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LA THÈSE A ÉTÉ
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Petroleum effects on mixed function oxidases and cortisol balance in the North American eel, *Anguilla rostrata*.

by

Minelia Flena Ledezma de Nava

A Thesis
presented to the University of Ottawa
in partial fulfillment of the
requirements for the degree of
Master of Science
in
Department of Biology

Ottawa, Ontario, 1979

December, 1979.

UNIVERSITÉ D'OTTAWA
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I dedicate this thesis to my husband, Carlos, whose kindness and understanding made the accomplishment of this study much easier.

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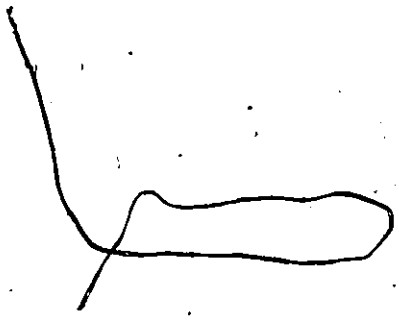

ABSTRACT

American eels, Anquilla rostrata, were force-fed ^{14}C-naphthalene-spiked crude oil for 5 days at a range of doses. Distribution, uptake, and fate of the petroleum hydrocarbon was followed during and after the dosing period. Marked uptake of the ^{14}C-naphthalene occurred by 3 days of dosing, but decreased to low levels by one week of depuration. A compartmentalization of the label was observed both during and after exposure. Water and organic solvent soluble phases occurred with enhancement of the water-soluble fraction particularly for bile. Bile is indicated as a major route of excretion utilizing liver detoxification mechanisms.

Examination of hepatic microsomal enzymes showed benzo[a]pyrene hydroxylase (BaPH) and cytochrome P-450 to be markedly induced by three days of exposure. A significant increase in the protein content of hepatic postmitochondrial fraction of oil-exposed fish was also observed.

Oil exposure in Anquilla rostrata resulted in a generalized adrenal corticoid stress response. Ingestion of crude oil also caused a higher breakdown rate of cortisol when compared to control fish, which appears to be a response to the induction of hepatic microsomal enzymes. Ex-

perimentally increased plasma cortisol levels did not significantly affect the levels of cytochrome P-450 and BaPH in the liver.

RESUME

Des anguilles d'Amérique, Anguilla rostrata, furent nourries pendant cinq jours une gamme de doses de pétrole brut marqué de naphthalène-C^{14}. La distribution, l'absorption et le sort des hydrocarbures furent suivis pendant et après le dosage. Une absorption prononcée du naphthalène-C^{14} eut lieu après trois jours de dosage mais diminus à des concentrations faibles après une semaine de dépuration. Une compartimentalisation du marqueur fut observée pendant et après le dosage. Des phases solubles dans l'eau et dans un solvant organique furent observées, avec un accroissement dans la bile de la fraction soluble dans l'eau. La voie principale d'excrétion, utilisant des mécanismes de détoxification du foie, semblerait être la bile.

L'étude des enzymes des microsomes hépatiques a montré une induction prononcée du benzo[a]pyrène hydroxylase (BaPH) et du cytochrome P-450 après trois jours de dosage. Un accroissement significatif du contenu protéique de la fraction postmitochondriale hépatique fut observé chez des poissons exposés au pétrole.

L'exposition au pétrole chez Anguilla rostrata donna lieu a une réponse généralisée au stress du cortisol et du tissu surrénal. L'ingestion du pétrole brut causa une aug-

mentation du taux de décomposition du cortisol, ce qui semblerait être une réponse à l'induction des enzymes hépatiques. L'augmentation expérimentale des niveaux de cortisol dans le plasma n'affecta pas significativement les concentrations de cytochrome P-450 et de NaPH du foie.

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GENERAL INTRODUCTION

It has become increasingly evident in recent years that complex mixtures of hydrocarbons are trace contaminants of the aquatic environment (Neff et al., 1976b). They enter the environment by three general pathways: biosynthesis, geochemical processes and anthropogenic activities (Clark and MacLeod, 1977). The synthetic activities of aquatic organisms result in a surprising variety of hydrocarbons that are released to the environment during metabolism or after death and decomposition of the organism (Blumer et al., 1969). Geochemical processes, such as weathering of soils and sediments, fallout of hydrocarbons from the atmosphere and diagenesis of organic matter, are also potential sources of hydrocarbons (Farrington and Meyers, 1975).

Petroleum hydrocarbons originating from human activities are quantitatively the most significant and are most easily identified. The major anthropogenic sources of oil pollution in the environment are those associated with marine transportation and terrestial run-off. Although much

of the public concern about petroleum pollution is a consequence of the effects of accidental release of petroleum hydrocarbons, these spectacular events contribute only a small fraction of the total oil that enters the aquatic environment. Land-based discharges such as those originating from refineries, waste oils, run-off and sewage, represent a major source of petroleum hydrocarbons in the environment and accounted for 41% of the total worldwide load of petroleum hydocarbons to the oceans for 1973 (National Academy of Sciences, 1975).

Activities associated with the tranportation of oil products represent the second most significant input of petroleum hydrocarbons to the aquatic environment. They include the release of waste fuels from vessels as well as the discharge of petroleum from washing of tankers (Clark and MacLeod, 1977). Oil spills resulting from accidental release of crude oil are also an important source of petroleum pollution associated with the transportation of oil products. Although major spills have been known to occur at sea, freshwater spills such as the Nepco 140, which took place in the Thousand Islands region of the St. Lawrence river in 1976, are also of major concern.

Offshore petroleum exploration and production activities contribute less than three percent of the total oil intake of the aquatic environment (Grossling, 1976). It includes discharges of petroleum through minor spills due to

production operations as well as major accidents such as ruptures of pipelines that carry the crude oil from production platforms to shore (Clark and MacLeod, 1977).

The impact of oil on the aquatic environment is governed by physical, chemical and biological factors in addition to the inherent complexity of crude oil and refined products. Different crude and refined oils, for instance, vary tremendously in their relative concentration of different compounds. As a result, they may show differences in solubility, dispersability and persistence in the water column (Neff et al., 1976a). It is generally accepted, however, that refined oils are usually more toxic than crude oil, first of all because many refined oils have a very high proportion of aromatic hydrocarbons and second, because they are usually less viscous than crude oil which results in a higher solubility in the water (Pice et al., 1977).

The different hydrocarbons commonly encountered in oil vary substantially in their toxicities. The aromatic fraction, however, has received the most attention as it has been known to account for most of the toxicity attributed to water-soluble fractions of different crude and refined oils.

Several studies have been carried out in order to establish the the toxicity of individual aromatic hydrocarbons in fish and invertebrates (Neff et al., 1976a; Caldwell et al., 1977; Wilber, 1969). In general, it can be said that monoaromatics appear to be the least toxic. Acute toxicity

increases with increasing molecular size until the 4 and 5 ring compounds are reached. The aromatics of higher molecular weight are not acutely toxic, however, they can be implicated in chronic toxicity since some are known to be potent carcinogens. In addition, they may contribute to long-term damage because of their tendency for long-term accumulation in body tissues (Neff et al., 1976b). Alkylation of the aromatic nucleus appears to increase the toxicity of the parent compound. Thus in both the benzene and naphthalene series toxicity increases with increasing degress of alkylation (Rice et al., 1977).

The impact of oil on the aquatic environment depends not only on the chemical complexity of the petroleum. Biological factors resulting in differences in sensitivity among species and individuals also play an important role in the expression of toxic effects of petroleum hydrocarbons. Aquatic organisms vary tremendously in their sensitivity to oil. Although no generalization can be established in view of the large differences observed, estuarine and benthic species are often, but not always, more tolerant than pelagic species (Neff et al., 1976a). In addition, it has been proposed that species and age-related differences in sensitivity to oil in fish may be related to their capabilities for metabolizing and excreting petroleum hydrocarbons (Lee et al., 1972a).

Attempts have been made to assess the impact of oil pollution on the developmental stage. At this time, generalizations about greater sensitivity to oil during early stages cannot be made. Some trends, however, have been observed: eggs appear to be more tolerant than other life stages, probably because of the protective shield offered by the surrounding chorion (Rice et al., 1977) ; larvae seem to be more vulnerable than adults. This is particularly true in most fish and crustacean species tested. Additional information, however, is required for many other groups of animals (Rice, 1973; Rice et al., 1977).

The mechanisms by which petroleum toxicity is mediated are not very well known. However, it appears that hydrocarbons preferentially associate with structures containing lipids such as plasma and nuclear membranes as well as organelle membranes. In addition it seems that many biological effects will depend on the charateristics of morphological and functional damage to normal membranes caused by the foreign compound (Stegeman, 1977).

Besides, any effect will of course vary with the concentration and exposure duration. Thus, extremely high levels of hydrocarbons will lead to interference with cellular and subcellular process resulting in the organism's death. A good example to illustrate this situation is the damage of the respiratory epithelia of gills after acute exposure of rainbow trout, Salmo gairdneri, to different hydrocarbon

mixtures (MacKeon and March, 1977). The destruction of the gill epithelium may result in blockage of gas exchange resulting in respiratory collapse and death of the fish. On the other hand, sublethal levels of hydrocarbons will also interfere with cellular and physiological processes but they won't cause immediate death, although death may follow due to indirect causes (Moore and Dwyer, 1974). For instance, exposure to petroleum of aquatic organisms is known to have a negative impact in suppressing immune response and disease resistance which may result in increased mortality of the organisms directly because of disease or indirectly because of reduced vitality (Hodgins et al., 1977). Whereas most studies of the biological effects of petroleum hydrocarbons in aquatic organisms have been directed mainly to the toxicity effects resulting from acute exposure, it is becoming evident that chronic low levels of oil pollution can cause related sublethal changes which are potentially dangerous to the ecosystem. For instance, disruption of behaviour, especially feeding and reproduction, may result in total elimination of a species from a geographical area. Once eliminated, the species can perhaps not repopulate the area because of the direct effects of continuing pollution or because its niche has been filled by a more tolerant species (Evans and Rice, 1974). It is known for example that different biological processes such as finding of food, escape from predators and sex attraction are often dependent on

chemical attraction based on very low levels of chemical substances dissolved in the water (Blummer, 1971). Aromatic hydrocarbons have been found to interfere with such processes resulting in disruption of feeding and reproduction of fish (Hasler, 1970).

Investigations to elucidate the effects of sublethal doses of petroleum hydrocarbons on organisms can be categorized as physiological studies, behavioral studies and investigations of growth and reproduction. In the case of physiological studies, research has been directed towards alterations in metabolism after exposure to petroleum hydrocarbons.

The effect of oil pollution on metabolism is often assessed by measuring changes in respiratory rate of individuals following exposure. In fish, respiration has been estimated by measurements of oxygen consumption or by a related index, the rate of opercular movement. It has been observed that the magnitude and nature of the respiratory response depends not only on the individual and species characteristics, but also on the nature and concentration of the hydrocarbons tested. For instance, the oxygen consumption of the goby, Chaenogobius heptacanthus, was measured after exposure to phenol, lamp oil and lubricating oil. It was observed that increases in concentrations of phenol or suspended oil resulted in a decrease in oxygen consumption while increasing concentrations of lamp oil caused an in-

crease in oxygen consumption (Syasuki, 1964, cited in Patten, 1977). Similarly, when the oxygen consumption of the sheepshead minnow, Cyprinodon variegatus, following exposure to four different oils was measured, it was found that the concentration required to elicit a change in respiratory rate was different among all the oils tested (Anderson et al., 1974).

In general, the respiratory response appears to be transitory, being most significant during or immediately after exposure and rapidly reaching control levels when animals are returned to clean waters. Transitory changes in breathing and coughing rates were observed by Thomas and Rice (1975) in pink salmon, Oncorhynchus gorbusha, exposed to the water-soluble fractions of Prudhoe Bay crude oil. Breathing and coughing rates rose to a maximum in 3 to 6 hours after beginning of experiment and then dropped back towards control rates. In addition, when the fish were returned to oil-free water, breathing and coughing rates rapidly returned to normal values.

Besides the observed changes in respiratory rate, some other metabolic and biochemical changes result from low levels of oil pollution. For instance, Sabo and Stegeman (1977) indicated that the killifish, Fundulus heteroclitus, from an oil polluted area showed marked differences in the rate of phospholipid synthesis as well as a decrease in the rate of triglyceride formation from acetate when compared to

control fish obtained from an "uncontaminated area". They also suggested that these changes may reflect an altered membrane structure either intracellularly or at the cell surface, since phospholipids along with cholesterol are major constituents of most membranes. Similarly, analysis of hepatic metabolism in the croaker, Stenotomus versicolor, exposed to petroleum hydrocarbons showed a significant reduction in the rate of acetate incorporation into lipid (Stegeman and Sabo, 1976).

Fish exposed to different levels of petroleum in the environment show various behavioral responses. Changes in locomotor and activity patterns appear to be a very common response induced by oil exposure. Reactions of juvenile coho (Oncorhynchus kisutch) and sockeye (Oncorhynchus nerka) salmon exposed to Prudhoe Bay crude oil at concentrations of 0.5 to 3.5 ppm were studied by Morrow (1973). At all concentrations, the fish swam at the surface of the water after 45 minutes of exposure. After 24 hours, the fish showed loss of equilibrium and reduced swimming activity. Salmon swam in a vertical (head up) position and did not recover. Similar swimming behaviour abnormalities were observed after exposure of juvenile chinook salmon, Oncorhynchus tshawytscha, and pink salmon to No. 2 diesel oil layered on the water at concentrations of 150-3,000 ppm. It was noticed, however, that if the fish were returned to clear water, a great percentage regained equilibrium (Cardwell, 1973).

Longnose killifish, Fundulus similus, subjected to the water-soluble fraction of No. 2 fuel oil at a concentration of 7 ppm for 20 minutes were also able to recuperate and regain equilibrium within 3 to 5 hours (Dixit and Anderson, 1977).

Changes in growth and reproduction of organisms have similarly been observed as a result of petroleum exposure; however, the literature available, particularly in fish, is very scarce. Rice et al. (1975) indicated that exposure of pink salmon to Prudhoe Bay crude oil at a concentration of 0.7 ppm resulted in a reduction in growth rate of alevins and fry. It was also found that alevin growth was inversely related to the water-soluble fraction concentration and this effect was more pronounced at later stages of development. Similarly, exposure of Pacific herring larvae, Clupea harengus pallasi, to 12 ppm of benzene dissolved in the water resulted in growth delay (Struhsaker et al., 1974).

Anderson et al. (1976) carried out a study to establish the influence of water-soluble fractions of No. 2 fuel oil and South Louisiana crude oil on the development rate and hatching success of embryos from three species of estuarine fish. Reduction of hatching success was observed for 2 species and the decrease in hatchability of eggs was apparently related to the contents of total hydrocarbons and total naphthalene in the exposure media. On the other hand, treatment of 3 year old rainbow trout for 6 to 7 months with Prudhoe Bay crude oil administered in the diet caused no ef-

fect on the mean hatching success. In addition, no statistically significant differences were found between the average survival of alevin of non-oil fed parents and that of oil fed parents (Hodgins et al., 1977).

In addition to the toxic effects previously mentioned, exposure to petroleum hydrocarbons in fish results in the activation of a multi-enzyme system localized in the microsomal fraction of cells kncwn as mixed function oxidases (MFO). In particular aryl hydrocarbon hydroxylases (AHH) are known to be highly inducible by a variety of petroleum products (Payne and Penrose, 1975; Burns and Sabo, 1975, James et al., 1977). The physiological implications of this induction however are not quite understood and our knowledge of the biotransformation of petroleum hydrocarbons in fish is rather limited. I have chosen to study this biotransformation in the American eel, Anguilla rostrata, because the species occurs in areas where it is likely to find different sources of chemical pollutants, including petroleum hydrocarbons. In addition, as a top predator and occasional scavenger (Tesch, 1977), the American eel is probably exposed to relatively high levels of foreign compounds accumulated along the food chain. From a commercial point of view, with the exception of salmon and trout, hardly any species of food fish has such a worldwide market as does the eel (Canada Fisherman, 1964). In canada, eels are fished commercially in the provinces of Quebec, Ontario and New

Brunswick. The national consumption, however, is very limited and most of the eel catch is exported to European countries such as Germany, Belgium, England and Denmark where they are considered a delicacy (Bedard, 1975). There are also practical reasons for using eels: first of all they are inexpensive and easy to obtain and secondly, eels can be kept under laboratory conditions without any major requirements in holding facilities.

