 L aerated aquaria three days before the toxicant flow was started. Mortalities were recorded every half hour for the first 12 hours of WSF exposure, then every hour for the next 48 hours and every eight hours for the next six days. All of the exposures were terminated after nine days. The criterion for death was the absence of opercular movement.

This entire bioassay was repeated a second time using four different toxicant levels in order to give an improved estimate of the incipient lethal level. Both experiments were conducted at a temperature of 11.6 ± 0.5 C and salinity of 31.6 ± 1.0°/oo.

Concentrations of aromatic hydrocarbons in the seawater of each aquaria were determined daily using the procedures described later. The fish were not fed during the experiments.

Design of Sublethal Effects Experiments

Experiment I

The first experiment was designed to investigate whether exposure of coho salmon to a Cook Inlet crude oil WSF affected the fatty acid composition of tissue phospholipids. Four groups of twelve OMP fed salmon (initial group mean weights ranging from 63.0 - 70.5 g) were placed in individual 60 L glass aquaria, two supplied with a diluted WSF of Cook Inlet crude oil and two with filtered, UV sterilized seawater at ambient temperature (Figure 2). After 18 days of exposure, the fish

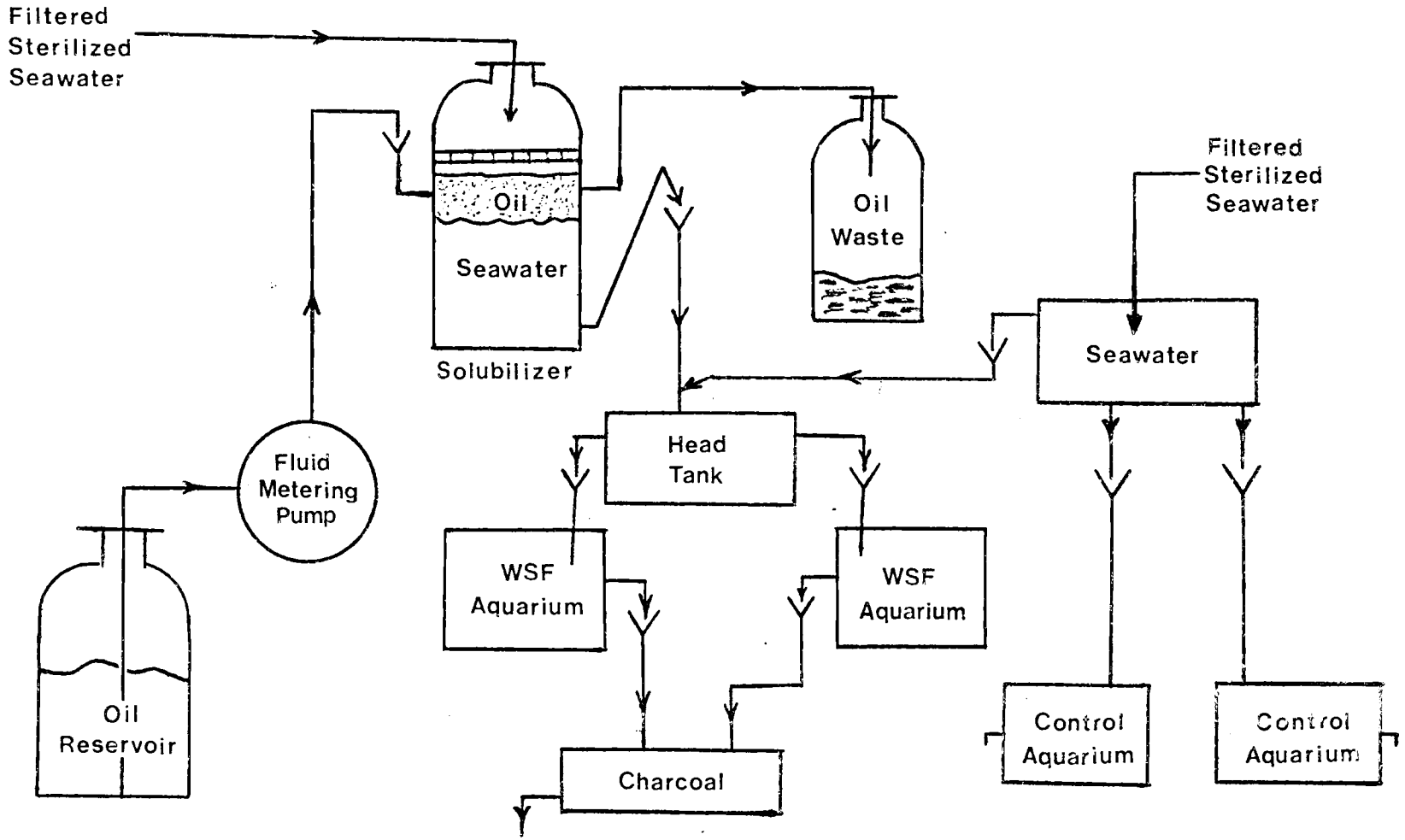

Figure 2. Flow diagram of the solubilizer system and diluting system used in Experiments I-III.

were killed by a blow to the head, weighed, and frozen at -5 C until the tissue lipids could be extracted. Tissues examined were the gills, brain, liver, and a muscle sample taken from just below the dorsal fin of each fish. Lipids were extracted from pooled samples of each tissue.

The water temperature in the aquaria averaged 12.8 C (12.0 - 14.0 C); the dissolved oxygen was 6.5 ppm (70% saturated); and the salinity averaged 31.7°/oo (29.9 - 32.1°/oo). Each aquarium was aerated and the photoperiod was 15 hours of light, nine hours of darkness. The fish were fed 1/8" OMP at a rate of 1% of their body weight per day. Water flow into each aquarium was 900 ml/min resulting in a fill time of 66 minutes. Seawater flow into the solubilizer was 750 ml/min and the solubilizer effluent was diluted by 1500 ml/min seawater in a head tank before entering the experimental (WSF) aquaria. Water samples were taken every other day from the WSF aquaria inlets and drains, and were analyzed to determine the aromatic hydrocarbon composition.

Experiment II

The second experiment was designed to test whether observed changes in the fatty acid composition of tissue phospholipids from salmon exposed to a crude oil WSF could be related to changes in AHH activity. The solubilizer system and diluting apparatus used in the first experiment was again used. Two groups of nine OTD fed fish (initial group mean weights of 75.5 g and 78.8 g) were placed in individual 60 L glass aquaria, one supplied with a diluted WSF of Cook Inlet crude oil and the other with filtered UV sterilized seawater at ambient temperature. The OTD was fed because it was thought that if the mechanism involved in

synthesizing polyunsaturated fatty acids (PUFA) was affected by exposing the salmon to hydrocarbons, it would be easier to detect if the fish were not able to obtain these fatty acids directly form their diet. The fish were fed the OTD at a rate of 1% of their body weight per day. After 30 days of exposure, the fish were stunned by a blow to the head and weighed. The livers from three fish in each tank were removed and immediatly processed to obtain the microsomal fraction. The microsomal pellets were used in AHH assays on the same day. The remainder of the fish were frozen at -5 C until the tissue lipids could be extracted. Extractions were performed on individual muscle, gill, liver, and brain tissue samples to facilitate statistical analysis of the data.

Water flow into each aquarium was 450 ml/min resulting in a fill time of 130 minutes. In this experiment, the toxicant concentration was increased because the results from the first experiment indicated no difference in the fatty acid composition between control and WSF exposed fish. Seawater flow into the solubilizer was 1,000 ml/min and the solubilizer effluent was diluted with 1,000 ml/min seawater in a head tank before entering the WSF aquarium. Water samples were taken every third day from the WSF aquarium inlet and drain and analyzed to determine the aromatic hydrocarbon composition.

The water temperature in the aquaria averaged 14.0 C (12.0 - 15.0 C); the salinity averaged 31.5^{o}/oo (29.9 - 31.8^{o}/oo); and the dissolved oxygen never fell below 80% saturation. Both aquaria were aerated and the photoperiod was 15 hours of light, nine hours of darkness.

Experiment III

The third experiment addressed the problem of whether exposure of coho salmon to a crude oil WSF affected the normal change in fatty acid composition of tissue phospholipids associated with a change in acclimation temperature. Four groups of nine OMP fed salmon (initial group mean weights ranging from 79.1 - 83.4 g) were placed in individual 60 L glass aquaria, two supplied with a diluted WSF of Cook Inlet crude oil and two with filtered, UV sterilized seawater (Figure 2). The fish were held at ambient water temperature (12 C) for one week; then the temperature of two of the aquaria (one control, one containing WSF) was gradually raised over a period of 12 hours to a temperature of 20.0 C (18.6 - 21.6 C) and held at that temperature for a period of 21 days. At the same time, the water temperature in the remaining two aquaria was gradually lowered over a period of 12 hours to 7.8 C (5.8 - 9.4 C) and held at that temperature for a period of 24 days.

At the end of each test period, the fish were stunned by a blow to the head and weighed. The livers from three fish in each tank were removed and immediately processed to obtain the microsomal fraction. The microsomal pellets were used in AHH assays on the same day. The remainder of the fish were frozen at -5 C until the tissue lipids could be extracted. Extractions were performed on muscle, gill, liver, and brain tissue of individual fish.

The fish were fed OMP at a rate of 1% of their body weight per day. The OTD was not used because the OTD fed fish in the holding tanks showed signs of bacterial infection.

Solubilizer conditions, WSF dilutions, aquaria fill times, and the photoperiod were the same as the second experiment. The dissolved

oxygen content of the aquaria never fell below 80% saturation and the salinity averaged 32.5°/oo (29.9 -- 32.8°/oo). Water samples were taken every third day from the WSF aquaria inlets and drains and analyzed to determine the aromatic hydrocarbon composition.

Determination of the Aromatic Hydrocarbon Composition of the WSF of Cook Inlet Crude Oil

The aromatic hydrocarbon composition of the WSF at the inlet and drain to each WSF aquarium was routinely monitored. Hexane was used to extract the hydrocarbons from two liters of seawater. A 1:100 (v:v) ratio of hexane:seawater was shaken for one minute in a glass separatory funnel. The phases were allowed to separate for a half hour and then the hexane layer was collected and evaporated under vacuum at 25 C in a rotary evaporator to a volume of 1.0 ml. An aliquot of the evaporated extract was chromatographed on a Hewlett-Packard Model 5711A gas chromatograph equipped with a dual flame ionization detector, temperature programming, an HP 7671A automatic sampler and an HP 3380A integrator. Stainless steel columns (7' x 1/8") packed with 10% SE-30 on 100/200 mesh chromosorb W HP were used. The detector temperature was 300 C, the injection port temperature was 250 C and nitrogen was used as the carrier gas at a flow rate of 15 ml/min. The columns were held at an initial temperature of 80 C for eight minutes, the temperature was then increased at a rate of 4 C/min to a final temperature of 170 C.

Peaks were identified by comparison of retention times with pure standards of each hydrocarbon (Chem Services, Inc., West Chester, PA.) and areas were quantitated by an internal standardization method using

a hexamethylbenzene standard.

The efficiency of the seawater extractions and the percent loss of individual hydrocarbons during rotary evaporation was determined using a quantitative composite standard. This standard consisted of pure aromatic hydrocarbons, in proportions similar to those found in the WSFs, dissolved in hexane. An exact volume of the standard was added to two liters of seawater; the mixture was then shaken and the hydrocarbons extracted and analyzed following the procedure described above. The concentrations of specific hydrocarbons reported were corrected for losses during analysis since replicates of the recovery efficiences were consistent (Table 2). The low recovery efficiencies were due to hydrocarbon loss during evaporation of the samples.

Lipid Analysis

Lipids were extracted from salmon tissues with chloroform:methanol (2:1, v:v) using the method of Folch et al. (1957). The crude extracts were washed with 0.034% $MgCl_2$ and then evaporated to dryness under vacuum on a rotary evaporator at 25 C. Acetone was added and the mixtures re-evaporated to remove residual water by co-distillation. The residue (total lipids) was dissolved in 5.0 ml chloroform and stored at -5 C in a teflon-capped culture tube that had been flushed with nitrogen.

A modification of the column chromatography method described by Caldwell et al. (1979) was used to separate approximately 10 mg of the total lipid extract into neutral and phospholipid fractions. The lipid was added to a champagne column (100 mm x 5 mm diameter) filled with fat-free silicic acid (Bio Rad, Bio-Sil A, 100-200 mesh) previously

Table 2. Recovery efficiencies for analysis of individual aromatic hydrocarbons in the WSF of Cook Inlet crude oil. Data given are means ± one standard deviation. N = 7.

Compound	Percent Recovery
Toluene	11.7 ± 1.8
Methylcyclohexane	9.4 ± 1.8
Ethylbenzene m-, p-Xylenes	37.0 ± 2.9
o-Xylene	40.3 ± 3.4
Naphthalene	62.7 ± 3.9
1,3,5-Trimethylbenzene[a]	49.2 ± 4.6
1,2,4-Trimethylbenzene	55.5 ± 5.0
p-Cymene[a]	57.8 ± 4.2
2-Methylnaphthalene	67.4 ± 4.4
Cumene	48.1 ± 4.9

[a]Principal compound, merged peaks.

conditioned with chloroform. Solvents were added to the column as follows: 8.0 ml chloroform; 5.0 ml chloroform:methanol (19:1, v:v); 5.0 ml chloroform:methanol (2:1, v:v); 20.0 ml methanol. The first two fractions, containing the neutral lipids, were discarded. The third and fourth fractions, containing the phospholipids, were collected in a round bottom flask and evaporated to dryness under vacuum in a rotary evaporator at 27 C. The residue was dissolved in 1.0 ml chloroform and stored at 3.0 C in a capped culture tube that had been flushed with nitrogen.

To determine the efficiency of the lipid separation, phospholipid phosphorus was measured in some of the total lipid samples and their corresponding neutral and phospholipid fractions, using the method of Parker and Peterson (1965). The ANSA reagent used in the procedure was prepared by grinding and mixing 30.0 g sodium bisulfite with 0.5 g recrystallized 1-amino-2-napthol-4-sulfonic acid (ANSA) (Fiske and Subbarow 1925) and 6.0 g sodium sulfite. This mixture was brought up to a volume of 250 ml with distilled water and stored in a brown bottle at room temperature. It was determined that 99% of the phosphorus applied to the column was recovered and 99% of the recovered phosphorus was in the combined phospholipid fractions. Possible contamination of the phospholipid fractions by neutral lipids was checked by silica gel thin layer chromatography (TLC) using hexane:chloroform:ether:acetic acid (160:20:20:2) as the solvent system. The spots were visualized by exposure of the plates to iodine vapor and identified by comparison with reference standards of a polar lipid mix, nonpolar lipid mix, and sphingolipid mix (Supelco, Inc.). The phospholipid fractions contained

negligible amounts of neutral lipids.

A modification of the method reported by Morrison and Smith (1964) was used to methylate the phospholipid samples. The organic solvents in the samples were evaporated in teflon capped culture tubes by a stream of nitrogen at room temperature. One ml methanol, 0.86 ml benzene, and 1.0 ml BF_3-methanol were added, and the sample vials were flushed with nitrogen, capped, and placed in a boiling water bath for 45 minutes. After cooling, 10.0 ml hexane and 5.0 ml water were added. The samples were vigorously shaken and placed in the refrigerator overnight to allow for complete separation of the phases. The hexane layer containing the methylated esters was pipetted off and evaporated to dryness under a stream of nitrogen at room temperature. The residues were dissolved in 1.0 ml hexane and stored at 3.0 C in capped gas chromatograph sampling bottles that had been flushed with nitrogen.

The methylated fatty acid esters were chromatographed on a Hewlett Packard Model 5711A gas chromatograph equipped as previously described. Columns were 6' x 1/8" stainless steel packed with 10% SP-2330 on 100/200 mesh Chromosorb W AW. The detector temperature was 300 C, the injection port temperature was 250 C and nitrogen was used as the carrier gas at a flow rate of 20 ml/min. The oven temperature was held at 180 C for 16 minutes then increased at a rate of 1.0 C/min to a final temperature of 210 C. Peaks were integrated and expressed as area percent of the total sample. Identification of the individual peaks was by comparison of retention times with prepared standards (Supelco, Inc.; RM-3 PUFA-1, PUFA-2); by plots of log retention times measured under isothermal conditions against carbon number (Ackman 1963); and by

separation of the fatty acid methyl esters by their degree of unsaturation on silver nitrate-silicic acid impregnated TLC plates. The $AgNo_3$ plates were prepared according to Privett et al. (1963) and Morris (1962) and developed in 30% ether in hexane. Detection of the bands of fatty acids was accomplished by spraying the plates with 2,7-dichlorofluorescein (0.2% in ethanol) and then viewing them under UV light. Individual bands were scraped off the plate and extracted from the silicic acid with absolute ether. The extracts were then evaporated to dryness under a stream of nitrogen and the residue dissolved in 1.0 ml hexane and analyzed on the gas chromatograph as described above.

Chromatography of quantitative methyl ester standards (Supelco Inc. RM-3, RM-6, GLC-50) confirmed that the detector response was proportional to mass for the range of fatty acid chain length employed (Horning et al. 1964) permitting expression of the analytical results for the fatty acids as weight percent. Quantitative analysis of the standards agreed with the stated composition data with a relative error less than 5% for major components (>10% of the total mixture) and less than 6.5% for minor components (<10% of the total mixture). The values for the precent relative error were the means of four determinations.

All of the lipid analytical work was completed within three weeks after the conclusion of each experiment. A sample of the linseed oil used in the OTD was methylated and analyzed in the same manner as described above.

Radioactive Assay of Aryl Hydrocarbon Hydroxylase Activity

Hepatic microsomes were prepared immediately after the fish were

sacrificed. Each liver was weighed, then homogenized by hand in a teflon-glass homogenizer with four volumes of ice-cold 0.25 M buffered sucrose (50 mM K-PO_4, pH 7.4). The homogenates were centrifuged at 9,000 x g for 15 minutes to sediment the mitochondria, nuclei, and cell debris. The 9,000 x g supernatant was then recentrifuged at 105,000 x g for 60 minutes to sediment the microsomal pellet. The pellets were rinsed and resuspended in 0.25 M buffered sucrose, to a final concentration equivalent to 1.0 g wet weight of liver/ml. All centrifugations were done at 5.0 C. Microsomal protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

A modification of the radioactive assay described by Van Cantfort et al. (1977) was used to determine the aryl hydrocarbon [benzo(a)pyrene] hydroxylase activity in the microsomal fractions. The 1.0 ml incubation mixture contained; 0.3 - 0.5 mg microsomal protein; 5.0 mM nicotinamide; 5.0 mM $MgCl_2$; 0.1 mM NADP; 2.0 mM glucose-6-phosphate; 1 I.U./ml glucose-6-phosphate dehydrogenase; and 50 mM K-PO_4 buffer (pH 7.4). Gruger et al. (1977) determined the optimum conditions for measuring AHH activity in coho salmon to be a pH of 7.4 and an incubation temperature of 25 C. All of the reagents were made up in 50 mM K-PO_4 buffer except the $MgCl_2$ which was dissolved in water.

The mixture was shaken for five minutes to allow for some conversion of $NADP^+$ to NADPH, then the reaction was started by adding 25 ul of ^{3}H-benzo(a)pyrene working stock, resulting in a 70 uM final concentration. Incubations were carried ou in duplicate with shaking at 25 C. The reaction was stopped after 20 minutes by the addition of 1.0 ml

0.15 M KOH in 85% dimethyl sulfoxide (DMSO). Controls consisted of assays containing boiled microsomes or no microsomes and zero-time incubations.

The stopped reaction mixture was transferred to a teflon-capped centrifuge tube and 5.0 ml hexane was added. The mixture was swirled for three minutes on a Vortex mixer and then centrifuged for five minutes to speed phase separation. The upper phase and interface containing the unmetabolized substrate were discarded, and the lower layer re-extracted. A 0.3 ml aliquot of the lower phase was acidified with 0.3 ml of 1 N HCl, diluted with 13.0 ml of ACS (Aqueous Counting Scintillant, Amersham Corp.), and counted on an LS 8000 Series Liquid Scintillation Counter using an external standard (^{137}Cs). A quench curve was constructed by counting a series of samples of known activity containing various amounts of water.

All of the sample counts were corrected for quenching and multiplied by the specific activity of the substrate to yield an enzymatic activity expressed as nmoles of metabolites formed per mg of microsomal protein per 20 minutes. The specific activity of the substrate can be used because all of the metabolites formed during the in vitro incubation are measured by this assay (Van Cantfort et al. 1977). The purification of the ^{3}H-benzo(a)pyrene resulted in a slight decrease in the specific activity and concentration of the stock solution form the expected values. To calculate the actual concentration of the ^{3}H-benzo(a)-pyrene stock solution, an aliquot was diluted 1:1000 in ethanol and the absorbancy at 296.5 nm was determined on a UV spectrophotometer using an acetone and ethanol blank. Using the molar absorption coefficient for

benzo(a)pyrene at that wavelength (57,500; Handbook of Chemistry and Physics) the concentration of the stock solution was calculated. The radioactivity of the diluted aliquot was also determined in order to calculate the specific activity of the solution. All determinations were done in duplicate. The specific activity of the ^{3}H-benzo(a)pyrene stock solution routinely used was 11.7 uCi/umole in a 2.8 mM solution.

Linearity of the AHH assay with time and protein concentration, and the dependence of activity on the concentration of the substrate was determined. The AHH activity was measured as described above except the incubation time, the amount of protein added to the incubation mixture, or the amount of substrate added, were varied. Three replicate assays were run using different pools of salmon livers.

Chemicals

Cook Inlet crude oil was supplied in gratis by Shell Oil Company. The following chemicals were obtained from Sigma Chemical Company: nicotinamide, nicotinamide-adenine dinucleotide phosphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and bovine serum albumin. Gas chromatograph column packings were purchased from Hewlett-Packard, Inc. and Supelco, Inc.. BF_3-methanol and 2',2'-dichlorofluorescein spray were purchased from Supelco, Inc.. ANSA was purchased from Eastman Chemical Co.. All solvents used for the lipid analyses were glass redistilled prior to use except hexane which was UV spectroquality grade (Burdick and Jackson Laboratories, Inc.). All additional chemicals were purchased from Mallinkrodt Chemical Works.

G-^{3}H-benzo(a)pyrene (56 Ci/nmole, Radiochemical Centre, Amersham)

was purified to lower the background counts by diluting 0.2 mCi with unlabelled benzo(a)pyrene to a specific radioactivity of 12.5 uCi/nmole, dissolving this mixture in 4.0 ml hexane and extracting it five times with 2.0 ml aqueous 1 M KOH/DMSO (13:17, v:v). The hexane layer was then evaporated under nitrogen at room temperature and the residue redissolved in 5.0 ml acetone and stored at -5 C in a capped bottle that had been flushed with nitrogen. This working stock solution was used within one week.

Statistics

Statistical comparisons were made using Student's two-tailed "t" test. Variances of the compared samples were determined to be equal.

RESULTS

Incipient Lethal Concentration of Total Aromatic Hydrocarbons in Seawater

A log-probit plot of time vs. percent survival of coho salmon exposed to four lethal concentrations of the WSF of Cook Inlet crude oil is shown in Figure 3. There were no deaths during the two week test period in any of the tanks containing a total aromatic concentration of 0.430 mg/l or less (Table 3). However, fish in the tanks containing 0.430 and 0.353 mg/l total measured aromatic hydrocarbons did show a loss of bouyancy control and a diminished response to tactile simulation (prodding). Befor death, the fish in the lethal concentrations of WSF exhibited a behavior pattern identical to that elicited by the application of a lethal dose of an anesthetic (Klontz and Smith 1968).

Median survival times obtained from the log-probit plots were graphed against the WSF concentration on a log x log scale (Figure 4) (Sprague 1969). The resulting shape of the curve and the absence of any deaths in the WSF aquaria containing 0.430 mg/l or less total measured aromatic hydrocarbons, indicates that the incipient lethal level for coho salmon at 11.6 C is approximately 0.60 mg/l total measured aromatic hydrocarbons.