In an attempt to understand the physiological significance of the MFO response to petroleum hydrocarbons in fish the following experimental approach was followed: in the first place, a study was carried out to establish some parameters in the uptake and release of crude oil from organs and body fluids of the eel. These parameters could then be used to correlate with high levels of hepatic microsomal enzymes observed in eels after petroleum exposure. Secondly, levels of hepatic MFO activity were determined after oral administration of crude oil and were related to length of exposure and dosing regimens. The enzymes measured were benzo[a]pyrene hydroxylase (BaPH) and cytochrome P-450. This is important because MFO's are known to act as the main machinery for the metabolism of petroleum hydrocarbons, therefore they may play an important role in determining the toxic effects of petroleum products.

Microsomal enzymes involved in the metabolism of foreign compounds are also known to take part in the metabolism

of steroid hormones (Conney, 1967). Therefore it was proposed that induction of MFO by petroleum may result in alterations in steroid balance. In order to test this hypothesis the dynamics of cortisol changes after exposure to crude oil was studied. This was done in order to establish whether or not petroleum induces a general cortisol stress response in eels. In addition, a separate study was carried out to assess the effect of MFO induction on the breakdown of cortisol.

Chapter I

UPTAKE AND CLEARANCE OF INGESTED ^{14}C-NAPHTHALENE PETROLEUM

1.1 INTRODUCTION

It is a generally accepted view that the ability of petroleum hydrocarbons to elicit any toxic effect is highly related to its capacity to enter and accumulate in the organism. Therefore absorption, distribution and release parameters are important factors determining the biological fate and effects of these compounds. Petroleum hydrocarbons are readily taken up into aquatic organisms. In addition, based on present data, there seem to be no restrictions on the type of hydrocarbon that can be absorbed (Stegeman, 1977).

Recent reports have discussed the fate of petroleum hydrocarbons in a variety of marine animals (Varanasi and Malins, 1977); however, there is not enough information on the accumulation and metabolic fate of petroleum in fish. Most studies have been carried out using a water-soluble fraction (WSF) under static bioassay conditions or using individual hydrocarbons. A very limited number of studies have looked at the uptake and clearance of petroleum hydrocarbons using ingestion as a route of administration. Several studies, however, have indicated that aquatic organ-

isms, including fish, ingest and retain hydrocarbons to which they are exposed. In addition, these hydrocarbons are transfered and retained by predators; therefore, even animals that have not been directly exposed to the hydrocarbons can become polluted by eating contaminated food (Blumer, 1971).

Studies done on fish indicate that although they readily take up hydrocarbons from the environment, substantial decreases in tissue oil concentrations occur once the animals are placed in clean water. This seems to indicate the existance of a metabolic machinery for the removal ot these compounds from body tissues. Support for this idea comes from studies indicating that several fish species extensively metabolize hydrocarbons to form both conjugated and non-conjugated derivatives. For instance, in a study carried out by Lee and co-workers (1977a) it was established that mudsucker (Gillichthys mirabilis), sculpin (Oligocothes maculosus) and sanddab (Citharichthys stigmaeus) were able to metaboilize naphthalene and 3,4-benzo[a]pyrene to dihydrodiols. In another study Varanasi et al. (1978) looked at the metabolism of ^{3}H-naphthalene administered through the diet in 3 species of fish. The hydrocarbons were extensively metabolized, and furthermore more than 80% of the radioactivity found in blood after 168 hr was due to metabolic products. The highest concentration of metabolites occurred in the gall bladder.

The distribution of accumulated hydrocarbons in various tissues may be influenced by a large number of factors such as lipid quantity and quality of the tissues, the degree of association with carriers in the plasma, the route of entry, and the extent of metabolism to which the hydrocarbon is subjected in the different tissues (Stegeman, 1977). According to previous studies, it appears that hydrocarbons and/or metabolites tend to accumulate predominantly in tissues that exhibit a high lipid content or represent apparent major metabolic and excretion sites. Numerous reports have indicated the tendency of different aromatic fractions to accumulate in visceral fat and brain (Korn et al. 1976; Whittle et al.., 1976; Melancon and Lech, 1978). Similarly, Roubal (1974) showed that substantial amounts of alkanes are accumulated not only in brain but also in spinal cord and lateral line nerves. In addition, Collier and Malins (cited in Malins et al., 1979) indicated that brain accumulated considerable amounts of naphthalene but unlike other organs it contained relatively low levels of bioconversion products. So, it appears that brain and other parts of the nervous system tend to accumulate significant levels of petroleum hydrocarbons, which probably relates to the loss of equilibrium and other behavioral abnormalities observed in fish after exposure to oil products (Dixit and Anderson, 1977).

Liver and gall bladder, organs associated with the metabolic breakdown and excretion of petroleum hydrocarbons, are also known to show very high levels of petroleum hydrocarbons. Different studies have indicated that gall bladder usually exhibits the highest levels of hydrocarbons among all organs examined. For instance, Melancon and Lech (1978) showed that short-term exposure of rainbow trout to naphthalene and methylnaphthalene resulted in bile concentrations of both chemicals of up to 300 times higher than that initially present in the water. In a similar experiment, they also found that long-term exposure of trout to naphthalene resulted in bile concentration for napthalene and metabolites of 300 µg/ml (approximately 13,000 times the water concentration of naphthalene). The high levels of hydrocarbons generally found in the bile of fish after exposure appear to be mainly due to the presence of metabolic products (Roubal et al., 1977; Melancon and Lech, 1978). The gall bladder is suggested to play an important role in the excretion mechanisms by which petroleum hydrocarbons are eliminated from body tissues in fish.

Liver has been reported to accumulate significant levels of petroleum hydrocarbons (Lee et al., 1972a; Korn et al., 1976). Furthermore, Roubal et al. (1977) indicated that intraperitoneal administration of selected hydrocarbons in coho salmon resulted in significant percentages of metabolic products at all times, irrespective of the hydrocarbon

administered. This finding is consistent with the known role of the liver in the metabolism of petroleum hydrocarbons and is probably due to the relatively high levels of aryl hydrocarbon hydroxylases present in this organ (Malins, 1977).

Studies on the accumulation and discharge of petroleum hydrocarbons in aquatic organisms have been largely restricted to alkanes and aromatic hydrocarbons. Present data indicate that substantial differences exist among species in the ability to retain and release various petroleum hydrocarbons. For instance, it has been found that fish and shrimps tend to accumulate aromatic hydrocarbons very rapidly. At the same time, they tend to release hydrocarbons more rapidly. On the other hand, clams and oysters tend to accumulate and release hydrocarbons more slowly. Some reports have shown a very low capacity of molluscs to metabolize aromatic hydrocarbons (Lee et al., 1972a) and this may explain in part the lower rate of release observed in oyster and clams.

Several studies have indicated that rates of uptake and release are also dependent on the nature of the hydrocarbon itself. For instance, Lee and co-workers (1972a) investigated the uptake and discharge of ^{14}C-naphthalene and ^{3}H-benzo[a]pyrene in mudsucker, sculpin and sanddab. They found that in all species naphthalene tended to accumulate more than benzo[a]pyrene. In addition, both hydrocarbons

were rapidly excreted in the urine; however, naphthalene and its metabolites were depurated at a faster rate than benzo[a]pyrene and its metabolites. Similarly, Roubal and co-workers (1977) administered ^{14}C-labelled benzene, naphthalene and anthracene to young coho salmon in the food and by intraperitoneal injection. It was found that regardless of the method of exposure accumulation in liver, brain and muscle was achieved in the following order anthracene > naphthalene > benzene. In other words, it appears that these aromatic hydrocarbons accumulate in key organs in relation to the number of benzenoid rings in the petroleum hydrocarbon molecule.

In another study, the uptake and release of substituted and non- substituted benzenoids and naphthalenes into muscle of coho salmon and starry flounder (Platichthys stellatus) was followed (Roubal et al., 1978). The results obtained indicated a strong tendency for coho salmon and starry flounder to accumulate alkyl-substituted aromatic hydrocarbons through the water column in preference to unsubstituted structures. Moreover, the hydrocarbon retention was found to increase in proportion to the extent of ring substitution of the hydrocarbon molecule. Thus, it appears that two structural properties (degree of alkylation and number of benzenoid rings) are related to the disposition of aromatic hydrocarbons in fish.

As mentioned previously, the ability of petroleum hydrocarbons to exert a toxic effect is related to its capacity to enter into the organism, the rate at which it is accumulated and the degree to which it is retained in the tissues following exposure (Neff et al., 1976a). In this regard, the absorption, distribution and metabolism of hydrocarbons are important factors that determine the expression of toxic effects elicited by these compounds, and must be accounted for in the interpretation of any biological findings.

The present study was undertaken to evaluate the dynamics of uptake, clearance, as well as tissue distribution, of petroleum hydrocarbons in the American eel, Anguilla rostrata following dietary exposure of crude oil. Analysis of the distribution and release from tissues and body fluids of hydrocarbons may give some understanding of the mechanisms by which fish clear petroleum hydrocarbons from their systems.

In chapter II, evidence is given about the stimulatory effect caused by the administration of crude oil on the activity of the MFO system. It was considered that absorption, release and distribution parameters of petroleum hydrocarbons should be established in order to best interpret the mechanisms as well as the physiological significance of such stimulation.

It is known that, in most cases, the metabolism of hydrocarbons results in the conversion of lipophilic compounds into water- soluble metabolites which can be excreted (Adamson, 1967). In the present study, a two-phase partition technique based on this principle was used to extract hydrocarbons from tissues and body fluids. Although no inferences can be made about the nature of specific metabolic products, this extraction procedure gives some idea about the levels of such products present in tissues and body fluids and the related degree of petroleum metabolism.

MATERIALS AND METHODS

1 Experimental Animals

Immature American eels (Anguilla rostrata LeSueur) of weights ranging from 70-100 grams were used as experimental animals. They were taken at the St. Lawrence river from an eel ladder placed at the Robert Sanders Generating Station at Cornwall, Ontario. Immediately after arrival in the laboratory, the eels were placed into a large tank with dechlorinated Ottawa City tap water for a period of 3 to 4 days. The purpose of this procedure was to allow for the removal of extra mucous and debris as well as elimination of sick animals. Eels were then transfered to 430 L fiberglass tanks fed with circulating dechlorinated tap water at a temperature ranging between 8 and 15°C, winter to summer, respectively. Photoperiod was approximately 12 hours of fluorescent light alternating with 12 hours of darkness (or low-intensity red light if observations were necessary at night). The animals were allowed to acclimate under the conditions described for a period of at least 3 to 4 weeks prior to use. The eels were not fed during the experimental period.

2 Experimental Procedure

The fate of petroleum hydrocarbons after ingestion was studied using a fresh crude oil obtained from Norman Wells, N.W.T. This oil is characterized by a relatively

high content of low boiling point hydrocarbons (Engelhardt, 1978). Two separate experiments were carried out to establish the uptake, distribution and release of hydrocarbons in fish following ingestion exposure of 10 and 100 µl oil/kg fish crude oil levels.

A simple technique was used to administer orally calculated amounts of crude oil spiked with ^{14}C-naphthalene (uniformly labelled; specific activity: 3.67 mCi/mmol naphthalene, Amersham/Searle, Arlington Heights, Ill.). The amount of labelled naphthalene used was previously calculated so that it would not alter the naphthalene composition of the crude oil. The spiked oil was mixed with beef-liver homogenate. A total volume of 0.1 ml was injected into the gut of the fish via the mouth. A 1.0 ml syringe was used, fitted with a gavage needle to avoid damage to the gut tissue. Careful handling ensured no regurgitation of the oil. Experimental animals were given single doses containing 0.5 µCi (in both 10 and 100 µl/kg) of ^{14}C-naphthalene for 5 consecutive days. Control fish were force-fed beef-liver homogenate only for the same period.

After dosing, fish were replaced into the holding tanks previously described. Five fish were sampled at each time period of 1,3 and 5 days after initiation of exposure. At the end of the 5 days of dosing, the remaining fish were transfered to another tank to follow the clearance of the oil dose. Again, groups of 5 fish were removed on days 3,6

and 12 of clearance. Control groups of 3 fish each were sampled simultaneously throughout the experimental period and were used to establish analytical and physiological baselines for oil-exposure-related experiments.

3 Analytical Methods

At each sampling, fish were anaesthetized in 2% MS222 solution (Ethyl-m-aminobenzoate methane sulfonic acid salt; Sigma Chemical Company) for approximately 3 minutes. The fish were killed by decapitation and samples of tissues and body fluids were taken. The tissues examined were liver, blood, gall bladder, heart, gills, white muscle, posterior kidney and brain. Extreme care was taken not to puncture the gall bladder during removal. Bile samples were taken. In addition, prior to excision, the liver was flushed with a perfusion fluid for fresh water eels (Shuttleworth, 1972) to remove blood trapped in the hepatic circulation. Each tissue was tested for radioactivity by the following procedure. A 200 mg sample was homogenized with 1 ml of distilled water using a motor-driven all-glass Potter-Elvehjem tissue grinder immersed in ice. Bile samples were not homogenized. After complete homogenization, an extraction with 4 ml of a 1:1 methanol-benzene solution (only spectral grade solvents were used) was carried out for 24 hours in teflon-stoppered test tubes on a rotator at a temperature of approximately 4°C. This extraction solution was then centrifuged at 1400

x g for 10 minutes. Aliquots of the organic and aqueous phases were tested separately for ^{14}C-activity. Radioactivity in the solvent phase was used to establish concentration of naphthalene spiked crude oil fractions while the aqueous layer was used to estimate values for total metabolites. Re-extraction of the residual pellet indicated complete elimination of solvent extractable radioactivity. In addition, only background activity was found in the NCS-digested pellets indicating that the extraction procedure for ^{14}C-naphthalene approached 100% efficiency. Recovery of known amounts of ^{14}C-naphthalene following the two-phase partition technique was carried out using labelled crude oil. The extraction procedure resulted in an organic/aqueous phase partition coefficient of zero, as no radioactivity above background levels was detected in the aqueous phase. A similar partition coefficient of the ^{14}C-naphthalene was found in bile and liver samples spiked with known quantities of the labelled oil.

One-half ml aliquots of the organic and aqueous layers were counted by liquid scintillation procedures, after mixing with 10 ml of Aquasol-2 (NEN, Canada) and Econofluor (Fisher Scientific, Canada), respectively. Determinations of ^{14}C-activity were done by counting samples in an LKB 1215 Rackbeta Scintillation counter. Quench-corrections were estimated using quench curves constructed with the patented Hat-trick calibration kit (LKB, Wallac, Finland).

RESULTS

The radioactivity levels in tissues and bile of the American eel A. rostrata during and following ingestion of a ^{14}C-naphthalene spiked crude oil at a dose of 10 µl/kg are shown in Fig. 1 and 2. Statistical differences among and within treatments were established using a two-factor analysis of variance ($P < 0.05$).

Two different patterns in uptake and release were observed for the solvent extractable fraction. Tissues such as liver, heart, blood, muscle, kidney, and gill showed a significant rise on the third day, followed by a drop on day 5 to day 1 levels. No further changes in radioactivity were observed between days 5 and 17 in these tissues. A second pattern, characterized by a later peak at the end of the dosing period (day 5), was observed for brain. This increase in radioactivity levels was followed by a gradual drop by day 8 (during the depuration period), resulting in tracer levels similar to those of day 1. Radioactivity levels remained unchanged throughout the rest of the experimental period.