Determination of Optimum Conditions for the Aryl Hydrocarbon Hydroxylase Assay

The activity of AHH increased linearly up to a protein concentration of approximately 1.8 mg/l (Figure 5). The rate of metabolite formation was approximately constant during the initial 20 minutes of

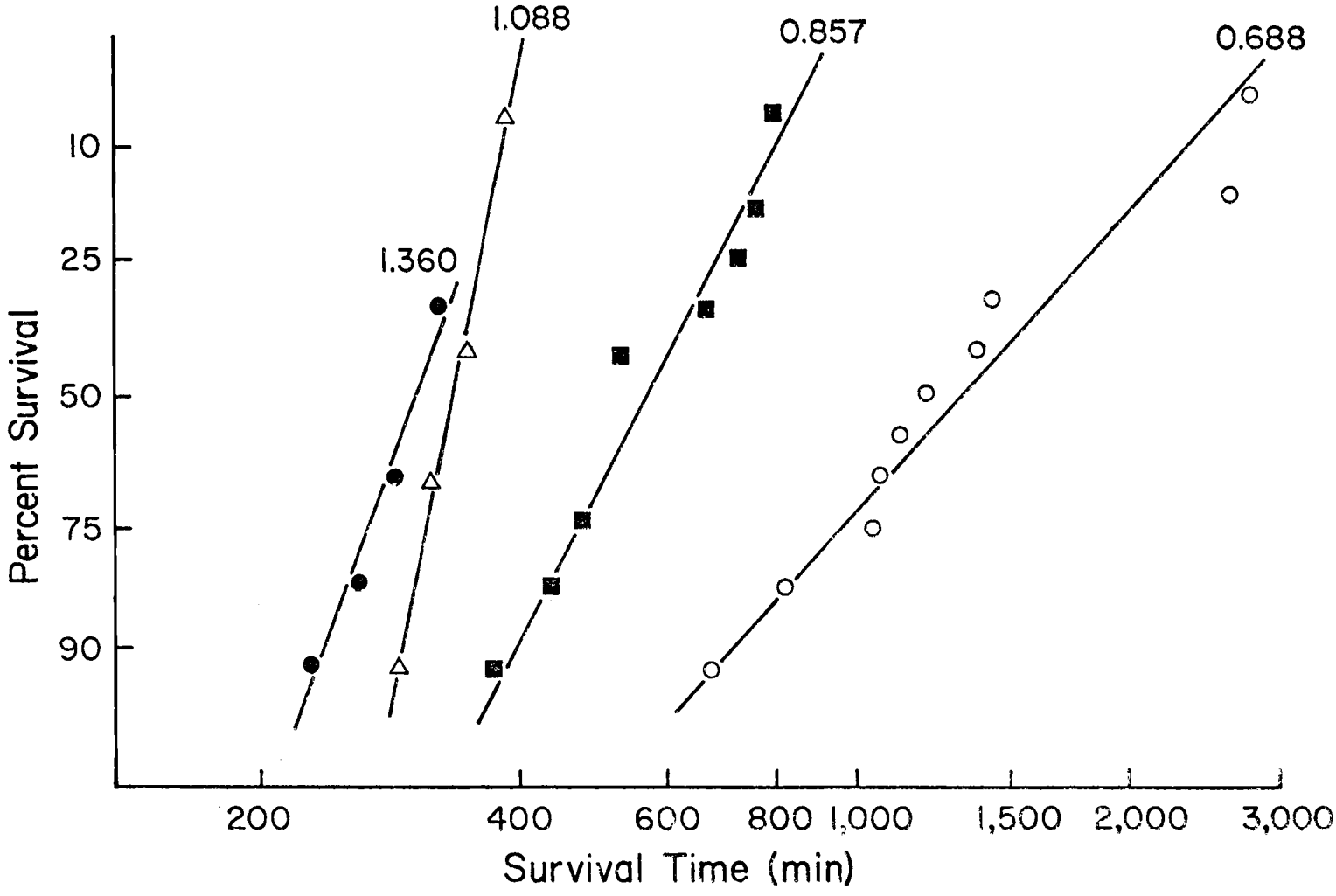

Figure 3. Relationship between percent survival and time for coho salmon at four concentrations of WSF. The concentrations are total measured aromatic hydrocarbons expressed in mg/l.

Table 3. Median survival time of coho salmon exposed to eight concentrations of WSF of Cook Inlet crude oil in a flowing water system.

Total measured aromatic hydrocarbons (mg/l)	Time to 50% death (minutes)
1.360	300
1.088	340
0.857	570
0.688	1,340
0.430	>12,960
0.353	>12,960
0.308	>12,960
0.265	>12,960

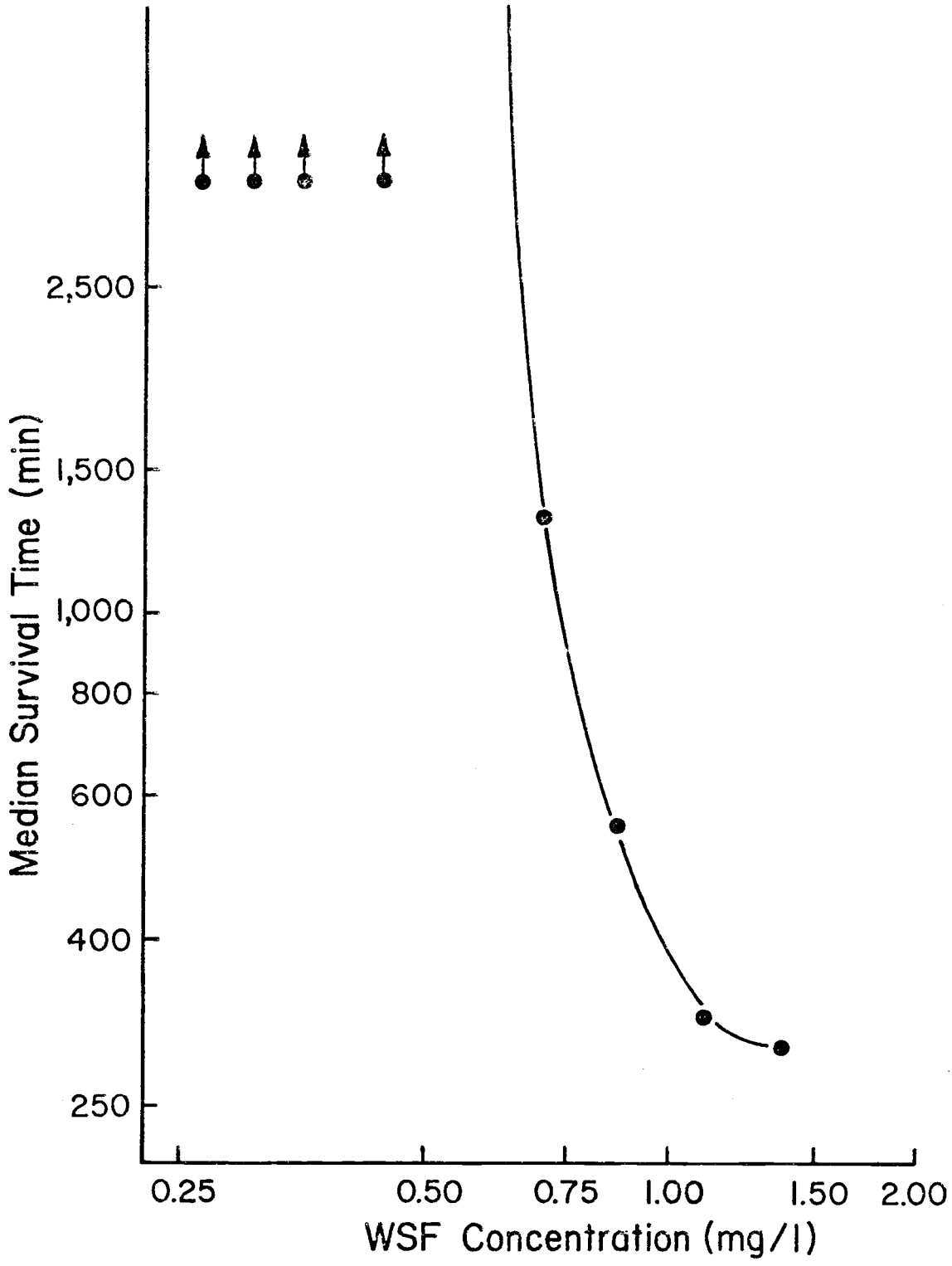

Figure 4. Relationship between median survival time of coho salmon and concentration of the total measured aromatic hydrocarbons in the WSF.

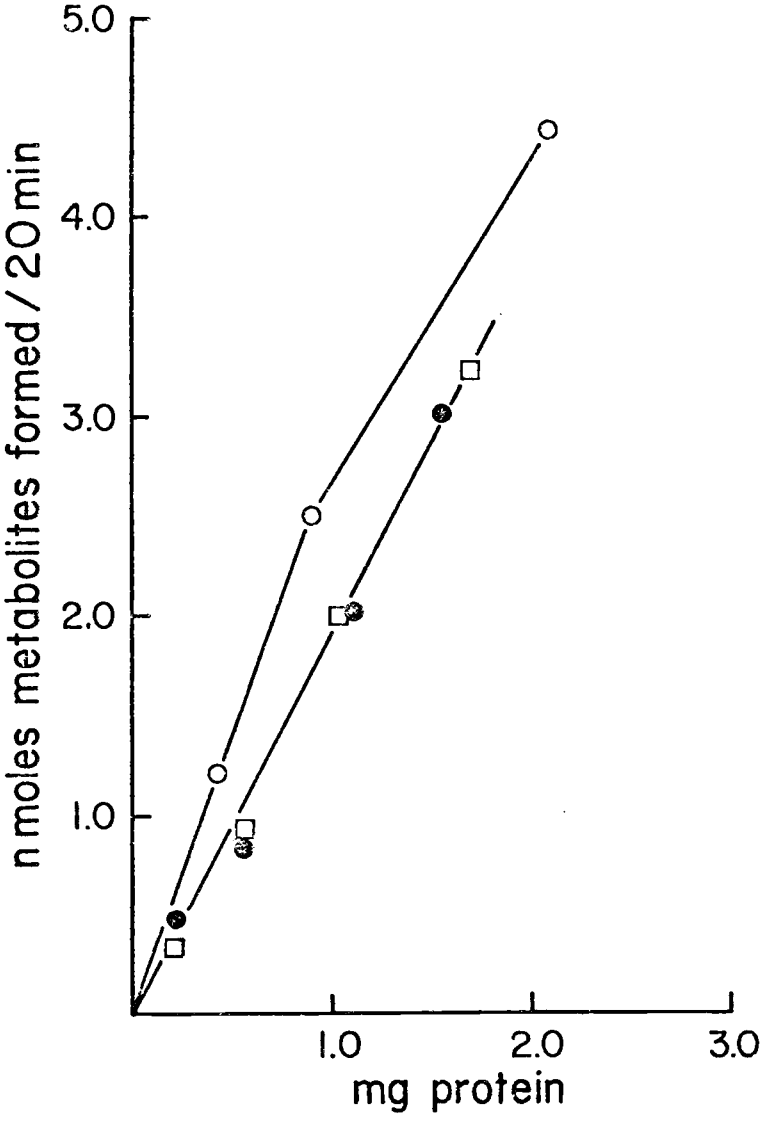

Figure 5. Linearity of the aryl hydrocarbon hydroxylase assay used with increasing protein concentration. Data points are determined from three assays using different pools of salmon livers. Differences in the activities is due to a difference in acclimation temperature of the fish used.

of incubation but essentially no more metabolites were formed after 20 minutes (Figure 6). The cessation of metabolite formation after 20 minutes may indicate inactivation of the enzymes, product inhibition, or a consumption of reducing equivalents. Figure 7 shows that a 30 uM concentration of ^{3}H-benzo(a)pyrene is saturating. Based on these preliminary results, a concentration of 70 uM ^{3}H-benzo(a)pyrene, an incubation time of 20 minutes, and a protein concentration ranging between 0.3 mg/ml and 0.5 mg/ml was chosen as the assay conditions for this study.

The three procedures used to determine control values for the assays yielded similar results. An average of these counts has been subtracted from all AHH activities reported.