The water-soluble fraction, which represents metabolized hydrocarbons, also showed two different patterns in uptake and clearance. With the exception of liver and blood, all tissues examined showed a significant increase in radioactivity at day 5 followed by a drop to day 1 levels during the depuration period at day 8. Bile levels also peaked

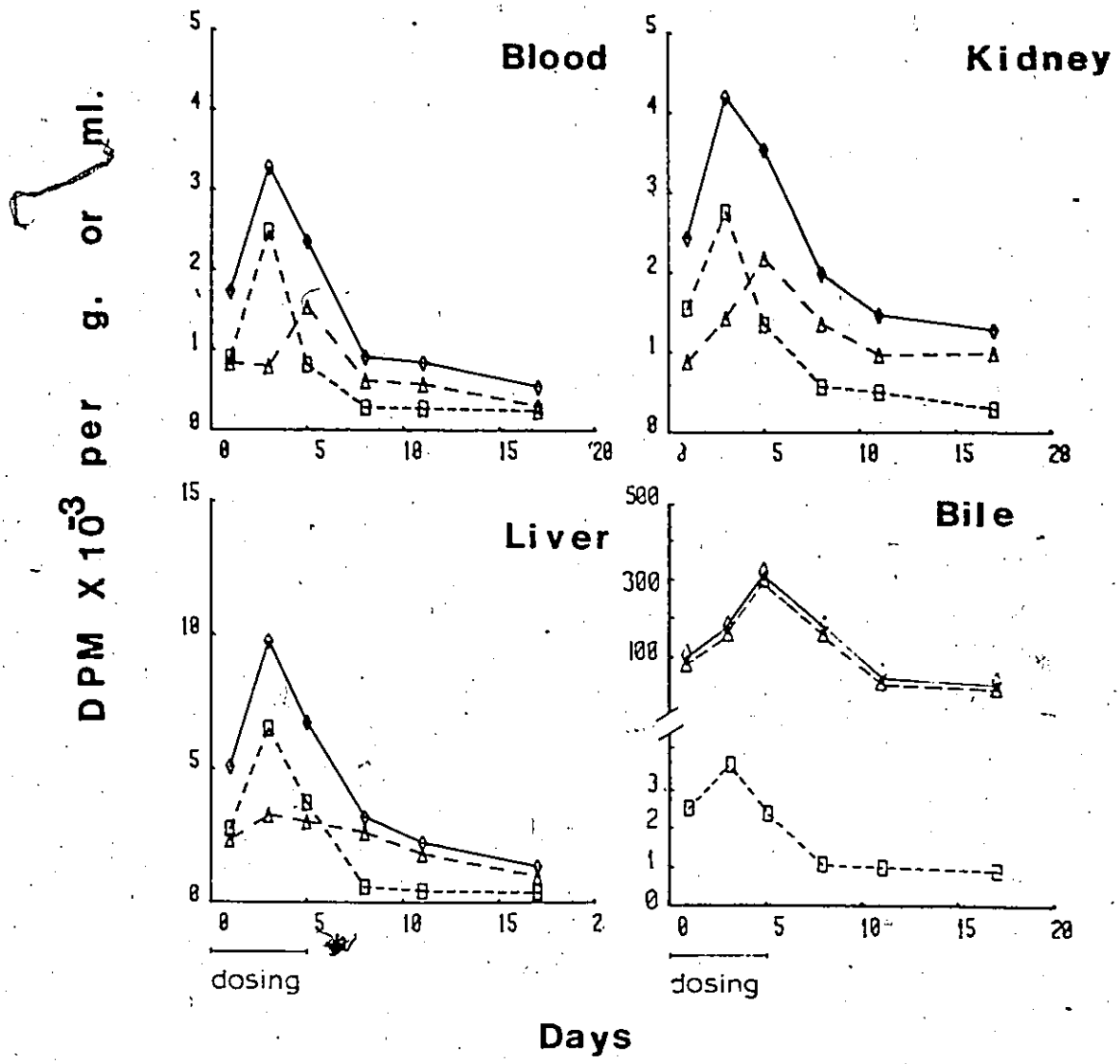
Radioactivity levels in selected tissues and bile of the American eel, Anguilla rostrata, during and following ingestion of a ^{14}C-naphthalene spiked crude oil at a dose of 10 µl/kg, given for five consecutive days (0.5 µCi/dose). Each point represents the mean of 5 fish.

◇——◇ , total radioactivity

□- - -□ , activity in organic solvent fraction

△- - -△ , activity in aqueous fraction

Figure 1: Tissue and bile radioactivity: 10µl/kg oil dose


Blood
Kidney
Liver
Bile
DPM X 10^-3 per g. or ml.
dosing
dosing
Days

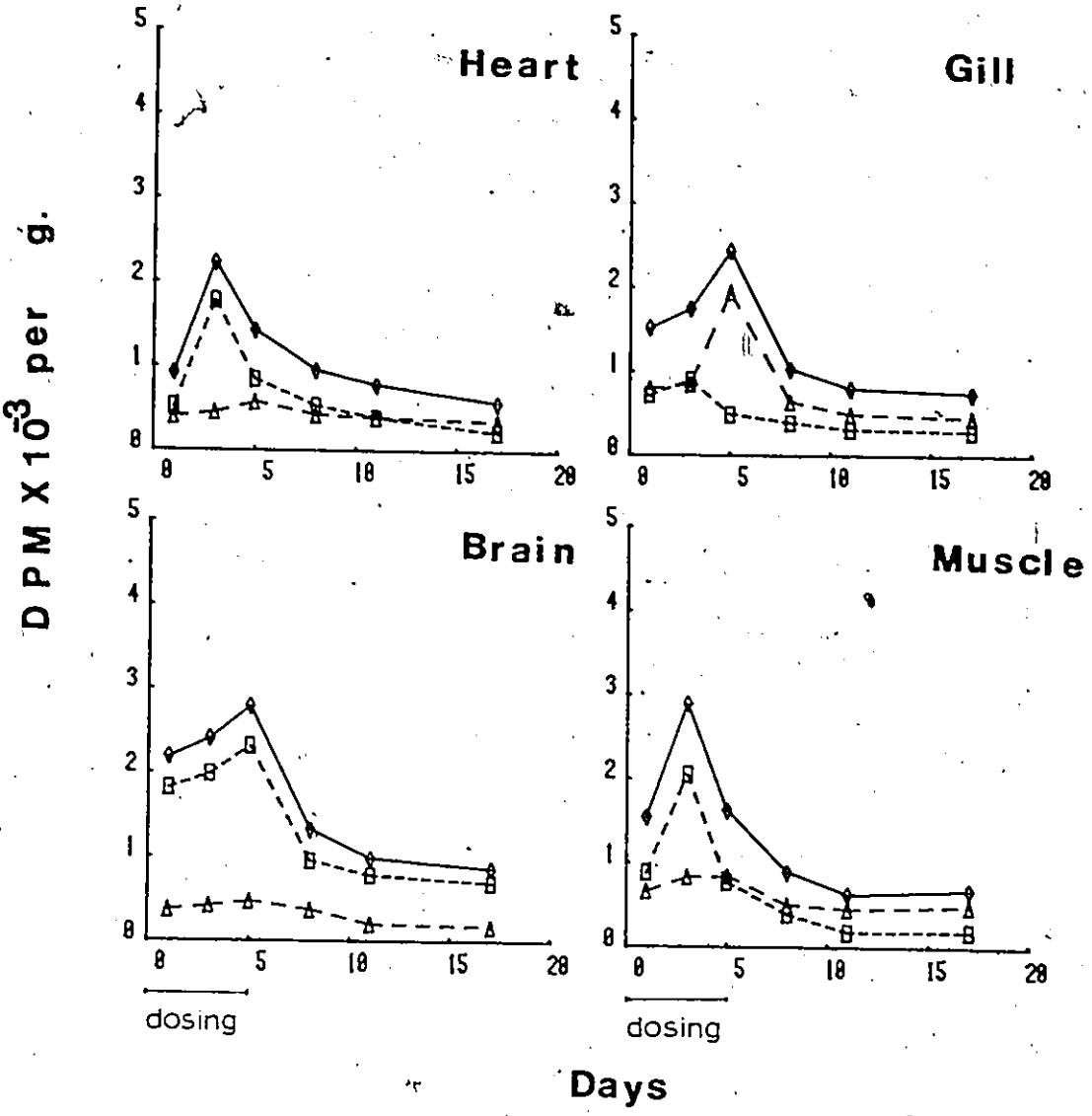
Radioactivity levels in selected tissues of the American eel, Anguilla rostrata, during and following ingestion of a ^{14}C-naphthalene spiked crude oil at a dose of 10 µl/kg given for five consecutive days (0.5 µCi/dose). Each point represents the mean of 5 fish.

◇——◇ , total radioactivity

□- -□ , activity in organic solvent fraction

△- -△ , activity in aqueous fraction

Figure 2: Tissue radioactivity: 10µl/kg oil dose


Heart
Gill
Brain
Muscle
DPM X 10⁻³ per g.
dosing
dosing
Days

at day 5. The rate of clearance, however, appeared to be slower than that of the tissues examined because maintained high levels are still observed on day 8. Similarly to what were found for the organic solvent fraction, liver and blood showed a significant increase in radioactivity during the dosing period at day 3. A delay of six days in clearance, however, was observed in the water-soluble fraction of these two tissues when compared to the organic solvent fraction; levels did not significantly drop until day 11.

Total radioactivity was calculated for bile and selected tissues. It represents the sum of tracer levels observed for the aqueous and organic solvent fractions. At all times, the total radioactivity levels in bile were significantly higher than in any other tissue examined. It showed approximately 45 times higher levels of maximum uptake than those observed for the liver which was the tissue exhibiting the highest tracer activity levels.

The radioactivity levels in selected tissues and bile of A. rostrata during and following ingestion of ^{14}C-naphthalene spiked crude oil at a dose of 100 µl/kg are shown in Fig. 3 and 4. In general, the patterns of uptake and release are similar to those previously described for the 10 µl/kg oil dose experiment. In addition, no significant differences in the tissue uptake of the labelled naphthalene were found between the 10 and 100 µl/kg exposed fish. This suggests a complete absorption of the label dur-

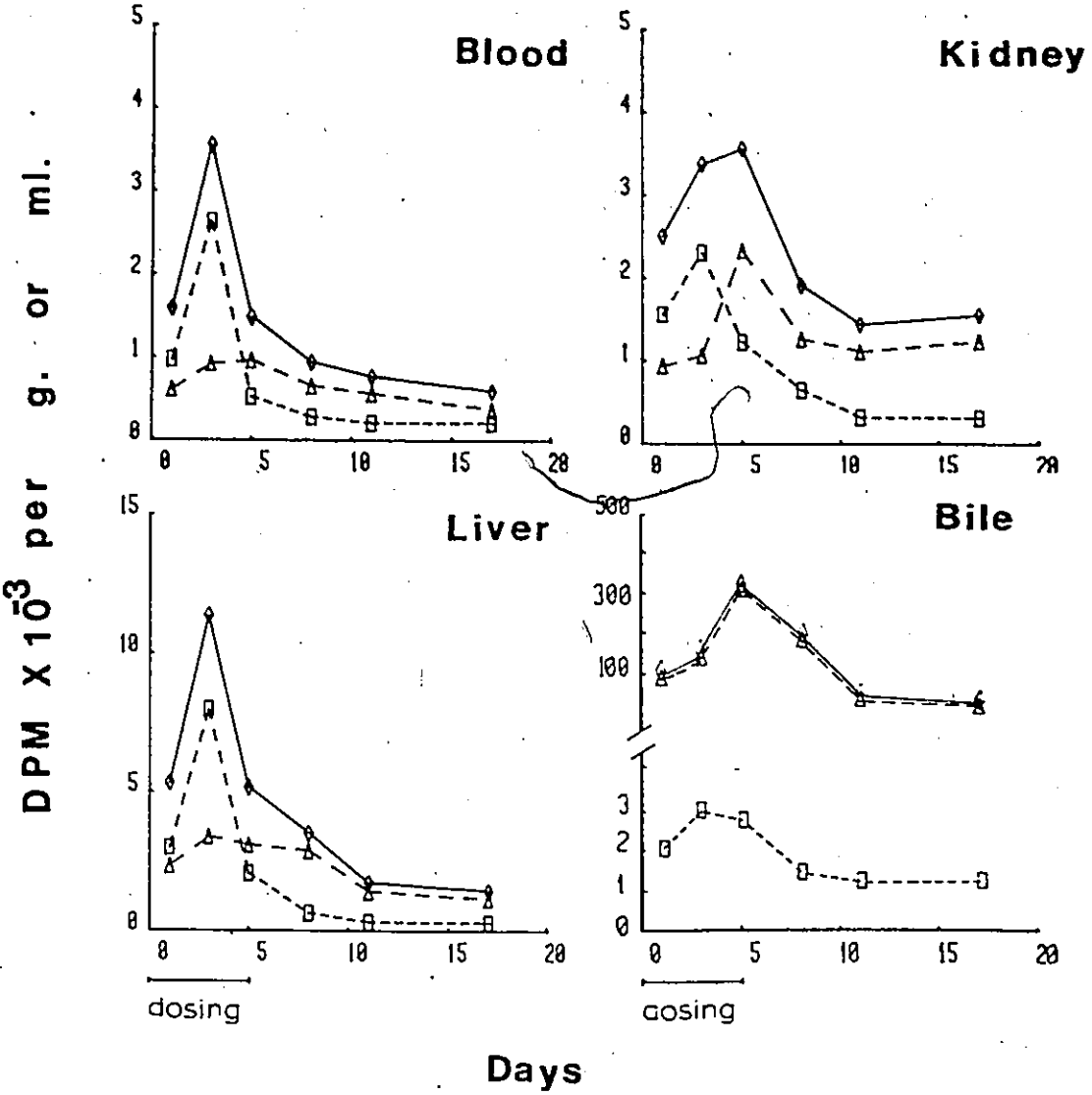
Radioactivity levels in selected tissues and bile of the American eel, Anguilla rostrata, during and following ingestion of a ^{14}C-naphthalene spiked crude oil at a dose of 100 µl/kg given for five consecutive days (0.5 µCi/dose). Each point represents the mean of five fish.

◇——◇ , total radioactivity

□- -□ , activity in organic solvent fraction

△- -△ , activity in aqueous fraction

Figure 3: Tissue and bile radioactivity: 100 µl/kg dose


Blood
Kidney
Liver
Bile
DPM X 10⁻³ per g. or ml.
dosing
dosing
Days

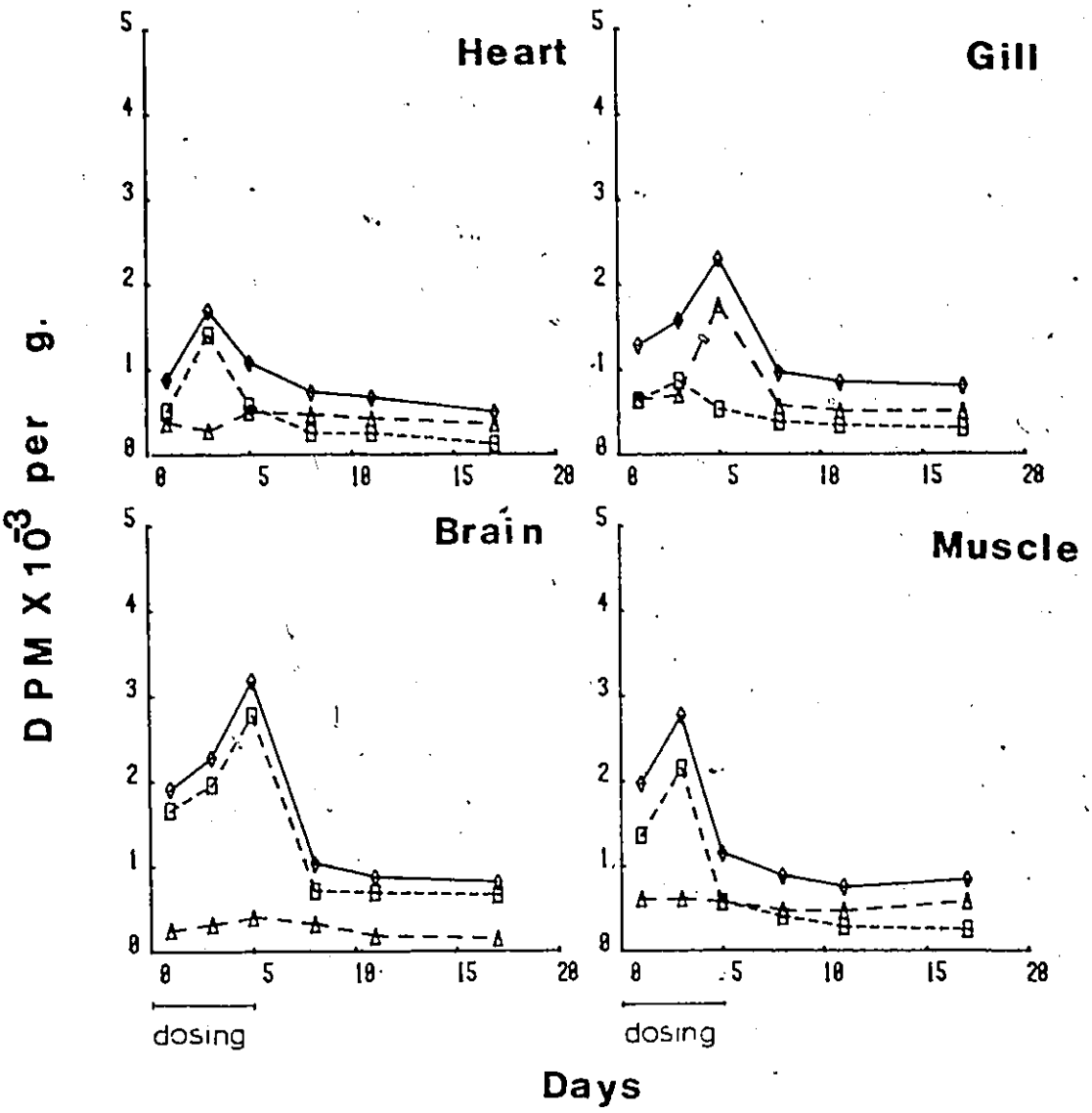
Radioactivity levels in selected tissues of the American eel, Anguilla rostrata, during and following ingestion of a ^{14}C-naphthalene spiked crude oil at a dose of 100 µl/kg given for five consecutive days (0.5 µCi/dose). Each point represents the mean of five fish.

◇——◇ , total radioactivity

□- -□ , activity in organic solvent fraction

△- -△ , activity in aqueous fraction

Figure 4: Tissue radioactivity: 100 µl/kg oil dose


Heart
Gill
Brain
Muscle
DPM X 10^-3 per g.
0
1
2
3
4
5
0
5
10
15
20
dosing
dosing
Days

ing the two dosing regimes. Therefore, based on specific activity of the original dose, the total hydrocarbon content of the tissues was derived. These nominal concentrations of petroleum hydrocarbons are presented in Table 1. From this table, it can be seen that there is a ten-fold greater content of hydrocarbons in bile and tissues after a 100 µl/kg dose compared to a 10 µl/kg exposure.

TABLE 1

Petroleum hydrocarbon distribution

Nominal concentrations(a) in µg/g of petroleum hydrocarbons in the American eel, A. rostrata, tissues and bile.

	At peak(c)		At 17 days	
Tissue(b)	10µl/kg	100µl/kg	10µl/kg	100µl/kg
Bile	2,717.0	28,044.0	176.2	1,171.0
Liver	87.9	1024.6	12.1	130.0
Kidney	31.8	322.3	11.6	140.6
Blood	29.5	321.1	4.9	53.4
Heart	20.0	153.0	5.1	44.3
Gill	22.0	207.2	6.7	72.7
Muscle	26.0	249.7	6.1	76.0
Brain	25.1	286.9	7.8	73.5

a = calculated on the basis of ^{14}C-naphthalene specific activity in crude oil doses
b = sample size of 5
c = maximum concentrations at 3 days, except at 5 days in bile, gills and brain.

DISCUSSION

The rapid uptake and release of hydrocarbons by the American eel, A. rostrata, was similar to the results obtained with other marine and fresh water fish (Lee et al., 1972; Melancon and Lech, 1978; Roubal et al., 1978). The findings indicate that ingestion of petroleum hydrocarbons results in absorption through the gut and deposition in key organs and tissues of treated fish. Tracer activity was mainly concentrated in gall bladder and liver which are organs asscciated with the metabolic breakdown and excretion of petroleum hydrocarbons (Korn et al., 1976). Accumulation of petroleum hydrocarbons in fish is known to reach considerable levels in tissues of high lipid content (Whittle et al., 1976); this probably explains the relatively high levels of tracer activity found in eel brain tissue after exposure to naphthalene spiked crude oil.

Ingested labelled naphthalene appeared to be readily taken into the body by way of the gut as indicated by the rapid increase in radioactivity seen in most tissues. The tracer levels in the bile were substantially higher than those found in all the tissues examined, suggesting a biliary route of hydrocarbon excretion in the eel. Hydrocarbon activity in the bile appears to be mainly due to metabolites since the activity was primarily found in the water-fraction of extracted samples. The finding of high levels of petroleum hydrocarbons in bile is consistent with other studies,

which also indicate the presence of high levels of hydrocarbons in the gall bladder of petroleum hydrocarbon exposed fish. For instance, Roubal et al. (1977) indicated that intraperitoneal administration of ^{14}C-naphthalene to coho salmon resulted in maximal ^{14}C activity in the bile. Further, seventy per cent of the radioactivity was due to the presence of naphthalene metabolites similar to the findings in the eel. Chromatography analysis of hydrocarbons in the bile of rainbow trout exposed to naphthalene and methylnaphthalene indicated that at least 65 to 70% of the activity recovered was due to metabolites. In addition, it was also found that most of the metabolites in the bile were more polar than the hydroxyl derivatives of naphthalene and methylnaphthalene and were probably present as conjugates (Melancon and Lech, 1978). Chromatographic analysis of hydrocarbons in the bile of 3 species of marine fish exposed to naphthalene indicated the presence of two major metabolic products: 1-hydroxynaphthalene and 1,2-dihydro-1,2-dihydroxynaphthalene (Lee et al, 1972a). Administration of naphthalene into rock sole (Lepidopsetta billineata) resulted in the appearance of 1-naphthyl glucoronide as a major metabolite in the bile. Moreover, 1-naphthol, 1,2-dihydro-1,2-di- hydroxynaphthalene, 1-naphthyl sulfate and 1-naphthyl glucoside were also present (Varanasi et al., 1978). Similar metabolites in the bile of A. rostrata are likely to have accounted for the observations of high water-

fraction radioactivity. The results of the study on the accumulation and release of petroleum hydrocarbons in A. rostrata are based solely on radiometric analysis, therefore it provides no indication on the molecular structures with which the ^{14}C was associated, however, the two phase partition technique used to extract bile and tissue samples gives some indication on the levels of parent and metabolized hydrocarbons. ^{14}C-labelled naphthalene was used to study the uptake and discharge of hydrocarbons in the eel because it was considered to be a good representative of the hydrocarbon mixture present in crude oil. In addition, polycyclic aromatic hydrocarbons such as naphthalene are known to account for most of the toxicity attributed to oil pollution (Blumer, 1971) and have been found to be very potent inducers of hepatic mixed function oxidases in fish (Bend et al., 1974; Philpot et al., 1976).

The existence of a detoxification mechanism is suggested by the high concentration of parent hydrocarbon and particularly metabolites found in the bile, as well as in the liver, of A. rostrata following oil ingestion. This is particularly important because the liver was also found to be a major site of aryl hydrocarbon hydroxylation in the eel (refer to Chapter II). In addition, in Chapter II, evidence is given about the ability of petroleum hydrocarbons to induce hepatic BaPH which probably causes an increased hydroxylation of aromatic petroleum hydrocarbons (Malins, 1977; Statham et al., 1978).

The accumulation of metabolic products is not restricted to the gall bladder and the liver. Relatively high radioactivity levels associated with the water soluble fraction were also found in other tissues such as kidney, gill and heart. It is not probable that these levels of metabolites are associated with the capacity of these tissues to metabolize petroleum hydrocarbons. A study of tissue distribution of BaPH in A. rostrata (Chapter II), indicated that the capacity of gill, kidney and heart to metabolize aromatic hydrocarbons is minimal. Presumably then, this water-fraction radioactivity in these tissues was attributable to a passive accumulation, or, especially in the case of kidney and gills, a selective excretion process. Definition of the excretion function in particular will require further study.

Substantial amount of radioactivity was also found in brain and muscle. Tracer activity, however, was associated mainly with the solvent fraction and radioactivity levels in the water-fraction were almost negligible. The low accumulation of hydrocarbon metabolites is consistent with other reports, indicating relatively low levels of petroleum bioconversion products in brain and muscles (Roubal et al., 1977; Collier and Malins cited in Malins et al., 1979).

When examining the dynamics of petroleum hydrocarbon content during the experimental period, brain seems to show a slower rate of release of hydrocarbon when compared to

most other tissues. Similar results have also been found by Neff and co-workers (1976b) when studying the uptake and release of petroleum hydrocarbon in Fundulus similus exposed to the water-soluble fraction of No. 2 fuel oil.

Further, evidence for the existence of an efficient mechanism for the removal of petroleum hydrocarbons in A. rostrata is offered by the decrease in tracer activity in blood despite continued ingestion of the labelled oil at this time. In addition, the depuration period resulted in significant decreases in hydrocarbon activity in most tissues examined. An exception, however, was the high tracer activity found in the bile. Thus, it seems that radioactivity lost in the various tissues during the clearance period was transferred to bile. This lends additional support to the hypothesis that the gall bladder is a major site for the excretion of petroleum hydrocarbons.

Chapter II

INDUCTION OF MIXED FUNCTION OXIDASES

INTRODUCTION

The exposure of an animal to foreign compounds can result in a wide range of effects, including an immediate toxic response, a delayed toxic response or no observable toxic effect at all. A major response to foreign chemicals is their biotransformation or metabolism into more polar metabolic products which can be excreted directly or converted into more water-soluble conjugated compounds and then excreted. The metabolic processes can be carried out in different tissues and organs. The hepatic system however, acts as the major site in the biotransformation of diverse chemicals such as industrial pollutants, pesticides, drugs and many other lipophilic substances. The general pattern of xenobiotic metabolism for most animals, including fish, can be divided according to Williams (1959) into two phases: phase I reactions in which biochemically reactive groups such as -OH, $-CO_2H$, $-NH_2$ and -SH are introduced or exposed within the molecular structure of the foreign compound. This phase comprises oxidation, reduction and hydrolysis. Phase II represents a synthetic reaction and includes conjugation. These reactions are considered to be the true de-

toxification mechanisms since the majority of conjugates are non-toxic water-soluble metabolites which are readily eliminated from the body (Parke, 1974).

Fish are members of the aquatic food chain and as such are subject to bioconcentration of a number of foreign or xenobiotic compounds. Attention has therefore been focused on the uptake, distribution and toxicity of foreign compounds in fish, and on the processes by which fish convert and excrete these compounds. Mechanisms for the detoxification of lipophilic compounds appear to be essential for fish survival, since it has been found that they do not readily excrete such substances through their gills (Adamson and Sieber, 1974).

Enzymes responsible for reduction of nitro- groups and azo-linkages are known to occur in different species of fish, including elasmobranchs and teleosts. Buhler and Rasmusson (1968a) examined the reduction of p-nitrobenzoic acid in 17 species of freshwater fishes. They found that all of the species tested were able to carry out this reaction, but to varying degrees. The nitro-reductase system was associated with the soluble fraction of liver homogenates; the enzyme required NADP and was inhibited by oxygen. A variety of fish species including both primitive (cyclostomes and elasmobranchs) fish and teleosts are able to carry out azo-reduction of neoprontosil to form sulphanilamide (Adamson, 1967), although the conversion rate is only approximately 1/10 of that found in mammals.

Oxidation of xenobiotics can occur by different pathways, including aromatic hydroxylation, aliphatic hydroxylation, o-dealkylation, n-dealkylation, deamination, n-oxidation and sulfoxidation. The majority of these reactions have been known to occur in several fish species both from *in vivo* and *in vitro* studies. Thus, hydroxylation of acetanilide and of aniline has been reported in rainbow trout (Buhler and Rasmusson, 1968b), the o-dealkylation of alkoxybiphenyls by trout liver, the o-dealkylation of phenacetin and 7-ethoxycoumorin, and n-dealkylation of aminopyrene and d-benzphetamine by the hepatic microsomal system of several species of fish (Sieber and Adamson, 1977). Liver microsomal preparations from numerous species of fish can also carry out the metabolism of parathion and related anticholinesterase insecticides by the conversion of a P=S group to a P=O group (Adamson, 1967).

Relatively little is known of the ability of fish to metabolize foreign compounds by hydrolysis. Although the reaction mechanisms have not been fully elucidated, Murphy (1966) found that sculpins, flounder, bullheads and sunfish could hydrolyse organophosphorus insecticides, but at a much slower rate than is found mammals.

The oxidation, reduction and hydrolysis of a lipophilic compound is frequently a preliminary to their further detoxification by conjugation. Fish excrete some endogenous substances such as bilirubin, steroids and thyroxine as con-

jugates, so it is perhaps not surprising that conjugation reactions involving foreign ccmpounds also take place (Parke, 1975). The six major conjugation reactions are: glucoronide formation, glycine conjugation, mercapturic acid synthesis, methylation, acetylation and ethereal sulfate synthesis. All of these reactions, with the exception of mercapturic acid synthesis, have been found to occur in fish (Adamson, 1967). Among the different conjugation pathways, glucosylation with glucoronic acid appears to be of singular importance in vertebrates. The enzymes involved in these transformations are responsible for the formation of glutathion conjugates from arene oxides, thus they are important in the ability of organisms tc convert potentially toxic epoxides to less toxic conjugated derivatives (Malins, 1977).

Bend et al. (1977) studied glutathione-s-transferase activity in the hepatic microsomal fraction of teleost, elasmobranch and invertebrate using different arene oxides as substrates. All species examined, including the American eel, were capable of converting these substrates; however, wide variation among individuals was observed. Similarly, Huang and Collins (1962) found that dogfish shark, goosefish and flounder were able to conjugate different isomers of aminobenzoic acid with glucurcnic acid, glycine and acetate.

Of all microsomal drug-metabolizing enzymes identified, the MFOs are the most widely distributed. They moder-

ate the metabolism of most foreign compounds and are present in the endoplasmic reticulum of animal cells. These enzymes show a requirement for NADPH and molecular oxygen for activity. For all MFO's so far studied, the following overall formula has been established:

$$RH + NADPH + O_2 + 2H^+ \longrightarrow NADPH^+ + H_2O + R\text{-}OH$$

Although the exact number of individual enzymes which carry out the vast array of activities assigned to the MFO system is not known, three components are required for maximal catalytic activity. They are: cytochrome P-450, NADPH-cytochrome c reductase and phospholipids. This electron transport system metabolizes a wide variety of drugs, aromatic hydrocarbons, pesticides and many other foreign compounds. In addition, it is well accepted that steroid hormones and fatty acids are physiological substrates of the hepatic MFO system (Conney and Burns, 1972; Schulte, 1974). The key enzyme of this system is cytochrome P-450, a complex of protein and heme which gets its designation from the fact that in the reduced form it binds carbon monoxide and then absorbs light most intensively at a wavelength of 450 nanometers. In the MFO, cytochrome P-450 serves as the terminal oxidase; it accepts electrons passed along by intermediates,

binds oxygen, and then delivers the oxygen to oxidize its substrate (Bock and Remmer, 1978). The function of the NADPH-cytochrome c reductase is to transfer electrons from NADPH to cytochrome P-450. The final result is the oxidation of the substrate with one atom of oxygen while the other atom usually combines with two atoms of hydrogen to form water (White et al., 1973). The phospholipid fraction facilitates the electron transfer from the reductase to cytochrome P-450 and the complex formation between the two components. In addition, this fraction enhances the binding of substrates to cytochrome P-450 (Bock and Remmer, 1978).