Experiment I

In the first experiment, the toxicant concentration at the inlet to the WSF aquaria averaged 0.480 mg/l total measured aromatic hydrocarbons during the 18 day test period. Due to aeration of the aquaria water and the concomitant loss of hydrocarbons through evaporation, the average concentration of total measured aromatic hydrocarbons that the fish were exposed to was 0.220 ± 0.112 mg/l which is 32% of the incipient lethal level determined previously at 11.6 C. The concentrations of the principal aromatic hydrocarbons measured in the inlet and drains of the aquaria containing the Cook Inlet crude oil WSF are listed in Table 4. The hydrocarbon data from the two aquaria were treated as one sample since there were no apparent differences in hydrocarbon content between the aquaria. Of the aromatic hydrocarbons analyzed, some of the gas chromatograph peaks were merged with minor peaks and integrated as one

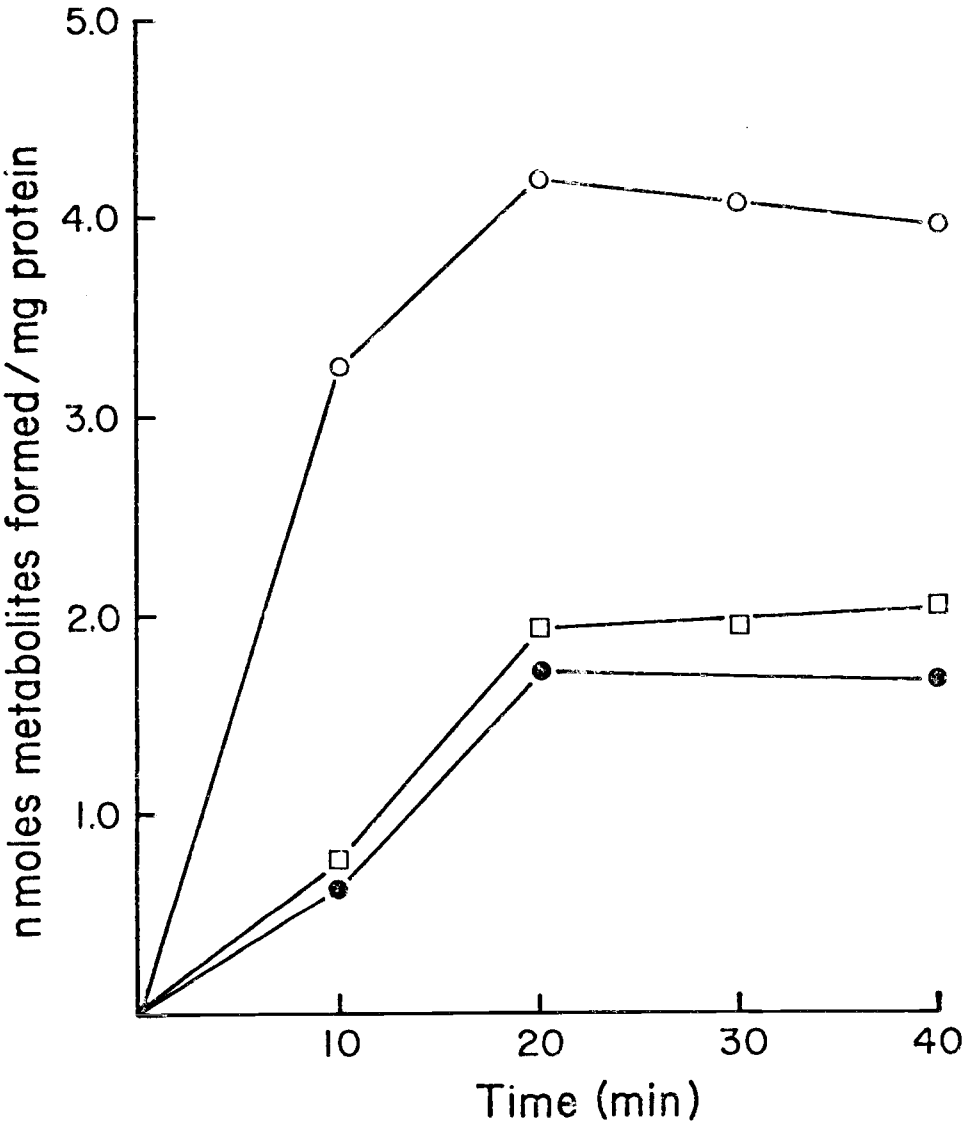

Figure 6. Relationship between aryl hydrocarbon hydroxylase activity and incubation time. Data points are determined from three assays using different pools of salmon livers. Differences in the activities is due to a difference in acclimation temperature of the fish used.

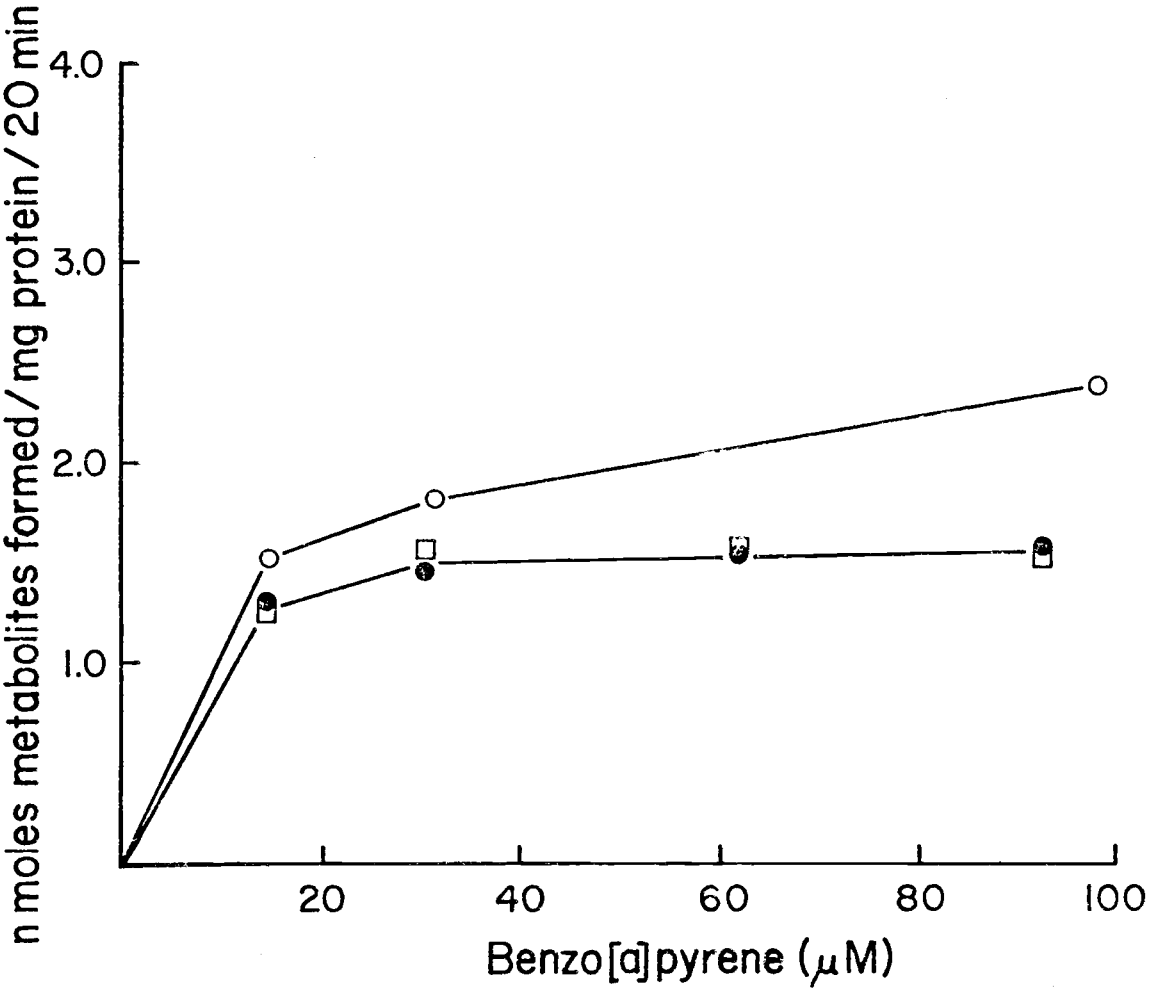

Figure 7. Relationship between aryl hydrocarbon hydroxylase activity and substrate concentration. Data points are determined from three assays using different pools of salmon livers. Differences in the activities is due to a difference in acclimation temperature of the fish used.

Table 4. Concentrations of the principal aromatic hydrocarbons in the WSF of Cook Inlet crude oil. Experiment I; 12.8 C water temperature.

Compound	Concentration (mg/l)		
	Inlet	Drain	% Loss
Toluene	0.301 ± 0.043[a]	0.147 ± 0.089[b]	51.2
Methycyclohexane	0.057 ± 0.019	0.026 ± 0.003	54.4
Ethylbenzene m-, p-Xylene	0.043 ± 0.006	0.023 ± 0.009	46.5
o-Xylene	0.029 ± 0.003	0.017 ± 0.006	41.4
Naphthalene	0.011 ± 0.001	0.007 ± 0.002	36.4
1,3,5-Trimethylbenzene[c]	0.010 ± 0.001	0.004 ± 0.002	60.0
1,2,4-Trimethylbenzene	0.008 ± 0.001	0.004 ± 0.002	50.0
p-Cymene[c]	0.007 ± 0.002	0.004 ± 0.002	42.8
2-Methylnaphthalene	0.006 ± 0.001	0.004 ± 0.001	33.3
Cumene	0.002 ± 0.000	0.001 ± 0.000	50.0
Total measured aromatics	0.480 ± 0.066	0.220 ± 0.122	

The hydrocarbon data from the two WSF aquaria were combined and treated as one sample since there were no apparant differences in the hydrocarbon content between the aquaria. Benzene, a major aromatic hydrocarbon component of the WSF was not measured due to a benzene contamination of the hexane used in the extractions.

[a]Mean ± one standard deviation. N = 6.

[b]Mean ± one standard deviation. N = 5.

[c]Principal compound, merged peaks.

peak. The major component of these merged peaks is noted in the tables. The gas chromatograph peaks of ethylbenzene and m- and p-xylene were also merged and integrated as one peak. Benzene, a major aromatic hydrocarbon component of the WSF was not measured due to a benzene contamination of the hexane used in the extractions.

The concentration of the individual hydrocarbons in the test WSF decreased with increasing alkyl-substitution of the aromatic rings and increasing number of rings, with the exception of naphthalene which was present at a higher concentration than the trimethylbenzenes and p-cymene. The loss of hydrocarbons through evaporation also decreased slightly with increasing alkyl-substitution and increasing number of rings (Table 4). There were no detectable aromatic hydrocarbons in the seawater inlets to the control aquaria.

The fatty acid composition of phospholipids extracted from the muscle, gills, liver and brain of the control and WSF exposed coho salmon are shown in Tables 5 and 6. Of the fatty acids analyzed, some of the gas chromatograph peaks were merged with minor peaks and integrated as one peak. The major component of these merged peaks is noted in the tables. The gas chromatograph peaks of 18:3ω3 and 20:1ω9 (eicosaenoic acid) have also been merged and integrated as one peak. Except in the brain samples, nervonic acid (24:1ω9) tended to be an odd shaped peak making it difficult to integrate the area and thus the results (percent composition by weight) are variable. In the brain samples, 24:1ω9 was a distinct, well formed peak and could be precisely integrated.

There were no apparent differences between the fatty acid data from fish in the two tanks receiving the same treatment; thus, for comparison

Table 5. The fatty acid composition of phospholipids extracted from muscle and gill tissue of control and WSF exposed coho salmon (Experiment I, 12.8 C acclimation temperature). Data given are weight percent as fatty acid methyl esters ± one standard deviation.

Fatty Acid	Muscle		Gill	
	Control (2)[a]	WSF (1)	Control (2)	WSF (2)
14:0	0.6 ± 0.1	0.6	1.0 ± 0.1	1.0 ± 0.1
16:0	21.1 ± 1.9	19.8	21.8 ± 0.9	23.0 ± 0.8
16:1ω7[b]	1.5 ± 0.2	1.6	2.9 ± 0.5	3.3 ± 0.0
18:0	7.3 ± 0.8	6.8	6.7 ± 0.6	7.3 ± 0.1
18:1ω9	11.0 ± 1.9	8.7	19.1 ± 0.0	18.5 ± 0.0
18:2ω6	4.9 ± 0.2	5.5	6.3 ± 0.2	6.1 ± 0.3
18:3ω3 20:1ω9	1.2 ± 0.2	1.1	1.3 ± 0.1	1.0 ± 0.4
20:4ω6	2.5 ± 0.6	2.3	3.8 ± 0.0	3.6 ± 0.2
20:5ω3	5.0 ± 0.5	5.5	3.8 ± 0.3	4.2 ± 0.1
22:5ω3	1.8 ± 0.1	1.9	1.3 ± 0.1	1.4 ± 0.0
22:6ω3	41.0 ± 4.9	45.6	25.7 ± 1.7	27.7 ± 0.9
24:1ω9[b]	1.6 ± 0.2	—	5.1 ± 4.7	1.6 ± 0.2
others	0.5	0.6	1.2	1.3

[a]Number of pools of ten fish.

[b]Principal compound, merged peaks.

Table 6. The fatty acid composition of phospholipids extracted from the livers and brains of control and WSF exposed coho salmon (Experiment I, 12.8 C acclimation temperature). Data given are weight percent as fatty acid methyl esters ± one standard deviation.

Fatty Acid	Liver		Brain	
	Control (2)[a]	WSF (2)[a]	Control (2)[b]	WSF (2)[b]
14:0	0.5 ± 0.1	0.7 ± 0.0	0.4 ± 0.2	0.3 ± 0.0
16:0	18.9 ± 1.1	19.2 ± 0.6	16.2 ± 1.5	15.9 ± 0.4
16:1ω7[c]	1.3 ± 0.1	1.4 ± 0.1	3.7 ± 0.1	3.1 ± 0.0
18:0	6.0 ± 0.3	6.1 ± 0.1	10.9 ± 1.0	10.3 ± 0.9
18:1ω9	11.0 ± 0.3	11.0 ± 0.4	24.7 ± 4.0	24.6 ± 0.7
18:2ω6	6.9 ± 0.4	6.8 ± 0.7	1.2 ± 0.1	1.0 ± 0.0
18:3ω3 20:1ω9	1.2 ± 0.1	1.1 ± 0.3	2.4 ± 0.1	2.3 ± 0.1
20:4ω6	5.3 ± 0.2	5.2 ± 0.3	1.0 ± 0.1	1.3 ± 0.3
20:5ω3	5.1 ± 0.2	5.5 ± 0.3	4.4 ± 0.2	4.7 ± 0.6
22:1ω9[c]	——	——	1.1 ± 0.1	2.4 ± 0.7
22:5ω3	1.9 ± 0.1	2.3 ± 0.3	2.3 ± 0.0	2.3 ± 0.0
22:6ω3	38.7 ± 0.3	38.3 ± 0.8	23.2 ± 2.3	22.2 ± 0.6
24:1ω9[d]	1.7 ± 0.3	1.3 ± 0.1	5.2 ± 1.7	6.1 ± 0.3
others	1.5	1.1	3.3	3.5

[a]Number of pools of five fish.

[b]Number of pools of ten fish.

[c]Principal compound, merged peaks.

[d]Principal compound, merged peaks in liver samples only.

between control and WSF exposed fish, these fatty acid data were treated as part of the same sample (Tables 5 and 6). In each of the four tissues analyzed, there were no statistically significant differences between the mean weight percent of individual fatty acids from the WSF exposed fish compared to controls.

Although not statistically significant, 16:0 and 18:1ω9 decreased and 22:6ω3 increased in the muscle sample from WSF exposed fish when compared to controls. The gill tissue from these fish also showed a slight increase in 22:6ω3, and an increase in 16:0. The mean weight percents of individual fatty acids from the livers of the two groups of fish were nearly identical.

After the second day in the experimental aquaria, both the control and WSF exposed fish stopped consuming the OMP. At the termination of the experiment, fish in all of the test aquaria showed a weight loss (Table 7). Before the start of the experiment, coho salmon in the holding tanks had a liver to body weight ratio of 0.85 - 1.1 and a microsomal protein to liver ratio of 0.4 - 12.8. Both control and WSF exposed fish showed a slight decrease in the microsomal protein to liver ratio at the end of the experiment (Table 7).

One control fish died during the 18 day test period. No deaths occured in the tanks containing the fish exposed to the WSF of crude oil. The dead fish showed some signs of fin rot on its caudal fin, indicating the possibility of bacterial infection.

Experiment II

In the second experiment, the toxicant concentration at the inlet

Table 7. Weight parameters of coho salmon exposed to a WSF of Cook Inlet crude oil. Weight data given are means ± one standard deviation.

Acclimation temperature	Aquaria	Final group mean weight of fish (g)	% Weight loss[a]	Weight of liver (g)	Total amount of protein in liver microsomal fraction (mg)	mg Liver microsomal protein per g liver tissue	g Liver weight per g final weight of fish (%)
Experiment I							
12.8 C	control	62.84 ± 13.06 (10)[b]	9.5	0.46 ± 0.17 (10)	3.44 ± 0.84 (6)	7.9 ± 1.5 (6)	0.7 ± 0.1 (10)
	WSF	65.15 ± 7.24 (10)	7.9	0.49 ± 0.06 (10)	3.80 ± 0.87 (5)	7.7 ± 1.5 (5)	0.8 ± 0.2 (10)
Experiment II							
14.0 C	control	55.87 ± 8.43 (5)	17.2	0.39 ± 0.33 (5)	2.76 ± 0.47 (3)	5.3 ± 0.2 (3)	1.0 ± 0.2 (5)
	WSF	73.32 ± 4.80* (5)	10.5	0.64 ± 0.08 (5)	3.95 ± 1.47 (3)	6.0 ± 1.5 (3)	0.9 ± 0.1 (5)
Experiment III							
20.0 C	control	57.11 ± 9.31 (4)	15.9	0.56 ± 0.24 (3)	3.03 ± 1.69 (3)	5.1 ± 0.9 (3)	0.9 ± 0.3 (3)
	WSF	62.98 ± 10.94 (6)	20.4	0.48 ± 0.14 (3)	2.69 ± 0.57 (3)	5.8 ± 0.9 (3)	0.7 ± 0.1 (3)
7.8 C	control	56.57 ± 4.91 (6)	17.9	0.38 ± 0.07 (6)	1.37 ± 0.30 (3)	3.6 ± 0.3 (3)	0.8 ± 0.1 (6)
	WSF	61.07 ± 10.78 (6)	14.8	0.63 ± 0.07** (6)	3.06 ± 0.05** (3)	4.9 ± 0.8 (3)	1.0 ± 0.1* (6)

WSF exposed fish were significantly different from control fish at *$0.05>P>0.01$; **$0.01>P$.

[a] % weight loss = $\frac{\text{initial group mean weight} - \text{final group mean weight}}{\text{initial group mean weight}} \times 100$.

[b] Number of individual fish in the sample.

to the WSF aquarium averaged 0.702 mg/l total measured aromatic hydrocarbons during the 30 day test period. Due to aeration of the aquaria water and the concomitant evaporation of hydrocarbons, the average concentration that the fish were exposed to was 0.259 ± 0.080 mg/l (Table 8) which is 43% of the incipient lethal level determined previously at 11.6 C.

The relative concentration and loss by evaporation of individual hydrocarbons in the WSF followed the same pattern as was observed in the first experiment. There were no detectable aromatic hydrocarbons in the seawater inlet to the control aquarium.

The mean AHH activity of the WSF exposed fish was very similar to that determined for the control fish (3.48 and 3.02 nmoles of metabolites formed per mg protein per 20 minutes respectively)(Table 9). There was no statistically significant difference between the means of the two groups or between AHH activities determined for male and female fish. Results from the duplicate AHH assays of the individual fish livers agreed within 8% or less ((difference between duplicates/mean of duplicates) x 100).

The fatty acid composition (by weight percent) of phospholipids extracted from the muscle, gills, liver, and brain of the control and WSF exposed OTD fed coho salmon are shown in Tables 10 and 11. Four of the fatty acids from WSF exposed fish (16:1ω7 in the muscle and 14:0, 16:1 ω7, and 18:0 in the gill) had statistically significantly different mean weight percents when compared to the corresponding controls. For three of the acids (16:1ω7 in the muscle and 14:0 and 16:1ω7 in the gill) the actual difference between means, and the weight percent that each

Table 8. Concentrations of the principal aromatic hydrocarbons in the WSF of Cook Inlet crude oil. Experiment II; 14.0 C water temperature.

Compound	Concentration (mg/l)		
	Inlet	Drain	% Loss
Toluene	0.459 ± 0.081[a]	0.169 ± 0.066[b]	63.2
Methylcyclohexane	0.074 ± 0.019	0.000 ± 0.000	100.0
Ethylbenzene m-, p-Xylene	0.062 ± 0.006	0.028 ± 0.005	54.8
o-Xylene	0.047 ± 0.010	0.023 ± 0.002	51.0
Naphthalene	0.018 ± 0.001	0.009 ± 0.002	50.0
1,3,5-Trimethylbenzene[c]	0.016 ± 0.002	0.007 ± 0.001	56.2
1,2,4-Trimethylbenzene	0.013 ± 0.001	0.007 ± 0.001	46.2
p-Cymene[c]	0.011 ± 0.001	0.007 ± 0.002	36.4
2-Methylnaphthalene	0.008 ± 0.001	0.005 ± 0.000	37.5
Cumene	0.006 ± 0.005	0.002 ± 0.001	66.6
Total measured aromatics	0.702 ± 0.107	0.259 ± 0.080	

Benzene, a major aromatic hydrocarbon component of the WSF, was not measured due to a benzene contamination of the hexane used in the extractions.

[a]Mean ± one standard deviation. N = 8.

[b]Mean ± one standard deviation. N = 4.

[c]Principal compound, merged peaks.

Table 9. Specific activities of hepatic microsomal aryl hydrocarbon hydroxylase in coho salmon exposed to a WSF of Cook Inlet crude oil.

Acclimation temperature	Aquaria	WSF concentration[a]	Activity[b] per mg microsomal protein	Activity per g liver	Activity per g body wt.
Experiment II					
	control		3.02 ± 0.20[c]	15.92 ± 1.66	0.15 ± 0.01
	WSF	0.259 ± 0.080	3.48 ± 0.34	21.24 ± 6.96	0.19 ± 0.07
Experiment III					
	control		0.62 ± 0.24	3.10 ± 1.06	0.03 ± 0.02
	WSF	0.119 ± 0.027	1.30 ± 0.35*	7.33 ± 0.98*	0.05 ± 0.01
	control		4.59 ± 0.71	16.33 ± 1.03	0.11 ± 0.02
	WSF	0.346 ± 0.47	2.70 ± 1.00	13.25 ± 5.67	0.14 ± 0.07

WSF exposed fish were significantly different from control fish at *0.05>P>0.01 with 4 d.f.

[a]Concentration expressed in mg/l of the total measured aromatic hydrocarbons from the drains of the aquaria.

[b]Activity is defined as nmoles of metabolites formed in 20 minutes.

[c]Mean ± one standard deviation of the average of duplicate analyses of three separate fish. Duplicate analyses agreed within 8% or less.

Table 10. The fatty acid composition of phospholipids extracted from muscle and gill tissue of control and WSF exposed OTD fed coho salmon (Experiment II, 14.0 C acclimation temperature). Data given are weight percent as fatty acid methyl esters ± one standard deviation.

Fatty Acid	Muscle		Gill	
	Control (3)[a]	WSF (3)	Control (3)	WSF (3)
14:0	0.5 ± 0.1	0.5 ± 0.1	1.1 ± 0.1	0.8 ± 0.1*
16:0	19.2 ± 0.6	21.4 ± 2.0	23.7 ± 1.0	23.1 ± 1.5
16:1ω7[b]	1.0 ± 0.1	1.4 ± 0.1*	2.3 ± 0.3	2.8 ± 0.2*
18:0	7.9 ± 0.5	7.5 ± 0.4	9.4 ± 0.3	8.0 ± 0.3**
18:1ω9	9.9 ± 0.2	9.8 ± 1.5	22.0 ± 0.1	20.5 ± 1.9
18:2ω6	5.8 ± 0.7	5.9 ± 0.4	6.8 ± 0.7	6.1 ± 0.5
18:3ω3 20:1ω9	4.4 ± 3.1	7.1 ± 5.2	4.3 ± 1.1	4.7 ± 2.9
20:4ω6	3.0 ± 1.0	1.8 ± 0.2	3.1 ± 0.3	3.3 ± 0.2
20:5ω3	5.2 ± 0.4	5.2 ± 0.0	2.9 ± 0.7	4.0 ± 0.2
22:5ω3	1.8 ± 0.2	1.7 ± 0.1	1.4 ± 0.2	1.6 ± 0.2
22:6ω3	38.6 ± 1.4	36.2 ± 5.8	20.5 ± 1.6	23.3 ± 4.0
24:1ω9[b]	1.5 ± 0.2	0.9 ± 0.5	1.1 ± 0.9	1.1 ± 0.6
others	1.2	0.7	1.4	0.6

WSF exposed fish were significantly different from control fish at *0.05>P>0.01; **0.01>P with 4 d.f.