Mixed function oxidases were first described in mammalian liver microsomes. Several studies, however, indicate that this enzyme system is widely distributed in nature. In mammals, cytochrome P-450 has been found in all species thus far examined. It occurs at varying concentrations in microsomes of liver, kidney, small intestine, lung, adrenal cortex, skin, testes, placenta and several other tissues (Wattemberg and Leong, 1968). During the last 15 years a great deal of work has been done on the ability of marine organisms to oxidize foreign compounds. It was originally thought that aquatic vertebrates do not require special mechanisms to dispose of lipid soluble substances. It was quite accepted, for example, that fishes process foreign compounds by the elimination into the external environment through the activity of the gills. Several studies have

shown, however, that microsomal enzymes that metabolize foreign compounds are present not only in fish but also in many other marine and fresh water species. Payne (1977) carried out a species survey for aryl hydrocarbon hydroxylases (AHH) in animals from the coastal North-west Atlantic. Activity was found in the twelve fish species tested, some crustaceans an elasmobranch, an annelid, and an echinoderm. Planktonic crustaceans such as copepods have also been known to metabolize naphthalene and benzo(a)pyrene (Lee, 1975).

In fish, the liver appears to be the principal organ of metabolism of lipophilic compounds. It demonstrates the highest activity for AHH with other tissues showing low or negligible activity. Some activity has also been detected however in gill and kidney (Payne and Penrose, 1975; Lee et al., 1972b). The rate of metabolism of drugs and other foreign compounds varies widely in different species and strains of animals and may also be affected by the age of the individual, its sex, diet and environment. Chemicals present in the food and the environment, however, appear to be the most important factors regulating the metabolism of lipophilic compounds by drug-metabolizing enzymes. By far, the most extensively studied characteristic of the microsomal MFOs is their ability to be induced by a large number of organic compounds.

The induction of hepatic microsomal enzymes is important pharmacologically for it may result in marked altera-

tions in the toxicity of lipophilic compounds. In some cases increase in enzyme activity could result in more rapid detoxification of foreign compounds. In other situations, however, metabolism leads to the formation of a more toxic metabolite; enzyme induction may consequently increase toxicity (Parke, 1975). In addition, since the MFO system also catalyzes the oxidation of naturally occurring compounds such as steroids, emphasis has been and should be given to this additional physiological effect of induction mechanisms by foreign substances. Although the mechanisms by which induction occurs are not quite understood, it is widely accepted that the increased level of MFO enzymes is due to an increase rate of synthesis of these proteins (Bock and Remmer, 1978). Since the levels of proteins in animal tissues in steady state are generally regulated both by synthesis and degradation, the term induction may be defined operationally according to Schimke and Doyle (1970), as an increase in the ratio of the rate of protein synthesis over the rate of protein degradation, that is, a turnover ratio.

The induction phenomena observed in liver following treatment with lipophilic compounds involves many processes besides the increase in cytochrome P-450. So far, they have not been elucidated, nevertheless a few significant features may be pointed out:

1) Although several hormones can alter the activity of drug metabolizing enzymes, the stimulation of NADPH-depen-

dent enzymes in liver microsomes by lipophilic compounds does not appear to be hormonally controlled. Activation in rats occurs even after adrenalectomy, hypophysectomy, or thyroidectomy, so that stimulation of the hypophyseal-adrenocortical system or the thyroid is not involved (Bock and Remmer, 1978). Furthermore, induction mechanisms appear to be independent of extrahepatic factors since benzo[a]pyrene hydroxylation can be activated by perfusion of isolated rat liver with 3,4-benzpyrene (Juchau et al., 1965).

2) The stimulatory effect of phenobarbital and polycyclic aromatic hydrocarbons on liver microsomal activity cannot be explained on the basis of an altered affinity of the enzyme for the substrate. Phenobarbital and 3,4-benzpyrene increase the maximal velocity of p-nitroanisole demethylation and hexobarbital oxidation without causing any effect on the Michaelis constants (Rubin et al., 1964).

3) Chemical inducers appear to act at a cellular level as induction of aryl hydrocarbon hydroxylase has also been demonstrated in mammalian cell cultures (Nebert and Gelboin, 1968).

4) Foreign compounds appear to produce their stimulatory effect by increasing the rate of synthesis of microsomal enzymes. It has been found that the induction of cytochrome P-450 and NADPH-cytochrome c reductase can be prevented by inhibition of protein synthesis at the transcriptional or

translational level (Gelboin, 1971, Jacob et al., 1974). Activation by foreign compounds is inhibited by the administration of puromycin (which blocks the transfer of amino acids from aminoacyl s-RNA to microsomal protein) and by actinomycin-D, an inhibitor of messenger RNA synthesis. In addition, incorporation studies with radioactive amino acids in vivo and in vitro have suggested an overall increase in the rate of hepatic protein after administration of chemical inducers (Conney, 1967).

5) Several observations indicate that the accumulation of liver proteins after treatment with lipophilic compounds is partly promoted by a decrease in protein catabolism. A prolongation in the half-lives of microsomal NADPH-cytochrome c reductase and cytochrome b5 was clearly demonstrated with ^{14}C guanidine-arginine (Kuriyama, 1969) indicating that phenobarbital can delay the catabolism of hepatic proteins.

The stimulatory effect of foreign compounds on liver microsomal enzyme activity was first observed in 1954 by Brown and co-workers, while studying the dietary factors that influence the activity of hepatic aminoazo dye N-demethylase. Since then the number of known inducers has expanded considerably making it quite difficult to establish generalization about the nature of the inducing agents. They apparently share no other property than a non-polar region in the molecule which permits lipid solubility at a physiological pH (Schulte, 1974). In addition, they are

substrates or become bound to the microsomal enzyme (Parke, 1975).

The stimulatory effect produced by reducing agents depends on the dose administered and the induced enzyme levels are maintained as long as the inducer is present in the body. Therefore, foreign compounds such as DDT, which tends to accumulate in body fat, will maintain high enzyme levels for a relatively long time (Bock and Remmer, 1978).

When a wide variety of polycyclic aromatic hydrocarbons (PAH) differing in molecular size and geometry were tested as enzyme inducers, it was found that the active hydrocarbons possessed an optimum molecular size ranging from 75 to 150 angstroms. Molecular coplanarity was also found to be an important factor in determining the PAHs as inducing agents (Arcos and Conney, 1961). These requirements suggest the importance of steric fit between the hydrocarbon and the biological structure upon which it acts.

Based on differences in their profile of biological effects, the inducers of MFO are classically subdivided into two groups. The first, represented by phenobarbital, stimulates a wide number of heterogeneous degradative pathways and augmentation of smooth endoplasmic reticulum. This group includes different compounds such as DDT, cholesterol, pentobarbital, chlorobutanol etc. (Conney, 1967). Phenobarbital-type inducers enhance the metabolism of drugs by microsomal enzymes at four different levels:

a) by increasing total liver mass

b) by increasing microsomal protein

c) by increasing specific activity of microsomal enzymes

d) by increasing amount of NADPH cytochrome c reductase and amount of cytochrome P-450 (Gram and Gillette, 1971).

The second group of inducers represented by 3-methylcholanthrene (3-MC) or 3,4-benzo[a]pyrene, stimulate a more limited number of oxidation pathways, the smooth endoplasmic reticulum is not increased and the cytochrome induced differs from that of control by its substrate specificity and spectral properties. In addition, 3-MC does not alter the amount of NADPH-cytochrome c reductase (Parke, 1974). The delay between exposure and maximum enzyme induction is of the order of hours for 3-MC and of days for phenobarbital. Further, in contrast to phenobarbital which produces striking effects on the ultrastructure of the liver, 3-MC does not cause any detectable morphological alterations (Gram and Gillette, 1971).

Both the levels of activity of the various drug metabolizing enzymes and the extent to which they may be induced are under genetic regulation, as may be seen from comparisons of different species or different strains of a given species. Large differences in basal levels of hepatic microsomal oxidases and their induction by phenobarbital have been observed in different strains of rabbits and rats (Parke, 1975). Similarly great differences in the ability of 3-MC and phenobarbital to induce aryl hydrocarbon hydrox-

ylases have been found among rats, guinea pigs and rabbits. Studies of AHH activities in fish revealed that extremely wide variation exists among individuals of apparently similar genetic backgrounds and raised under similar environmental conditions (Malins, 1977).

Increased AHH activity by exposure to petroleum hydrocarbons has been reported for different aquatic species (Burns and Sabo, 1975; Payne and Penrose, 1975). Comparatively, polycyclic aromatic hydrocarbons have also been found to activate aryl hydrocarbon hydroxylases. Increased enzyme activity was found in sheepshead and little skate (Raja crinacea) after administration of 3-MC and dibenzanthracene respectively (James, et al., 1977; Bend et al., 1976). Benzo[a]pyrene hydroxylase (an AHH enzyme) is highly induced in fish by exposure to petroleum products, therefore a practical application of MFO measurement has been suggested. Thus, levels of AHH have been proposed as an alternative for monitoring petroleum pollution in the marine environment. However, since different chemicals, other than petroleum occur as aquatic pollutants and may act as AHH inducers, care must be taken when correlating oil pollution with enhanced AHH levels.

It is clear now, that both aquatic and terrestrial animals metabolize a diversity of chemicals including different pollutants both in vivo and in vitro and that the hepatic system plays a major role in this process. The meta-

bolic response to those chemicals constitutes in part, a detoxification mechanism. It has recently been shown, however, that reactions mediated by microsomal enzymes are associated with the formation of metabolically active epoxides which are known as very potent chemical carcinogens. Different experiments have shown that exposure of fish to a number of pure organic chemicals such as benzopyrene (which is metabolized by the MFO system) increases the incidence of tumors, (Harschbarger, 1977). In addition there is some evidence indicating an increased frequency of tumors among fish from very polluted waters. It has been suggested for example that epidermal papillomas observed in the European eel, Anguilla anguilla, are associated with the presence of pollutants, including chemical carcinogens, in the aquatic environment (Peters, 1975).

Despite these facts, detailed investigations on the biotransformation of foreign compounds by fish have begun just recently and our knowledge in the area is still rather limited. The present study was designed to characterize first of all, an MFO response in the American eel, Anguilla rostrata ; thus a tissue distribution was carried out in order to assess the relative contribution of different tissues to the metabolism of foreign compounds. Determination of optimum pH and temperature were also made. This aspect was considered quite important in view of the differences among species reported by several authors (Parke, 1975; Buh-

ler and Rasmusson, 1968). MFO activity was measured as cytochrome P-450 and aryl hydrocarbon (benzo[a]pyrene) hydroxylase.

A study was also carried out to determine the ability of crude oil to induce MFO activity. Induction was related to time of exposure, clearance parameters, and dosing regimes.

MATERIALS AND METHODS

1 Experimental Design

Immature yellow eels (Anguilla rostrata LeSueur) of weights ranging from 70-100 gm were obtained from the St. Lawrence River at Cornwall, Ontario. (For detailed information on experimental conditions and design, refer to chapter I). Animals were not fed during the experimental period. Benzo[a]pyrene hydroxylase (BaPH) activity in the eel was characterized by establishing the enzyme tissue distribution as well as optimum pH and temperature for activity. Liver, posterior kidney, gill, white muscle, intestine, heart, blood, and brain were tested for enzyme activity. The effect of pH on hepatic AHH activity was assessed with the use of a tris-chloride buffer system. Optimum temperature for BaPH in the eel was determined by measuring enzyme activity at different incubation temperatures.

Experiments were carried out to demonstrate induction of mixed function oxidases after oral exposure to 10, 100 and 500 µl/kg of Norman Wells crude oil. In the 3 experiments, measurement of MFO levels in oil-exposed and control fish were carried out simultaneously. Experimental animals were force-fed daily for 5 days with 0.1 ml of a crude oil and beef-liver homogenate preparation while control fish were fed the same volume of beef-liver homogenate only. (For additional information of feeding procedure refer to Chapter I). Enzyme activity was measured after 1,3 and 5

days. In addition, MFO activity in eels was also determined at the third, sixth and twelfth day of clearance.

2 Enzyme Preparations

Fish were anaesthetized in a 2% MS222 solution. Livers were flushed in situ as previously described in Chapter I. Since bile has been known to inhibit aryl hydrocarbon hydroxylases in fish (Balk et al., personal communication), care was taken to avoid contamination of liver tissue by leakage from the gall bladder. Excised livers were blotted dry with filter paper and weighed. A liver portion of known weight was placed into four volumes of ice-cold 0.05M Tris-chloride 0.25M sucrose pH 7.4 buffer. Liver tissue was diced with a pair of scissors and homogenized in a motor-driven glass Potter-Elvehjem homogenizer. After homogenization, the homogenate was centrifuged in a refrigerated centrifuge (Sorvall RC2-B) at 9,000 x g and 5°C for 10 minutes to remove cell debris, nuclei, mitochondria and lysosomes. The resulting postmitochondrial supernatant was then decanted into plastic tubes immersed in ice. A portion of this supernatant was used for the estimation of BaPH. The remainder of the supernatant was re-centrifuged in a preparative ultracentrifuge (Beckman LS-50) at 100,000 x g for 60 minutes to precipitate the microsomal fraction. Microsomes were further washed by resuspending the pellet in ice-cold 1.15% KCl and re-centrifuged at 100,000 x g for 30 addi-

tional minutes. The microsomal pellet was assayed for cytochrome P-450 content.

3 Enzyme Assays

Whenever assays were not carried out immediately after preparation of subcellular fraction, whole livers were wrapped in aluminum foil and frozen at -70°C. It was previously established that under these conditons, determination of AHH activity and cytochrome P-450 content after a month resutled in a 10% and 15% loss in specific activity respectively. The activity of AHH in the liver was assayed in the supernatant fraction using the method of Nebert and Gelboin (1968). The protein concentration in the supernatant used for the assay was standardized at 5 mg/ml.

The concentration of extracted hydroxylated benzo[a]pyrene (3-OH BaP) in the reaction mixture was determined fluorimetrically using a Turner 430 - spectro-fluorometer, with activation at 396nm and fluorescence measured at 522 nm. Quinine sulfate and 3-hydroxybenzo[a]pyrene (kindly supplied by Dr. A.R. Patel of the National Cancer Institute, Carcinogenesis Research Program, Bethesda, Maryland) were used as instrument calibration and analytical standards respectively. Concentrations were calculated on the basis of the amount of hydroxylated benzo[a]pyrene per mg of supernatant protein as well as per gram of body weight. Cytochrome P-450 concentration was determined in liver microsomes. The

washed microsomal pellet was resuspended in ice-cold 100mM Tris-chloride buffer at pH 7.4, and diluted to a standard volume of 1 mg protein per ml. This preparation was used for the determination of cytochrome P-450 content. Cytochrome P-450 levels were measured by the method of Omura and Sato (1964) and the modification developed by Johannesen and De Pierre (1978) to correct for contamination by hemoglobin and methemoglobin. Cytochrome P-450 was analyzed optically in CO-bound, dithionite-reduced samples vs. CO-bound reference, with a Pye-Unicam SP8-100 U.V. recording spectrophotometer. Cytochrome P-450 content was determined using an OD450-475 extinction coefficient of 105 /mM/cm (Johannesen and De Pierre, 1978). Concentrations were expressed as nmoles of cytochrome P-450 per mg of microsomal protein. Protein levels in the supernatant and microsomal fractions were determined by the method of Lowry and co-workers (1951). Crystalline bovine albumin was used as the reference standard (Sigma Chemical Company).