[a]Number of individual fish in the sample.

[b]Principal compound, merged peaks.

Table 11. The fatty acid composition of phospholipids extracted from the livers and brains of control and WSF exposed OTD fed coho salmon (Experiment II, 14.0 C acclimation temperature). Data given are weight percent as fatty acid methyl esters ± one standard deviation.

Fatty Acid	Liver		Brain	
	Control (2)[a]	WSF (2)[a]	Control (1)[b]	WSF (1)[b]
14:0	0.3 ± 0.1	0.4 ± 0.0	0.3	0.2
16:0	18.2 ± 2.4	18.0 ± 0.3	15.2	16.4
16:1ω7[c]	0.9 ± 0.3	1.3 ± 0.2	3.2	3.5
18:0	7.9 ± 0.8	7.5 ± 0.3	10.9	9.6
18:1ω9	11.1 ± 0.5	12.1 ± 0.2	26.3	26.9
18:2ω6	6.4 ± 0.6	5.8 ± 0.1	1.0	1.1
18:3ω3 20:1ω9	3.9 ± 3.6	8.9 ± 1.9	2.4	3.0
20:3ω6	0.4 ± 0.0	0.2 ± 0.1	0.3	0.3
20:4ω6	6.5 ± 0.8	4.5 ± 0.9	0.9	1.3
20:5ω3	4.9 ± 3.3	7.2 ± 0.2	4.3	4.4
22:1ω9[c]	——	——	1.1	1.4
22:5ω3	1.7 ± 0.7	2.1 ± 0.1	2.5	2.5
22:6ω3	35.3 ± 7.4	29.9 ± 0.4	22.2	22.1
24:1ω9[d]	1.1 ± 0.4	0.9 ± 0.2	6.6	6.2
others	1.3	1.2	2.8	1.1

[a]Number of individual fish in the sample.

[b]Number of pools of three fish.

[c]Principal compound, merged peaks.

[d]Principal compound, merged peaks in liver samples only.

contributed to the total, was very small. The fourth acid (18:0) significantly decreased in the gill tissue, from a mean of 9.4 in the controls, to 8.0 in the WSF exposed fish. However, the only corresponding increases seen in the treated fish (20:5ω3 and 22:6ω3) were not significantly different from the controls.

Although not statistically significant, 22:6ω3 and 20:4ω6 slightly decreased in both muscle and liver tissues of WSF exposed fish while 18:3ω3; 20:1ω9 increased. Both 18:1ω9 and 20:5ω3 also showed slight increases in the liver and 16:0 increased in the muscle and brain samples.

Both the control and WSF exposed fish consumed the OTD diet at a rate of 1% of their body weight daily for the first 14 days of the experiment. After this time, both groups stopped eating. The effect of food deprivation on the fish can be seen by the large percent weight loss and the decreases in the microsomal protein to liver ratios from the pretest values (9.4 - 12.8) (Table 7). The fatty acid composition of the linseed oil used in the OTD is listed in Table 12.

There were three deaths in the control group during the experiment-on the 14th, 23rd, and 27th days--and no deaths in the WSF group.

Experiment III

In the third experiment, the toxicant concentration at the inlet to the WSF aquaria averaged 0.702 mg/l total measured aromatic hydrocarbons. Due to aeration of the aquaria water and the evaporation of the hydrocarbons, the average concentrations that the fish were exposed to were 0.119 ± 0.027 mg/l in the 20.0 C aquarium (28 day test period), and 0.340 ± 0.047 mg/l in the 7.8 C aquarium (31 day test period) (Table 13). These

Table 12. Fatty acid composition of the linseed oil used in the Oregon Test Diet. Data are expressed as weight percent of the fatty acid methyl esters ± one standard deviation. N = 4.

Fatty Acid	Weight Percent
14:0	0.03 ± 0.01
16:0	5.52 ± 0.09
18:0	3.44 ± 0.03
18:1ω9	20.78 ± 0.01
18:2ω6	16.17 ± 0.04
18:3ω3	53.94 ± 0.10
20:3ω3	0.10 ± 0.00
others	0.03

Table 13. Concentrations of the principal aromatic hydrocarbons in the WSF of Cook Inlet crude oil. Experiment III; 20.0 C and 7.8 C water temperature.

Compound	Concentration (mg/l)				
	Inlet to both aquaria	20.0 C water temperature		7.8 C water temperature	
		Drain	% Loss	Drain	% Loss
Toluene	0.459 ± 0.081[a]	0.085 ± 0.024[b]	81.5	0.221 ± 0.026[c]	51.8
Methylcyclohexane	0.074 ± 0.019	0.000 ± 0.000	100.0	0.000 ± 0.000	100.0
Ethylbenzene m-, p-Xylene	0.062 ± 0.006	0.013 ± 0.001	79.0	0.034 ± 0.006	45.2
o-Xylene	0.047 ± 0.010	0.011 ± 0.001	76.6	0.026 ± 0.003	44.7
Naphthalene	0.018 ± 0.001	0.005 ± 0.001	72.2	0.011 ± 0.003	38.9
1,3,5,-Trimethylbenzene[d]	0.016 ± 0.002	0.001 ± 0.001	93.7	0.009 ± 0.001	43.7
1,2,4-Trimethylbenzene	0.013 ± 0.001	0.002 ± 0.000	84.6	0.008 ± 0.001	38.5
p-Cymene[d]	0.011 ± 0.001	0.002 ± 0.001	81.8	0.007 ± 0.003	36.4
2-Methylnaphthalene	0.008 ± 0.001	0.002 ± 0.001	75.0	0.005 ± 0.001	37.5
Cumene	0.006 ± 0.005	0.000 ± 0.000	100.0	0.001 ± 0.001	83.3
Total measured aromatics	0.702 ± 1.107	0.119 ± 0.027		0.346 ± 0.047	

Benzene, a major aromatic hydrocarbon component of the WSF, was not measured due to a benzene contamination of the hexane used in the extractions.

[a]Mean ± one standard deviation. N = 8.

[b]Mean ± one standard deviation. N = 3.

[c]Mean ± one standard deviation. N = 4.

[d]Principal compound, merged peaks.

concentrations represent 20% and 57% respectively of the incipient lethal level determined previously at 11.6 C. The relative concentrations of hydrocarbons in the WSF followed the same pattern as was observed in the first two experiments. The loss of hydrocarbons due to aeration of the aquaria water was much greater in the 20.0 C WSF aquarium compared to the 7.8 C WSF aquarium. There were no detectable aromatic hydrocarbons in the seawater inlet to the control aquaria.

The 20.0 C acclimated WSF exposed fish had significantly greater hepatic microsomal AHH activity, when expressed on a per mg protein or per g liver basis, than the corresponding controls (Table 9). The AHH activities of the 7.8 C acclimated WSF exposed fish were not statistically significantly different from the corresponding controls.

The AHH activities of both control and WSF exposed fish significantly increased with a decrease in acclimation temperature (Table 9). When expressed on a per mg protein, per g liver, or per g body weight basis, the mean AHH activities of 7.8 C acclimated control fish were respectively, 7.4, 5.3, and 3.7 time greater than their 20.0 C counterparts. When expressed on the same basis, the mean AHH activities of 7.8 C acclimated WSF exposed fish were respectively, 2.1, 1.8, and 2.8 times greater than the corresponding 20.0 C acclimated fish.

There were no apparent differences between AHH activities determined for male and female fish from any of the groups. Results from the duplicate AHH assays of the individual fish liver microsomes agreed within 8% or less ((difference between duplicates/mean of duplicates) x 100).

Tables 14 - 17 list the fatty acid composition of phospholipids of muscle, gill, brain, and liver tissues extracted from control and WSF

Table 14. The effect of acclimation temperature on the fatty acid composition of coho muscle tissue phospholipids from control and WSF exposed fish (Experiment III). Data given are weight percent as fatty acid methyl esters ± one standard deviation.

Fatty Acid	Control		WSF	
	20.0 C (3)[a]	7.8 C (3)	20.0 C (3)	7.8 C (3)
14:0	0.7 ± 0.2	0.5 ± 0.0	0.7 ± 0.0	0.7 ± 0.2
16:0	24.1 ± 2.4	19.9 ± 0.5*	24.0 ± 3.2	21.5 ± 0.8
16:1ω7[b]	1.3 ± 0.3	1.2 ± 0.1	1.4 ± 0.4	1.2 ± 0.1
18:0	9.5 ± 0.6	6.6 ± 0.4**	8.9 ± 0.5	6.7 ± 0.3**
18:1ω9	12.1 ± 1.0	8.8 ± 1.0*	12.2 ± 4.3	9.9 ± 2.1
18:2ω6	6.2 ± 0.8	5.7 ± 0.6	5.7 ± 0.6	6.3 ± 1.3
18:3ω3 20:1ω9	1.4 ± 0.2	1.3 ± 0.2	1.9 ± 0.5	1.1 ± 0.4
20:4ω6	2.5 ± 0.3	2.2 ± 0.3	2.3 ± 0.4	2.3 ± 0.2
20:5ω3	4.7 ± 0.2	5.4 ± 0.3*	4.7 ± 0.2	4.6 ± 0.3
22:5ω3	1.7 ± 0.2	1.8 ± 0.1	1.6 ± 0.1	1.8 ± 0.1
22:6ω3	33.3 ± 3.5	46.0 ± 1.7**	34.3 ± 3.7	41.9 ± 2.9*
24:1ω9[b]	2.7 ± 2.1	0.1 ± 0.0	1.4 ± 1.5	1.2 ± 0.1
others	0.0	0.5	0.9	0.8
PUFA:SMFA[c]	0.99	1.67	1.04	1.41

Cold acclimated fish were significantly different from warm acclimated fish at *0.05>P>0.01; **0.01>P with 4 d.f..

[a]Number of individual fish in the sample.

[b]Principal compound, merged peaks.

[c]PUFA:SMFA = ratio of the sum of polyunsaturated fatty acids to the sum of saturated and monounsaturated fatty acids.

Table 15. The effect of acclimation temperature on the fatty acid composition of coho gill tissue phospholipids from control and WSF exposed fish (Experiment III). Data given are weight percent as fatty acid methyl esters ± one standard deviation.

Fatty Acid	Control		WSF	
	20.0 C (2)[a]	7.8 C (3)	20.0 C (2)	7.8 C (3)
14:0	0.9 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
16:0	26.3 ± 1.0	21.6 ± 0.8*	25.7 ± 1.2	22.0 ± 0.9
16:1ω7[b]	2.6 ± 0.2	2.8 ± 0.1	3.0 ± 0.4	3.0 ± 0.2
18:0	10.5 ± 1.7	7.5 ± 0.4	9.6 ± 0.5	7.0 ± 0.7*
18:1ω9	20.6 ± 1.8	18.3 ± 0.3	10.4 ± 2.6	18.8 ± 0.5
18:2ω6	5.4 ± 1.5	8.2 ± 0.6*	5.9 ± 1.4	8.0 ± 0.8
18:3ω3 20:1ω9	1.4 ± 0.2	1.3 ± 0.4	1.4 ± 0.1	1.7 ± 0.5
20:4ω6	3.4 ± 0.1	4.1 ± 0.3	3.8 ± 0.5	4.1 ± 0.7
20:5ω3	2.6 ± 0.1	4.0 ± 0.3**	2.8 ± 0.3	4.1 ± 0.7
22:5ω3	1.0 ± 0.1	1.6 ± 0.1**	1.2 ± 0.1	1.6 ± 0.3
22:6ω3	23.4 ± 2.2	27.0 ± 0.1*	24.1 ± 5.7	27.6 ± 1.7
24:1ω9[b]	0.9 ± 1.2	1.7 ± 0.1	1.5 ± 0.1	0.7 ± 0.9
others	1.0	1.1	0.0	0.5
PUFA:SMFA[c]	0.58	0.83	0.60	0.83

Cold acclimated fish were significantly different from warm acclimated fish at *0.05>P>0.01;**0.01>P with 3 d.f..

[a]Number of individual fish in the sample.

[b]Principal compound, merged peaks.

[c]PUFA:SMFA = ratio of the sum of polyunsaturated fatty acids to the sum of saturated and monounsaturated fatty acids.

Table 16. The effect of acclimation temperature on the fatty acid composition of coho brain tissue phospholipids from control and WSF exposed fish (Experiment III). Data given are weight percent as fatty acid methyl esters.

	Control		WSF	
	20.0 C (1)[a]	7.8 C (1)	20.0 C (1)	7.8 C (1)
14:0	0.25	0.22	0.25	0.29
16:0	16.00	16.43	17.94	15.44
16:1ω7[b]	3.53	3.10	3.56	3.19
18:0	10.52	10.05	10.78	10.12
18:1ω9	29.59	22.75	26.69	24.57
18:2ω6	1.08	0.96	1.14	1.10
18:3ω3 20:1ω9	2.35	2.19	2.02	2.50
20:3ω6	0.30	0.30	0.30	0.31
20:4ω6	1.01	1.00	1.04	1.03
20:5ω3	3.77	4.32	3.64	4.20
22:1ω9[b]	1.46	1.20	0.93	1.21
22:5ω3	2.07	2.26	2.21	2.41
22:6ω3	20.20	25.94	22.56	22.58
24:1ω9	7.08	5.56	6.36	6.30
others	0.73	3.72	0.58	4.75
PUFA:SMFA[c]	0.46	0.69	0.50	0.64

[a]Number of pools of three fish.

[b]Principal compound, merged peaks.

[c]PUFA:SMFA = ratio of the sum of polyunsaturated fatty acids to the sum of saturated and monounsaturated fatty acids.

Table 17. The fatty acid composition of phospholipids extracted from the livers of control and WSF exposed coho salmon (Experiment III; 7.8 C acclimation temperature). Data given are weight percent as fatty acid methyl exters ± one standard deviation.

Fatty Acid	Control (3)[a]	WSF (3)
14:0	0.5 ± 0.0	0.6 ± 0.2
16:0	19.1 ± 1.4	19.3 ± 0.5
16:1ω7[b]	1.2 ± 1.1	1.3 ± 0.2
18:0	5.7 ± 0.4	5.8 ± 0.4
18:1ω9	11.3 ± 0.8	11.4 ± 0.4
18:2ω6	8.4 ± 0.7	7.9 ± 0.5
18:3ω3 20:1ω9	1.3 ± 0.2	1.4 ± 0.2
20:3ω6	0.3 ± 0.1	0.3 ± 0.0
20:4ω6	6.1 ± 0.1	5.7 ± 0.5
20:5ω3	5.9 ± 0.7	5.1 ± 1.0
22:5ω3	2.3 ± 0.2	2.1 ± 0.4
22:6ω3	36.0 ± 1.2	36.9 ± 0.6
24:1ω9[b]	1.3 ± 0.1	1.2 ± 0.3
others	0.6	1.0

[a]Number of individual fish in the sample.

[b]Principal compound, merged peaks.

exposed fish acclimated to 20.0 C and 7.8 C. The changes in the composition due to temperature acclimation were quite large and significant in the tissues analyzed. The usual pattern of an increase in polyunsaturates with a decrease in acclimation temperature could be seen. For example, in the muscle tissue of control fish, the mean weight percent of 20:5ω3 and 22:6ω3 significantly increased and the mean weight percent of 16:0, 18:0, and 18:1ω9 significantly decreased with a decrease in acclimation temperature (Table 14). These same fatty acid changes were also seen in the muscle tissue of WSF exposed fish; however, only the mean weight percents of two of the fatty acids (18:0 and 22:6ω3) were statistically significantly different.

In the gill tissue of control fish, significant increases were seen in 18:2ω6, 20:5ω3, 22:5ω3, and 22:6ω3, and significant decreases in 16:0 and 18:0 with a decrease in acclimation temperature (Table 15). Again, these same fatty acid changes were also seen in the gill tissue of WSF exposed fish; however, only the decrease in the mean weight percent of 18:0 was statistically significant.

The overall increase in unsaturation with a decrease in temperature is illustrated by comparison of the ratios of the sum of the polyunsaturated fatty acids to the sum of saturated and monounsaturated fatty acids (PUFA:SMFA). With a decrease in acclimation temperature, these ratios increased in the muscle tissue from 0.99 to 1.67 in the control fish and from 1.04 to 1.41 in the WSF exposed fish. In the gill tissue they increased from 0.58 to 0.83 in the control fish and from 0.60 to 0.83 in the WSF exposed fish.

The fatty acids which showed the largest differences in mean weight

percent with an increase in acclimation temperature in brain tissues from control animals were 18:1ω9 and 24:1ω9 which decreased and 22:6ω3 which increased (Table 16). In the brain tissue samples from the WSF exposed fish, 18:1ω9 and 16:0 decreased, and 20:5ω3 increased. The PUFA: SMFA ratios in the brain tissues also increased with a decrease in acclimation temperature, from 0.46 to 0.69 in control fish and from 0.50 to 0.64 in the WSF exposed fish. There were no liver fatty acid samples from the warm acclimated fish due to the high rate of mortality of the experimental animals.

In all of the tissues sampled, there were only two statistically significant differences between individual fatty acid mean weight percents from WSF exposed fish compared to their corresponding controls. These were 16:0 which increased and 20:5ω3 which decreased in the muscle tissue from the 7.8 C acclimated fish. Although not statistically significant, 22:6ω3 alsoe decreased in muscle and brain tissue from these fish but increased in the fish acclimated to 20.0 C. In the brain samples there was also a decrease in 18:1ω9 and an increase in 16:0 in the 20.0 C acclimated fish. These same fatty acids changed in the opposite direction in the 7.8 C acclimated fish. The mean weight percent of individual fatty acids from the livers of the 7.8 C acclimated control and WSF exposed fish were nearly identical (Table 17).

The fish consumed the OMP daily at a rate of 1% of their body weight until the water temperature in the aquaria was altered (on the 7th day of exposure). After this time, they stopped eating. The effect of food deprivation on the fish can be seen by the large percent weight loss, and the decrease in microsomal protein to liver ratios from their

pretest values (9.4 - 12.8)(Table 7). There were no significant differences between control and WSF exposed fish when comparing that ratio; however, WSF exposed 7.8 C acclimated fish had liver and total microsomal protein weights approximately double the weights of the 7.8 C controls. Trends in the control values indicate a decrease in liver weight and total microsomal protein with a decrease in temperature. WSF exposed fish values show the opposite trend but to a lesser degree.

There were four deaths in the 20.0 C control aquarium and two deaths in the 20.0 C WSF aquarium between the 9th and 16th day after the temperature had been raised. There were no deaths in the 7.8 C aquaria.

DISCUSSION

The cellular location, molecular components, and cofactor requirements of the MFO and fatty acid desaturase systems are strikingly similar. Both of these hepatic microsomal electron transport systems contain a hemoprotein and require a reduced pyridine nucleotide and oxygen as cofactors (Nebert et al. 1975, Schenkman et al. 1976). Recent research indicates that interactions between the two systems may exist. For example, Jansson and Schenkman (1975) found that an increase in MFO activity, resulting from injection of rats with phenobarbital, was accompanied by a 50% decrease in stearyl CoA desaturase activity. These results indicate that the interaction may be competitive. However, Ninno et al. (1979) found that administration of 150 ppm dieldrin (an MFO inducer) in the diet of rats resulted in a substantial and persistent increase in the activity of Δ9 desaturation of palmitic acid, suggesting the possibility of co-induction of the two microsomal systems. Thus, the nature of the interaction which may occur is not clear.

The biological significance of an interaction between these two enzyme systems could be considerable. It is well documented that the fatty acid composition of fish lipids is altered during thermal acclimation (Caldwell and Vernberg 1970, Knipprath and Mead 1965). The mechanism responsible for this change has not been determined, but recent experiments indicate that the fatty acid desaturation system may be involved (Ninno et al. 1974, DeTorrengo and Brenner 1976). It is generally believed that the changes seen in lipid composition in thermally acclimating fish are essential for maintaining optimal membrane function (Hazel 1973, Prosser 1973). It follows that any interference with

control of the fatty acid composition of the membrane phospholipids might prove deletrious to the fish.

A recent study by Caldwell et al. (1979) investigated the possible effect that the MFO inducer Aroclor 1254 might have on the fatty acid composition of thermally acclimating staghorn sculpins. They found significant increases in the fatty acid saturation level in fish acclimated to both 7 C and 19.2 C, 12 days after administering the PCB. The authors suggested that the changes seen in the Aroclor treated fish may adapt them, with respect to the biophysical properties of the cell membrane, to a temperature approximately 13 C above their actual acclimation temperature. The study, however, did not present any direct evidence for induction of the MFO system.

The studies reported here represent a continuation of their research, employing coho salmon as the test organism and petroleum hydrocarbons as the xenobiotic. In these studies, salmon were exposed to a water-soluble fraction of Cook Inlet crude oil and the fatty acid composition of tissue phospholipids, and the hepatic aryl hydrocarbon hydroxylase activities were determined in order to investigate whether any observed changes in the fatty acid composition could be related to changes in MFO activities. Grueger et al. (1977) exposed coho salmon for six days to a 150 ppb WSF of Prudhoe Bay crude oil and found induction of AHH (an MFO system) suggesting that experiments with petroleum would be a suitable test system. In the present experiments, salmon exposed to the oil WSF were also thermally acclimated to investigate whether the normal change in fatty acid composition of tissues was affected, as was seen in the sculpin study by Caldwell et al. (1979).

Table 18 summarizes the changes in fatty acid composition seen in several experiments with WSF exposed coho salmon. The table includes all differences greater than 1% of the total fatty acid composition by weight, though, statistically, the majority of the differences were not significant. The sample sizes were small and some of the standard deviations large, which may have masked statistically siginificant differences between the means.

Changes seen in the fatty acid composition of a particular tissue are not consistent between experiments. For example, the results from the first experiment indicate a possible shift in the fatty acid pattern towards an increase in unsaturation in muscle tissue phospholipids as a result of WSF treatment. Both 16:0 and 18:1ω9 decreased while 22:6ω3 increased. Together, these three fatty acids total approximately 72% of the total fatty acid composition by weight. This pattern, however, is not seen in the muscle tissue from fish in any of the other experiments. In fact, in the second experiment, and the third experiment at the 7.8 C acclimation temperature, muscle lipids increased with respect to 16:0 and 18:1ω9 and decreased with respect to 22:6ω3, changes which would increase the overall saturation of the lipids. This result is similar to that seen by Caldwell et al. (1979) with PCB treated sculpins.

Another example of these inconsistencies between experiments is seen in the liver. In the second experiment, though not statistically significant, liver phospholipids showed an increase in 18:1ω9, 18:3ω3, 20:1ω2 and 20:5ω3 and decreases in 22:6ω3 and 20:4ω6, associated with WSF exposure. However, the mean weight percent of individual fatty acids from the liver of the WSF exposed and control fish in the first and third

Table 18. Summary of the changes in the fatty acid composition of tissue phospholipids extracted from control and WSF exposed coho salmon. Only the means showing differences greater than 1% of the total fatty acid composition by weight were noted.

Acclimation Temperature	Fatty Acid	Tissue: Muscle	Gill	Liver	Brain
Experiment I					
12.8 C	16:0	-[a]	+[b]		
	18:1ω9	-			
	22:6ω3	+	+		-
	24:1ω9		-		
Experiment II					
14.0 C	16:0	+			+
	18:0		-**		-
	18:1ω9		-	+	
	18:3ω3 20:1ω2	+		+	
	20:4ω6	-		-	
	20:5ω3		+	+	
	22:6ω3	-	+	-	
Experiment III					
20.0 C	16:0			no sample	+
	18:1ω9				-
	22:6ω3	+			+
	24:1ω9	-			
7.8 C	16:0	+*			-
	18:1ω9	+			+
	22:6ω3	-			-
	24:1ω9		-		

The differences between the means were statistically significant at *0.05>P>0.01; **0.01>P.

[a](-) = mean of WSF exposed fish less than mean of control fish.

[b](+) = mean of WSF exposed fish greater than mean of control fish.

experiments were virtually identical.