RESULTS

1 Physical characteristics of benzo[a]pyrene hydroxylase

Incubation of BaP with 10,000 x g supernatant fraction from various tissues demonstrated that Anguilla rostrata could catalyze the hydroxylation of this polyaromatic hydrocarbon (Table 2). Liver preparations yielded the greatest activity but some minimal activity was also associated with other tissues such as kidney, gill and heart at 1%, 0.6% and 0.6% respectively, as compared to liver levels. Activity was not detectable in any of the other tissues examined.

The effect of pH on BaP hydroxylation by A. rostrata liver supernatant fraction is shown in Fig. 5. A peak was sharply defined at pH 7.5. Eel hepatic BaP hydroxylase activity was tested at several incubation temperatures (Fig. 6). Maximum hydroxylation of benzo[a]pyrene occurred when incubations were carried out at 27°C. Enzyme activity decreased at higher temperatures and only traces of activity remained at 40°C. The amount of hydroxylated BaP also decrease at lower temperature; the inhibitory effect however, was more marked at the high temperature range.

2 Induction of mixed function oxidases

The stimulatory effect of petroleum hydrocabon ingestion on hepatic BaP hydroxylase activity, expressed as

TABLE 2

Tissue BaP hydroxylase activity

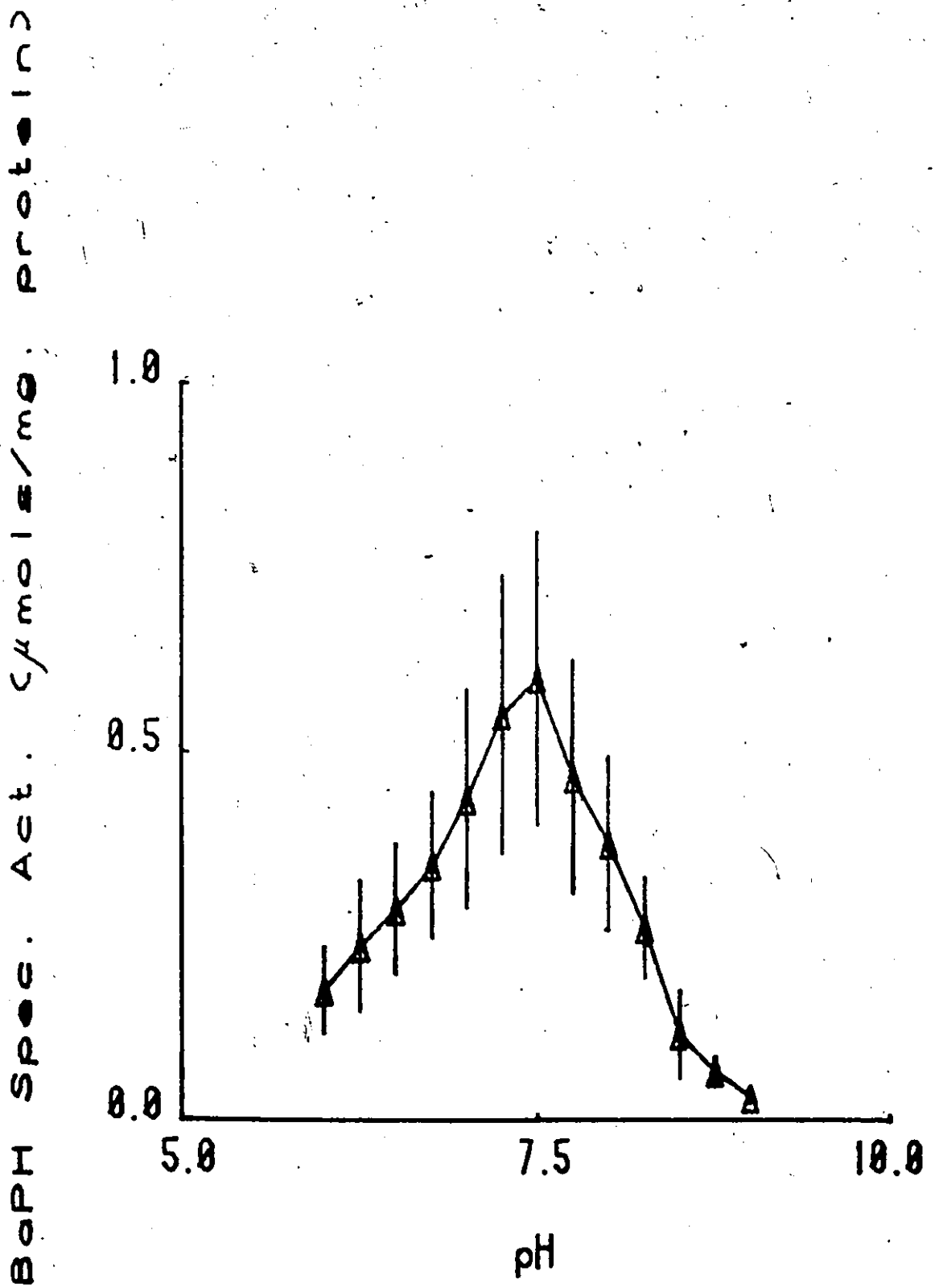
Tissue distribution of benzo[a]pyrene hydroxylase activity in the American eel, Anguilla rostrata.

Tissue(a)	BaPH specific activity(b)
Liver	0.320 ± 0.1500
Kidney	0.003 ± 0.0010
Gill	0.002 ± 0.0009
Muscle	ND(c)
Intestine	ND
Heart(d)	002 ± 0.0006
Blood	ND
Brain(d)	ND

a = sample size of 5
b = µmoles of hydroxylated BaP/mg of supernatant protein per 30 min. incubation
c = not detectable (less than 0.002 nmoles)
d = samples pooled to give 2 replicates before homogenizing

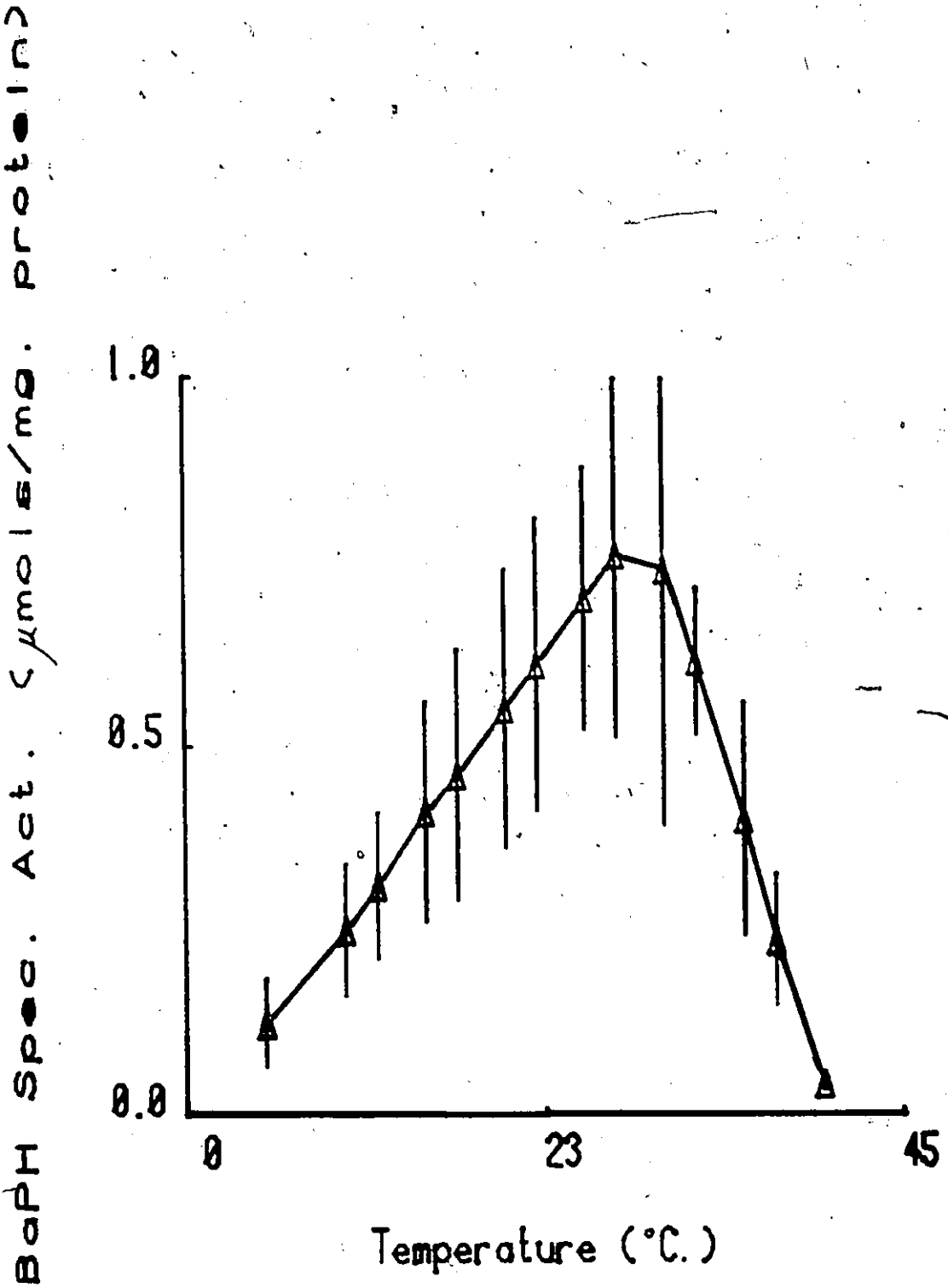
Relationships of pH and benzo[a]pyrene hydroxylase activity (expressed as µmoles of hydroxylated BaP/mg of supernatant protein/30 min.) in eel liver supernatant fractions using a tris-chloride buffer system. Each point represents the mean of 5 fish ± standard deviation.

Figure 5: Effect of pH on BaP hydroxylation


BaPH Spec. Act. (μmoles/mg. protein)
1.0
0.5
0.0
5.0
7.5
10.0
pH

Effect of incubation temperature on benzo[a]pyrene hydroxylase activity (expressed as µmoles of hydroxylated BaP/mg of supernatant protein/30 min.) in eel liver supernatant fractions. Each point represents the mean of five fish ± standard deviation.

Figure 6: Effect of temperature on BaP hydroxylation


BaPH Spec. Act. (μmoles/mg. protein)
1.0
0.5
0.0
0
23
45
Temperature (°C.)

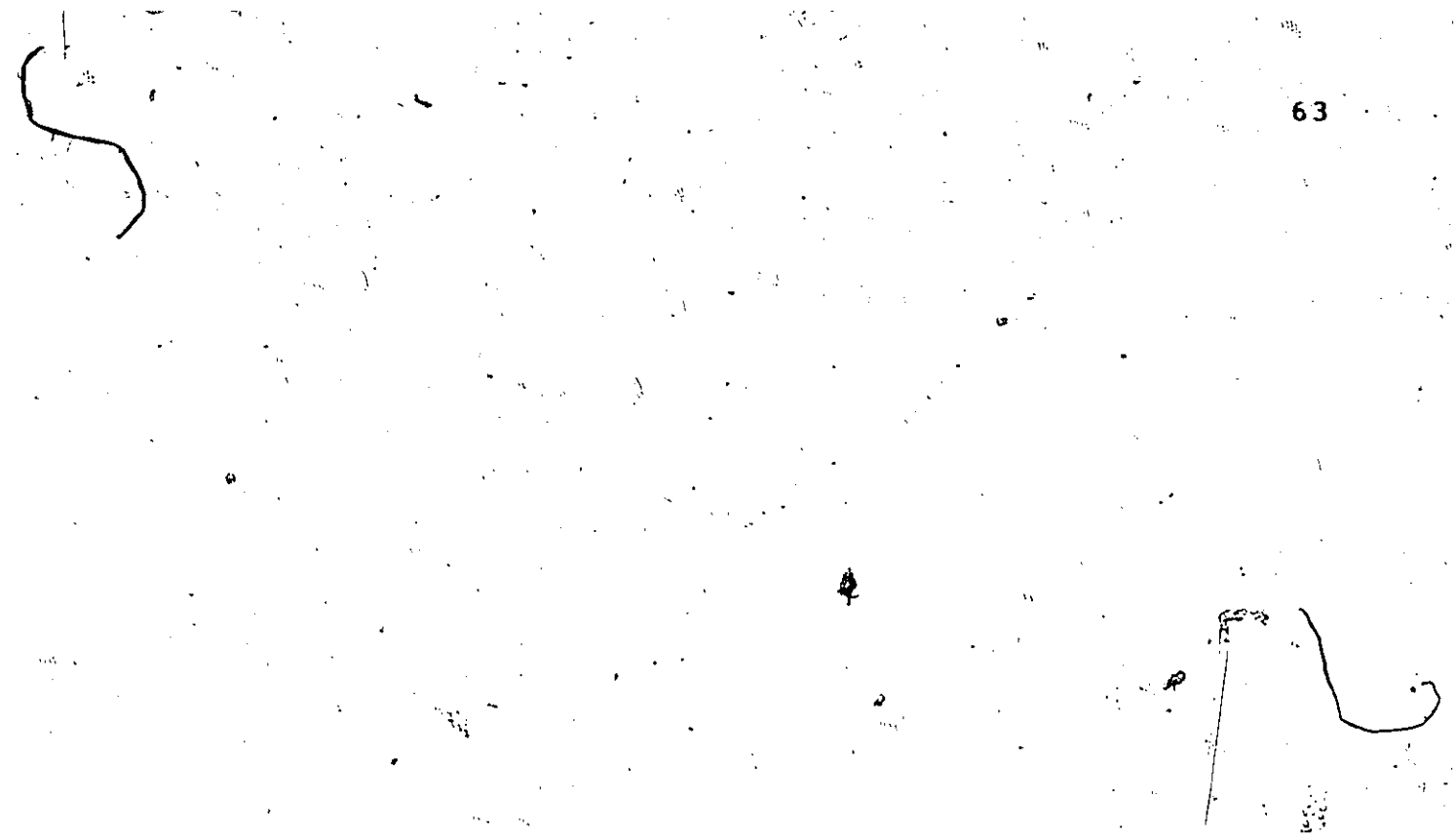
umoles/mg supernatant protein, in Anguilla rostrata is shown in Fig. 7. Statistical differences among and within treatments were established using a two-factor analysis of variance ($P < 0.05$).

A significant increase in enzyme activity was observed at day 3 during the 10 µl/kg dose experiment, followed by a gradual decrease resulting in activity levels similar to those of control fish by day 11. No further changes in the enzyme levels were found between day 11 and 17 in the oil-dosed group.

During the 100 µl/kg dose experiment, BaP hydroxylase levels showed a rise on day 3 fcllowed by a slight drop by day 5. These levels however, were both (at days 3 and 5) significantly higher than those observed for the control group. Induced levels in enzyme activity were observed throughout the experimental period.

Marked differences in enzyme activity levels between 10 and 100 µl/kg oil-exposed fish were observed at days 3, 11 and 17, in all cases, however, 100 µl/kg values were higher than those observed in the 10 µl/kg experiment.

A significant rise in enzyme activity was observed at day 3 during the 500 µl/kg dose experiment. This increase was followed by a drop to control fish levels on day 5. No differences in the amounts of hydroxylated BaP were found from days 5 to 17. With the exception of day 1, the enzyme levels in the 500 µl/kg oil exposed fish were significantly lower

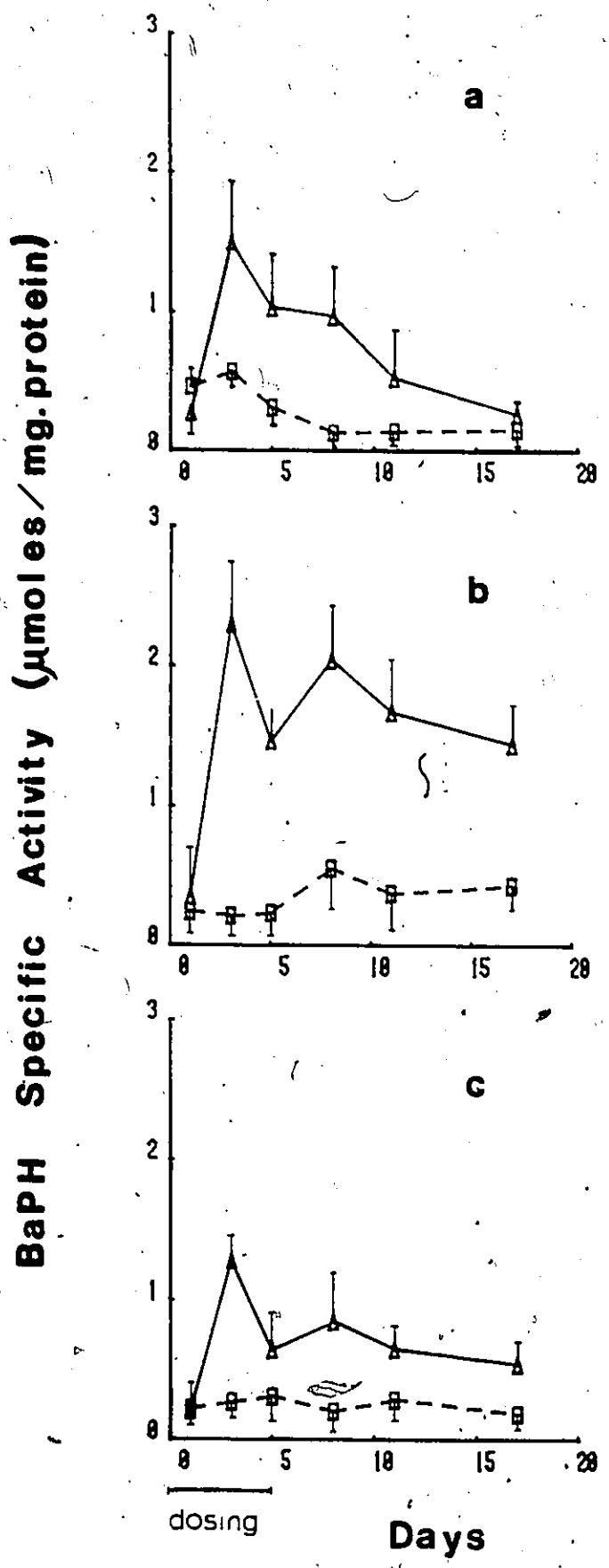


Effect of petroleum hydrocarbon exposure on hepatic BaP hydroxylase activity, expressed as umoles of hydroxylated BaP per mg supernatant protein, in A. rostrata at three dose levels: (a) 10 µl/kg; (b) 100 µl/kg; (c) 500 µl/kg. Values are means ± standard deviation.

△——△ , oil-exposed (n = 5)

□- -□ , controls (n = 4)

Figure 7: Petroleum exposure vs. BaPH (µmoles/mg. super. prot.)


BaPH Specific Activity (μmoles/mg.protein)
a
b
c
0
1
2
3
0
5
10
15
20
dosing
Days

than those observed in the 100 µl/kg oil-dosed eels. No diferences in enzyme activity however, were found between the 500 µl/kg and the 10 µl/kg oil-dosed fish.

The effect of petroleum hydrocarbon ingestion on hepatic BaP hydroxylase activity, expressed as µmoles of BaP/gm body weight, is shown in Fig. 8. With the exception of the 500 µl/kg experiment, enzyme activity followed the same dynamics along experimental time independently of whether it is calculated on the basis of the amount of supernatant protein or per gram body weight. When enzyme activity was expressed on the basis of microsomal protein a significant rise in the activity of BaPH was observed on day 3. In contrast, when the activity was expressed per gram of body weight, the rise in BaPH activity was not seen until day 8.

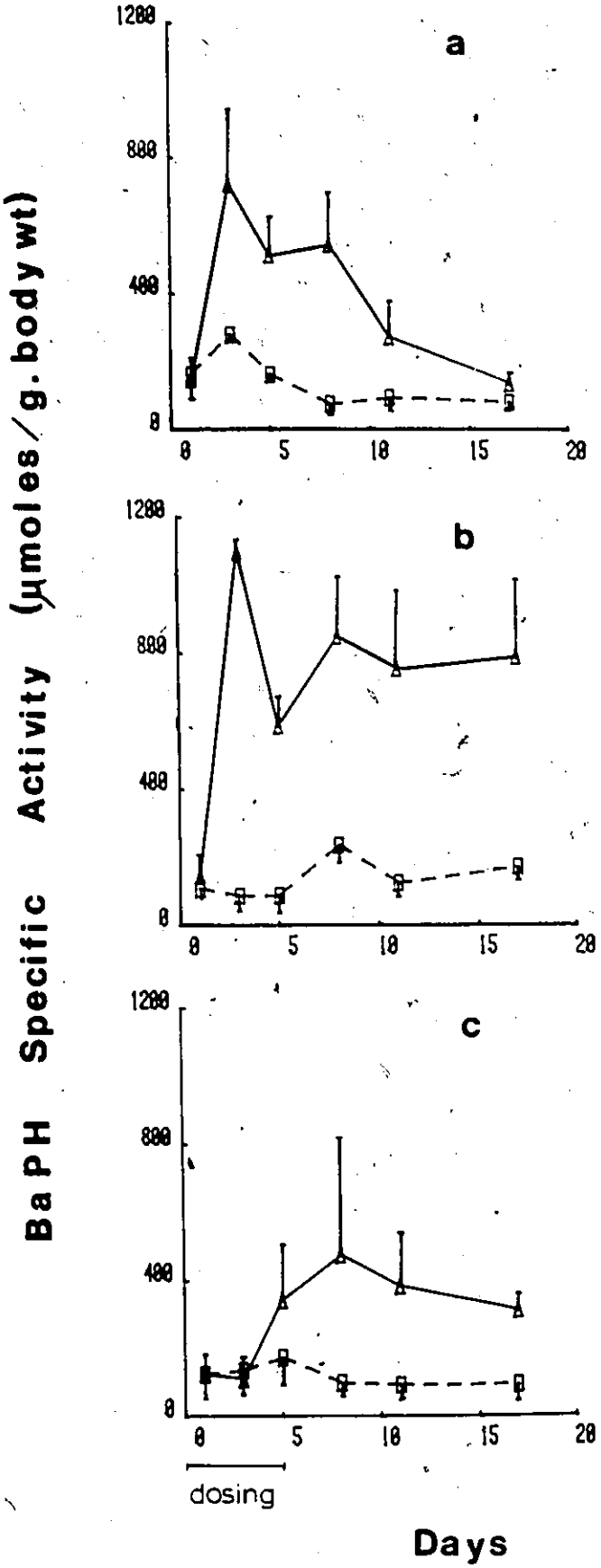
The effect of crude oil ingestion at doses of 10 and 100 µl/kg on hepatic cytochrome P-450 content of A. rostrata is presented in Table 3. Statistical differences among and within treatments were established using a two-factor analysis of variance ($P < 0.05$). Ingestion of crude oil resulted in a significant increase in the cytochrome P-450 content on day 3 of exposure, independent of the exposure level used. Oil-exposed fish showed over 2 times higher P-450 content when compared to hepatic levels in control fish. Induced levels in cytochrome P-450 were observed throughout the experimental period.

Effect of petroleum hydrocarbon exposure on hepatic BaP hydroxylase activity, expressed as µmoles of hydroxylated BaP/gm body weight/30 min., in Anguilla rostrata at three dose levels: (a) 10 µl/kg; (b) 100 µl/kg; (c) 500 µl/kg. Values are mean ± standard deviation.

△——△ , oil-exposed (n = 5)

□- - -□ , controls (n = 4)

Figure 8: Petroleum exposure vs. BaPH (µmoles/gm body wt.)


BaPH Specific Activity (μmoles/g.body wt)
1200
800
400
0
a
b
c
0
5
10
15
20
dosing
Days

The protein content in hepatic postmitochondrial supernatant fraction of A. rostrata during and following ingestion of crude oil at doses of 10, 100 and 500 µl/kg is shown in Table 4. Overall, ingestion of crude oil resulted in a significant increase in the protein content of hepatic postmitochondrial supernatant fraction regardless of the exposure level. No significant differences in protein concentration were found among fish exposed to different oil concentrations. In the 3 dosing sequences, maintained high protein content was observed throughout the experimental period.

TABLE 3

Effect of oil ingestion on hepatic cytochrome P-450

Cytochrome P-450 content in the liver of A. rostrata during and following ingestion of crude oil at doses of 10 and 100 µl/kg, given for 5 consecutive days. Each point represents the mean of four controls and five experimental fish ± standard deviation.

	Cytochrome P-450 (nmoles/mg protein)			
	10 µl/kg		100 µl/kg	
Days	Control	Oil-exposed	Control	Oil-exposed
1	0.16 ± 0.05	0.19 ± 0.08	0.17 ± 0.04	0.22 ± 0.06
3	0.23 ± 0.08	0.39 ± 0.09	0.25 ± 0.09	0.45 ± 0.13
5	0.19 ± 0.05	0.43 ± 0.11	0.21 ± 0.03	0.49 ± 0.15
8	0.21 ± 0.07	0.44 ± 0.14	0.19 ± 0.06	0.47 ± 0.11
11	0.22 ± 0.08	0.42 ± 0.15	0.24 ± 0.08	0.48 ± 0.12
17	0.18 ± 0.04	0.40 ± 0.12	0.26 ± 0.07	0.49 ± 0.15

TABLE 4

Effect of oil ingestion on hepatic protein content

Protein content in hepatic postmitochondrial supernatant fraction of the American eel, A. rostrata during and following ingestion of crude oil at doses of 10, 100, and 500 µl/kg given for 5 consecutive days. Each point represents the mean of four control and five experimental fish ± standard deviation.

	10 µl/kg		100 µl/kg		500 µl/kg	
Days	Control	Oil-treated	Control	Oil-treated	Control	Oil-treated
1	34 ± 12	38 ± 9	34 ± 7	38 ± 10	38 ± 8	41 ± 9
3	33 ± 9	44 ± 10*	35 ± 11	49 ± 11*	37 ± 7	47 ± 13 *
5	30 ± 11	41 ± 14 *	35 ± 13	42 ± 12*	39 ± 10	41 ± 7 *
8	34 ± 10	47 ± 13 *	32 ± 10	44 ± 13 *	35 ± 9	46 ± 11 *
11	37 ± 8	45 ± 11 *	33 ± 8	46 ± 15 *	33 ± 7	46 ± 13 *
17	32 ± 9	40 ± 12 *	34 ± 7	45 ± 13 *	37 ± 8	43 ± 10 *

*- indicates significant difference from control means; $P<0.05$.

DISCUSSION

Numerous studies have demonstrated that the hepatic microsomal enzyme systems of fish can carry out a variety of mixed function oxidase and NADPH-dependent reduction reactions (Adamson, 1967; Buhler and Rasmusson, 1968a; Chambers and Yardbrough, 1976). Among these, aryl hydrocarbon hydroxylases are known to occur in hepatic microsomes of marine and fresh water fish and have received considerable attention in relation to their role in hydroxylation of petroleum hydrocarbons.

Evidence is given in the present study on the ability of the hepatic systems of A. rostrata to metabolize foreign lipid compounds. The results also indicate that it is a cytochrome P-450-linked monooxygenase system which is responsible for the hepatic metabolism of petroleum hydrocarbons in particular. This study of tissue distribution of BaPH in A. rostrata suggest the liver as the main site of petroleum hydrocarbon metabolism, as indicated by the minimal levels of enzyme activity found in all the other tissues examined. Similar results have been found in other fish species, such as rainbow trout, brown trout and capelin (Mallotus villosus) (Pedersen and Hershberger, 1974; Payne and Penrose, 1975).

Several reports have shown that the major features of the MFO system of fish generally resembles that of mammals with regard to subcellular localizaton and co-factor re-

quirements (Chambers and Yardbrough, 1976). Fish liver microsomal enzymes have, however, much lower temperature optima than do the corresponding mammalian systems (Adamson, 1967) and are almost inactive at 37°C. Measurements of optimum temperature for mixed function oxidases in the stingray, Dasyatis sabina, obtained from the coast of Florida, however, have been reported to reach up to 40°C (James et al., 1979). The results presented here indicated that BaPH in the American eel, Anguilla rostrata, has similar characteristics to those reported in other fish, showing optimal pH and temperature characteristics in the region of pH 7.5 and 29°C, respectively.

The experimental ingestion of petroleum hydrocarbons by A. rostrata at doses of 10, 100 and 500 µl/kg resulted in elevated levels of BaPH. High BaPH activity is consistent with other reports, indicating that this mixed function oxidase can be induced by a variety of different compounds including crude oil (Payne and Penrose, 1975; Yardbrough and Chambers, 1977). In addition, high levels of BaPH activity has been found to occur in fish living in contaminated waters, suggesting that the levels of MFO enzymes can be linked to environmental contamination (Stegeman, 1978; Payne, 1976) and may be used as a biological index of such contamination.

The time course study of the induction of BaPH by petroleum hydrocarbons showed a typical time dependent in-

crease in MFO activity. Independently of the crude oil dose to which A. rostrata was exposed, BaPH activity, as calculated on the basis of the amount of supernatant protein, was significantly increased by the third day of dosing. The maximum levels of enzyme activity as well as the enzyme levels during depuration time, however, are quite different according to the doses used.

A number of reports have indicated that the induction of microsomal enzymes is associated with an increased de novo synthesis of hepatic protein (Gelboin, 1971). In the present study, ingestion of crude oil by A. rostrata at doses of 10, 100 and 500 µl/kg resulted in a significant increase in the protein content of the hepatic postmitochondrial supernatant fraction which perhaps accounts for the elevated enzyme activity reported for the oil-exposed fish.

Comparison of BaPH enzyme levels found for the 10 and 100 µl/kg oil-exposed fish indicates the existence of a dose-dependent response. Enzyme activity for the 500 µl/kg treated fish do not follow this trend, but exhibit a lower enzyme level, when compared to those of the 100 µl/kg exposed fish. Furthermore, continuous exposure for 5 days at 500 µl/kg resulted in a significant drop in BaPH specific activity followed by a new increase when the animal entered in the depuration period. Comparatively, in a study carried out by Ahokas and co-workers (1976), it was found that pike (Esox lucius) from highly polluted water showed a decreased

MFO capacity which was associated with a release of NADPH cytochrome c reductase from the endoplasmic reticulum. This resulted in an impaired effect on the activity of cytochrome P-450-linked oxidations. Whether or not long-term ingestion of high levels of petroleum hydrocarbons could have the same negative effect on A. rostrata will require further investigation. It is important to point out that when enzyme activity for the 500 µl/kg exposed fish was expressed per gram of body weight, maximal BaPH activity was observed only during the depuration period. This suggests a hepatotoxic effect due to the high exposure level.