Changes in fatty acids are also inconsistent when comparing several tissues from one experiment. For example, in muscle and liver tissues of fish from the second experiment, the percentages of 18:3ω3 and 20:1ω2 increased while those of 20:4ω6 and 22:6ω3 decreased as a result of WSF exposure. However, in the gill tissue from the same fish, 22:6ω3 increased as did 20:5ω3. The fish from this experiment were fed the OTD which contained essentially no ω3 PUFAs other than linolenate. It was thought that if the mechanism involved in the synthesis of PUFA was affected by exposing the salmon to crude oil, it would be easier to detect if the fish were not able to obtain these fatty acids from their food. In fact, there were more differences between the mean weight percent of individual fatty acids from control compared to WSF exposed OTD fed fish. However, there was no consistent pattern to the changes. Also, of the four fatty acids from this experiment which showed statistically significant changes as a result of WSF treatment (16:1ω7 in the muscle, and 14:0, 16:1ω7, and 18:0 in the gill), the actual difference between the means for three of the fatty acids was less than 0.5% of the total composition. Thus, the effect of the differences on the overall fatty acid pattern would have to be considered functionally insignificant.

Because of this lack of consistent differences between control and WSF exposed fish within a group of fish from one acclimation temperature or within one type of tissue from different experiments, it is concluded that exposure of coho salmon to the WSF of Cook Inlet crude oil did not markedly affect the fatty acid composition of the phospholipids, and

consequently is not likely to have significantly affected the biophysical properties of the membrane lipids.

The results from the third experiment indicate that crude oil exposure also did not affect the normal changes in fatty acid composition of tissues phospholipids associated with a change in acclimation temperature. The usual pattern of an increase in unsaturation, with a decrease in acclimation temperature, could be seen in the tissues of both control and WSF exposed fish, though more of the changes were statistically significant in the controls. For example, in the muscle tissue of cold acclimated control fish, 20:5ω3 and 22:6ω3 were significantly increased from the levels seen in the warm acclimated fish, while significant decreases were seen in 16:0, 18:0 and 18:1ω9. These same fatty acid changes were also seen in the muscle tissue of cold acclimated WSF exposed fish with respect to their warm acclimated counterparts; however, the mean weight percent of only two of the fatty acids (18:0 and 22:6ω3) were significantly different (Table 14).

Even though the magnitude of the changes in the individual fatty acids, in the cold acclimated compared to the warm acclimated WSF exposed fish, were not as large as those seen in corresponding control fish, the overall effect of an increase in unsaturation with a decrease in temperature was comparable to that seen in control fish. For example, the ratio of PUFA:SMFA in the muscle tissue increased with a decrease in acclimation temperature from 0.99 to 1.67 in the control fish and from 1.04 to 1.41 in the WSF exposed fish. In the gill tissues they increased from 0.58 to 0.83 in control fish and from 0.60 to 0.83 in the WSF exposed fish.

The PCB treated sculpins, in the study conducted by Caldwell et al.

(1979) also showed an increase in unsaturation with a decrease in acclimation temperature, even though in comparison to controls at both acclimation temperatures, the treated fish had significantly greater saturation levels.

No correlations were observed between changes in AHH activity and changes in fatty acid composition of tissue phospholipids. In the third experiment, only the WSF exposed fish acclimated to 20.0 C had significantly greater mean AHH activity than the corresponding controls (1.30 and 0.62 nmoles/mg protein/20 min. respectively); however, there were no statistically significant differences in the fatty acid composition between these two groups, nor even any indentifiable trend (Table 18). For example, in the muscle a slight decrease in 24:1ω9 and an increase in 22:6ω3 occurred, while in the brain 22:6ω3 and 16:0 increased and 18:1ω9 decreased.

Thus, it appears that in the 20.0 C acclimated WSF exposed fish, an increase in AHH activity did not affect the fatty acid composition of the phospholipids. This conclusion, however, does not exclude the possibility that interactions between the two microsomal systems did occur. The data obtained are insufficient to permit any conclusions regarding the possibility of an interaction between the MFO system and the metabolism of polyunsaturated fatty acids in oil exposed salmon. If an interaction had occurred, the effect on the fatty acid desaturase system may not have been evident in the fatty acids associated with the membrane phospholipids.

The desaturase system has not yet been fully characterized. Recent research indicates that in both rats and fish, a different enzyme may be

responsible for each of the Δ5, Δ6, and Δ9 desaturation reactions (Ninno et al. 1974). It also appears that exposure to 150 ppm dieldrin in the diet of rats affects these desaturases differentially. Ninno et al. (1974) found a persistent increase in the activity of the hepatic microsomal Δ9 desaturation of 16:0; a slight inhibition of the Δ5 desaturation of 20:2 ω9 and 20:3ω6; and no effect on the Δ6 desaturation of 18:2ω6 and 18:3ω3, associated with this treatment. The work by Jansson and Schenkman (1975) and Oshino (1972) with phenobarbital treated rats, also showed the Δ9 desturase system to be affected. The study by Caldwell et al. (1979) suggested that the Δ4 desaturation of 22:5ω3 to 22:5ω6 may have been inhibited by PCB administration. However, this response was inferred from changes in fatty acid patterns rather than by direct measurement of desaturation rates.

Thus it appears that interactions between the two microsomal enzyme systems may be dependent on the substrate to be desaturated. Possibly only Δ9 desaturations are affected by induction of the MFO system. It is not known whether a change in Δ9 desaturase activity would affect the level of 18:1ω9 (a major fatty acid component of membranes) in membrane phospholipids. No differences in mean percent weight were seen in this fatty acid in the 20.0 C acclimated fish with respect to AHH induction.

Another possibility is that no interaction occurred between the two electron transport system. This may have been due to the use of hydrocarbons as a xenobiotic. Selected compounds can differentially induce MFO activities, a result that has suggested to some that these responses may involve different cytochrome P450s (Stegeman 1978, Ullrich and

Kremers 1977). It is possible that the nature of the interaction between the MFO and the fatty acid desaturase systems may be dependent upon the specific P450 involved. For example, Jansson and Schenkman (1975) and Oshino (1972) found an inhibition of the stearyl CoA (9) desaturase system in rats treated with phenobarbital. Ninno et al. (1974) however, found an increase in the Δ9 desaturase activity of palmitic acid, in rats treated with dieldrin. Marine fish treated with Aroclor 1254 showed a significant decrease in the level of polyunsaturated fatty acids (Caldwell et al. 1979). It is possible that the cytochrome P450 induced by exposure of 20.0 C acclimated fish to hydrocarbons does not interact with the fatty acid desaturase system.

The lack of induction of AHH activity in the WSF exposed fish in the second experiment, and in the 7.8 C acclimated fish in the third experiment, could have been due to hydrocarbon concentrations which were too low to stimulate induction at those temperatures. However, this would not seem to be the case if comparisons are made with the results of Grueger et al. (1977). These investigators found induction of benzo-(a)pyrene hydroxylase activity in coho salmon following a six day exposure to 150 ppb of Prudhoe Bay crude oil WSF. In the present study, no induction of AHH activity was found in coho salmon exposed for 31 days to 350 ppb total measured aromatic hydrocarbons (Experiment III, 7.8 C acclimation temperature). Unfortunately, it is difficult to compare the AHH inducing level or toxic concentrations of hydrocarbons with other experiments due to the many variables that can effect the laboratory toxicity of oil to organisms. For example, the manner in which the WSF was obtained, the origin of the oil, the water quality, the time of year

the test was conducted, and whether the system was static or flowing are all important variables. Furthermore, until recently, there have been no standardized techniques for extracting and quantitating the hydrocarbons in seawater. Many of these variables differed between Grueger's experiments and those reported here.

Other factors such as water temperature, time of year, sex, diet, genetic differences and species will all change the response or degree of response of the animal to the toxicant. Grueger et al. (1977) found large individual variations in benzo(a)pyrene hydroxylase activity when exposing coho salmon to a WSF of crude oil. Kuhnhold et al. (1979) found that adult female flounders, exposed to 100 ppb water accomodated fuel oil for up to 60 days, showed no induction of either benzo(a)pyrene hydroxylase activity or aminopyrine demethylase acitvity, yet these same fish contained fuel oil hydrocarbons in their tissues. Payne and May (1979) exposed cunners (Tautogolabrus adspersus) for five days to seven different pure hydrocarbon compounds dissolved or accommodated in seawater. Five of the compounds tested were known mammalian MFO inducers. They found no induction of AHH activity in the liver microsomal fraction with any of the compounds tested.

In both the Kuhnhold et al. (1979) and Payne and May (1979) experiments, the water temperatures were low, 1-10 C and -1-2 C respectively, which may have contributed to the lack of induction of AHH activity observed. Stegeman (1979) determined that after treatment with benzo(a)-pyrene, warm (16.5 C) acclimated fish (Fundulus heteroclitus) had significant induction of AHH while similarily treated fish acclimated to 6.5 C had slightly less AHH activity than corresponding controls. I observed

this same response to temperature in the third experiment. The warm acclimated (20.0 C) WSF exposed salmon had induced levels of AHH while their cold acclimated (7.8 C) counterparts, which were exposed to a higher concentration of hydrocarbons, had lower (but not statistically significant) AHH activities than the corresponding controls. It appears that fish acclimated to warmer temperatures may be more responsive to AHH inducers than those acclimated to colder temperatures.

Dewaide (1970) determined that both roaches and trout acclimated to 5.0 C or 18.0 C, had higher basal levels of aminopyrine demethylase and aniline hydroxylase activities in the liver of the cold acclimated fish. Basal levels of both benzo(a)pyrene hydroxylase and aminopyrine demethylase activity increased in the marine fish Fundulus heteroclitus when the habitat temperature was lowered (Stegeman 1979). I also found an indrease in basal levels of benzo(a)pyrene hydroxylase in coho salmon with a decrease in acclimation temperature.

The fish in all three of the experiments lost weight. The largest difference in weight loss, between the control and WSF exposed fish, occurred in the second experiment. With the data that I obtained, I am not able to say whether starvation of the fish had any affect on the activity of the AHH enzymes, on the desaturation activity, or on the possible interaction between the two systems.

SUMMARY

Various test animals, exposed to chemicals known to induce monooxygenase activities, have shown alterations in certain aspects of lipid metabolism. This study was designed to investigate whether exposure of coho salmon (Oncorhynchus kisutch) to a potential aryl hydrocarbon hydroxylase inducer, a water-soluble fraction of crude oil, had an effect on the fatty acid composition of phospholipids from various tissues.

Salmon, acclimated to four water temperatures (20.0 C; 14.0 C; 12.8 C; 7.8 C), were exposed for periods ranging from 18 to 31 days to a crude oil water-soluble fraction concentration equal to 20%, 30%, 43%, and 53%, respectively of the incipient lethal concentration determined at 11.6 C. Based on the experiment employing temperatures of 20.0 C and 7.8 C, both control and WSF exposed salmon showed a significant increase in AHH activity with a decrease in acclimation temperature; whether expressed on a per mg protein, per g liver, or per g body weight basis. An increase in AHH activity due to exposure of the fish to the toxicant was observed in the 20.0 C acclimated fish only.

The fatty acid composition of the phospholipids extracted from muscle, gill, liver, and brain tissues from all the groups was determined. With minor exceptions, there were no significant differences in individual fatty acid mean weight percents between control and exposed fish at any of the hydrocarbon concentrations or in any of the tissues examined. One group of fish, acclimated to 14.0 C, was fed a semi-purified diet which contained no $\omega 3$ polyunsaturated fatty acids other then linolenate (18:3ω3). It was thought that if exposure to the toxicant

interfered in some manner with the ability of the fish to desaturate its fatty acids, the effect on tissue fatty acid patterns would be more pronounced in the fish fed this diet. These fish also showed no difference in the fatty acid composition of the phospholipids extracted from the tissues of exposed fish compared to their corresponding controls.

The changes in the fatty acid composition of the phospholipids due to acclimation temperature were quite large and significant in control fish, and to a slightly lesser degree in the WSF exposed fish. Cold (7.8 C) acclimated fish from each treatment contained a higher percentage of unsaturated fatty acids than those that were warm acclimated (20.0 C). In the gill and muscle tissues, this change with a decrease in temperature was due primarily to an increase in the mean weight percent of docosahexaenoic acid (22:6ω3) and a decrease in palmitic acid (16:0). The brain fatty acids from the WSF exposed fish showed a different pattern.

The studies reported here indicate that exposure of salmon (O. kisutch) to sublethal levels of petroleum hydrocarbons in water does not alter the fatty acid composition of tissue phospholipids or effect the normal change in fatty acid composition of phospholipids occuring with a change in acclimation temperature. The data obtained are insufficient to permit any conclusion regarding the possibility of an interaction between the MFO system and the metabolism of polyunsaturated fatty acids in oil exposed salmon.

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