An elevation in hepatic cytochrome P-450 content was observed in conjunction with higher BaPH activity for the 10 µl/kg and 100 µl/kg crude oil-exposed fish. This is in agreement with other reports which demonstrated a simultaneous induction of AHH and cytochrome P-450 in F. heteroclitus from an oil polluted area (Stegeman, 1978). Similar results were also found in rainbow trout after exposure to a variety of polyaromatic hydrocarbons (Statham et al., 1978). The levels of cytchrome P-450 found in control eels were similar to those previously reported for other fish species (Philpot et al., 1976). Induction of BaPH in many mammalian species is sometimes accompanied by a shift in the maximum absorption of cytochrome P-450 from 450 to 448 nm. This is attributed to a relative increase of a cytochrome of different spectral properties, the cytochrome P-448 (Alvares et

al.,1967). In this study, no differences in the optical properties of cytochrome P-450 indicating a P-448 were observed, similar to the results of Burns (1976) and Stegeman (1978) using F. heteroclitus.

This study has shown that ingeston of crude oil by the American eel, Anguilla rostrata, results in an enhanced hepatic MFO activity measured as BaPH and cytochrome P-450.

Chapter I

EFFECTS ON CORTISOL BALANCE

INTRODUCTION

Elevation of plasma adrenocorticosteroid concentration appears to be a generalized response to a variety of disturbances in many vertebrates (Selye, 1976). Circulating cortisol concentrations in fish have been shown to increase in response to stress factors such as handling, physical exercise, temperature changes, decreases and presence of pollutants in the water environment (Fagerlund, 1967; Schreck and Lorz, 1978; Di Michele and Taylor, 1978).

Fish respond to stress in a way which is basically similar to the mechanism in mammals. Exposure to stress results in the release of ACTH from the pituitary gland which stimulates the synthesis and release of cortisol from the interrenal tissue (Donaldson and Dye, 1975). Clear evidence of hypothalamic control of ACTH release in teleost has been indicated by Redgate (1974). Removal of the pituitary gland of a salmonid fish results in a decline in plasma cortisol concentration and a failure to respond to stress (Donaldson and McBride, 1967). Similar results have also been obtained in *Anguilla rostrata* after surgical removal of interrenal tissue (Butler *et al.*, 1969).

The increase in circulating corticoids along with elevated catecholamine levels is considered to be a primary effect of stress in fish. In addition, a large body of results seem to indicate that metabolic and osmoregulatory disturbances as a result of stressing situations in fish are brought about by changes in these hormone levels and relate to the role of cortisol in metabolism and osmo- and electrolyte regulation. Cortisol is known to have a pronounced glucocorticoid action involving stimulation of liver transaminases, and gluconeogenesis accompanied by elevated glucose levels in both muscle and liver (Chan and Woo, 1978). In the European eel (Anguilla anguilla), administration of cortisol results in significantly elevated blood glucose and liver glycogen probably due to an enhanced liver gluconeogenesis (Lidman et al., 1979) from non-carbohydrate substrates.

The regulatory effects of cortisol on lipid metabolism are not quite understood. As a result of the studies carried out on mammals, the corticosteroids have been assigned a lipolytic effect. Intraperitoneal administration of cortisol in the Japanese eel, Anguilla japonica, appears to increase fat utilization. In addition, cortisol injection into starved A. rostrata caused a rapid increase in plasma free fatty acid concentrations (Butler, 1973). Further, Lidman and co-workers (1979) indicated that administration of cortisol in A. anguilla for 14 days resulted in

decreased triglyceride titres in muscle and liver tissue, as well as in blood plasma. They also suggested that these results expressed an impaired re-esterification of triglycerides and/or an increased lipolysis as an effect of cortisol.

Cortisol has also proven to play an important role in the osmo- and electrolyte regulation by controlling the sodium influx across epithelial membranes. Potassium homeostasis has been shown to be affected by cortisol adinistration in the European eel (Lidman et al., 1979). By comparison, surgical adrenalectomy of A. rostrata, resulting in a significant reduction in plasma cortisol, failed to produce any significant changes in either plasma or epaxial muscle electrolyte concentration although a decrease in plasma magnesium was observed.

As mentioned before, circulating cortisol concentrations have been shown to increase in response to exposure to pollutants. To date, however, only a limited number of studies have dealt with the corticosteroid response to foreign compounds. Schreck and Lorz (1978) carried out a study on the effect of heavy metals in salmon. They indicated that a general, perhaps cortisol-mediated, stress response in coho salmon is observed depending upon the nature of the chemical under study. Thus, it was found that while copper exposure was followed by a large cortisol response, cadmium caused death without any change in circulating cortisol levels. In addition, acute exposure of sockeye salmon (Oncorhynchus

nerka) to copper produced a marked dose-dependent elevation in cortisol levels monitored over 24 hours. Similarly, Di Michele and Taylor (1978), indicated that exposure of Fundulus heteroclitus to naphthalene dissolved in water resulted in an increase in serum cortisol concentrations which were proportional to the naphthalene concentration to which the animals were exposed.

Not only absolute levels, but also the dynamics of circulating cortisol responses appear to depend on pollutant exposure levels. The initial phase is usually characterized by an immediate increase in cortisol for all the concentrations tested. This phase is generally followed by a period in which cortisol levels return to normal and the organism appears to regain homeostasis. With very high pollutant levels the return to normal is transient and a second increase in cortisol levels is observed. It has been observed that during this period, maintained high cortisol titers may lead to death. On the other hand, animals exposed to relatively low concentrations do not reach the stage of exhaustion, rather they appear to be able to accomodate and adapt to the stressor (Donaldson and Dye, 1975; Schreck and Lorz, 1978). It appears, then, that the cortisol response in fish elicited by foreign chemicals depends not only on the specific nature of the stress, but also on its concentration, as well as the length of time that the fish has been exposed to the compound (Lockhart et al., 1972).

Whereas a primary response to the presence of foreign compounds is an elevation of cortisol levels in the plasma of fish, it is important to consider the recent findings that exposure is frequently associated with an induced MFO activity. Several studies have been recently oriented to the examination of the possible physiological role of hepatic microsomal enzymes. A question of particular interest is whether the prime function of this system, known to catalize the hydroxylation of drugs and many other foreign substances, is to participate in the metabolism of endogenous compounds such as steroids and fatty acids. The cortisol response pattern would be complicated by possible consequent alterations in cortisol balance. It has been found, for example, that exposure of rainbow trout to PCB's, which results in enhanced hepatic microsomal enzyme activity, is associated with a marked decrease in corticosteroid levels (Sivarajah et al., 1978).

The possibility that the oxidative drug metabolizing enzymes play a role in the metabolism of steroids is an important question to consider for there is a close similarity between enzymes that oxidize drugs and enzymes that oxidize steroids. Both groups are localized in liver microsomes and require NADPH and oxygen for activity (Conney and Burns, 1963). Additional studies have demonstrated many other similarities, suggesting that steroid hormones are naturally occurring substrates for drug metabolizing enzymes in liver

microsomes. Further, it has been shown that these detoxifying enzymes are inhibited by carbon monoxide and this inhibition can be reversed by the absorption of monochromatic light at 450 nm. A similar inhibitory effect of carbon monoxide has been found in liver microsomal steroid hydroxylation. More specifically, the inhibitory effect of carbon monoxide on the 6β -, 7α -, and 16α -, hydroxylation of testosterone by liver microsomes can also be prevented by monochromatic light at 450 nm (Conney, 1967). The uniformly low Michaelis constants for the hydroxylation of testosterone, progesterone, and estradiol by liver microsomes also support the concept that steroids are normal substrates for oxidative drug metabolizing enzymes (Kuntzman, 1969).

The many similarities between the hepatic hydroxylation of drugs and steroids, prompted detailed studies on the effects of liver microsomal inducers on the metabolism of steroid hormones. Studies carried out during the last 20 years have shown that chronic administration of many drugs causes a non-specific increase in the metabolism of steroids by microsomal enzymes. Therefore, enhanced steroid hydroxylation in animals treated chronically with drugs would be an important consequence of the hypothesis that drug and steroids are metabolized by the same enzyme system. Treatment of rats with phenobarbital for several days increases the in vitro liver microsomal hydroxylation of testosterone (Conney and Klutch, 1963), progesterone (Kuntzman et al., 1966),

cortisol, corticosterone, and cortisone (Conney et al., 1965; Conney and Schneidman, 1964). Several structurally unrelated chemicals that stimulate the activity of drug metabolizing enzymes also activate steroid hydroxylase activity. Examples of such compounds include diphenylhydantoin, chlorcyclizine, phenadrine, phenylbutazone and several halogenated hydrocarbon insecticides (Conney and Burns, 1972). The administration of DDT (10 ppm) and dieldrin (2 ppm) for 1 week to male and female pigeons stimulates the metabolism of testosterone by liver microsomes (Peakall, 1967). In addition, Davison and Sell (1974) observed that reduction of hepatic microsomal enzymes after administration of DDT and dieldrin produces thinning of eggshells in the mallard duck.

Chronic drug administration has also been found to increase the metabolism and decrease physiological actions of steroids in vivo. The increased hydroxylation of progesterone caused by phenobarbital, chlordane and DDT is associated with a decrease in the anaesthetic action of progesterone and decreased amounts of this steroid and its metabolites in the brain and total body in the rat. Similar effects of phenobarbital treatment have been observed for other steroids such as deoxycorticosterone, androsterone, and testosterone (Kuntzman, 1969). Whereas normally, the administration of estradiol and estrone to immature rats increases uterine weight, pre-treatment with phenobarbital, chlordane, or dieldrin has been found to block the utero-

tropic action of these hormones (Conney and Burns, 1972). In humans, as well, treatment with phenobarbital, phenylbutazone, antipyrene and o,p'-DDD, stimulate the metabolism of cortisol to form 6β-hydrocortisol, normally a minor metabolite of cortisol. Similarly, treatment with N-phenylbarbital alters the relative amounts of testosterone metabolites excreted in the urine (Kuntzman et al., 1968). The observation that compounds which stimulate drug metabolizm also increase the excretion of 6β-hydrocortisol in man has lead to the suggestion that the level of urinary 6β-hydrocortisol in man may be used as a measure of enzyme induction (Kuntzman, 1969).

Not only can treatment with drugs alter the metabolism of steroids, but several reports indicate that treatment with steroids can alter the metabolism of drugs. Induction of hepatic MFO by steroids of all kinds including corticosteroids, androgens, estrogens and progestogens, together with various synthetic steroids have been shown to affect the metabolism of drugs by stimulating or inhibiting the activity of drug metabolizing enzymes of the liver (Conney, 1967). The administration of progesterone and its analog norethynodrel to rats during 1 to 2 hours inhibited the metabolism of hexobarbital and zoxazolamine by liver microsomes. On the other hand, enhanced metabolism of hexobarbital and zoxazolamine was observed when rats were treated with norethynodrel at 18 hours before sacrifice (Juchau and

Fouts, 1966). Administration of testosterone to female rats for several weeks increases the hepatic microsomal metabolism of hexobarbital and narcotic drugs. In addition, subcutaneous administration of several 19-nortestosterone derivatives produce an increased hepatic microsomal metabolism of hexobarbital and decrease zoxazolamine prostration time in mice (Novick et al., 1966).

Interestingly it has been found that adrenalectomy of the male rat decreases the rate of metabolism of hexobarbital and many other drugs but this activity can be restored by administration of cortisol or phenobarbital (Parke, 1975). In addition, removal of the adrenal gland or the hypophysis has been known to decrease hepatic NADPH-cytochrome c reductase and cytochrome P-450 reductase more that it decreases cytochrome P-450. Activity of these enzymes is restored by corticosterone, or ACTH, which sugests that the pituitary-adrenal system exerts a regulatory function on the hepatic drug metabolizing enzymes (Castro et al., 1970).

Our knowledge about the ability of pollutants to alter the metabolism of steroid hormones in fish is quite limited and most work has been done either in mammals or birds. Thus far, only one report seems to indicate that enhanced hepatic microsomal enzymes have an effect on circulating steroid levels in fish. It was found that exposure to a PCB (Aroclor 1254) administered intraperitoneally resulted in a marked induction of aminopyrene demethylase and cytochrome

P-450 in both trout and carp. In addition, induction of MFO enzymes was associated with a significant decrease in plasma concentrations of androgens, estrogens and corticoids (Sivarajah, 1978). The metabolic relationship, if any, has not been tested further.

Our knowledge about the effects of steroids on the mixed function oxidases of fish liver is also very limited. Hansson and Lidman (1978) investigated the effects of cortisol on some of the components of the MFO system in rainbow trout liver. They indicated that implantation of cortisol pellets, which resulted in elevated cortisol levels, caused a concomitant increase in NADPH cytochrome c reductase activity. Cytochrome P-450 levels, however, did not change after cortisol treatment.

From the previous information, it is clear that a relationship exists between the metabolism of steroids and that of lipophilic foreign compounds. Yet, our knowledge about the mechanisms behind that relation, as well as its physiological significance, is very scarce. Particularly in fish, very little is known about the perhaps mutual metabolic effects of foreign compounds and steroids. Steroid physiology alone in fish requires further study. It was considered worthwhile to investigate the effect of petroleum compounds in the form of crude oil, a very common pollutant of the aquatic environment, on the cortisol balance in the American eel, Anguilla rostrata. The present study was di-

vided into 3 phases. Firstly, attention was concentrated on establishing whether crude oil produces a general cortisol stress response in A. rostrata as it has been reported for many other pollutants in other species. Secondly, attempts were made to determine if the induced levels in MFOs observed after exposure to crude oil (refer to Chapter II), had any effect on the breakdown rate of circulating cortisol levels in fish. It was thought that this aspect would give some understanding about the physiological significance of the interaction between steroid hormones and the microsomal enzymes which carry out the metabolism of foreign compounds. Finally, since steroids have been known to induce drug metabolizing enzymes in mammals, an experiment was conducted in order to establish the inducing effect of cortisol administration on the levels or activities of hepatic microsomal enzymes, measured as cytochrome P-450 and AHH.

MATERIALS AND METHODS

1 Experimental

Immature yellow eels (Anguilla rostrata LeSueur) of weights ranging between 70 and 100 gm were obtained from the St. Lawrence River at Cornwall, Ontario (for detailed information on holding facilities refer to chapter II). Eels were not fed during the experimental period.

2 Effects of oil ingestion on plasma cortisol levels

This experiment was designed to determine whether oral exposure to Norman Wells crude oil in eels resulted in changes in circulating cortisol levels and if so to describe the dynamics of these changes. Since it is widely recognized that intense handling of fish results in elevated cortisol levels (Mazeaud et al., 1977), experiments were carried out in our laboratory to define the cortisol response of eels to handling (Engelhardt and Roy, pers. comm.). In that study, it was established that although handling resulted in elevated plasma cortisol titers, the fish seemed to adapt rapidly to the handling stress as measured by this index, and cortisol levels returned to control values in 3 to 4 days of continued handling and remained low with subsequent continued experimental handling. In the present study, a pre-handling period of 4 days, prior to the experimental run, was adopted. During this time, all animals to be used (control and experimental fish) were handled daily

using the same procedure to be used later during experimental time (catching eels with a net and using a sham force-feeding technique). At the end of this period, experimental fish were exposed to 10 and 100 µl/kg doses. Experimental and control fish were sampled simultaneously and at a restricted time of day. At each sampling time, animals were anaesthetized with 2% MS222 solution. Blood samples were obtained from the caudal vein with heparinized syringes. Blood sampling was carried out between 09.00 and 10.00 hours to avoid unrelated variations in plasma cortisol levels due to possible diurnal cycling. In addition, the time required for anaesthesia and blood sampling of each fish was never more than 5 minutes. The collected blood was transfered to heparinized centrifuge tubes and centrifuged at 1400 xg for 10 minutes to obtain plasma for later analysis.

Quantitative determinations of cortisol were done in duplicate by radioimmunoassay (RIA) (Immuno-Phase, Cortisol Radioimmunoassay test system, Corning Medical, Medfield, Mass.). Briefly, the cortisol RIA involves the liberation of cortisol from protein binding by a specific releasing agent, 8-anilino-naphthalene- sulfuric acid (ANS). An antibody specific for cortisol is reacted with a trace amount of ^{125}I-labelled antigen and a test plasma sample. The labelled and unlabelled antigen compete for the antibody binding site; therefore, the amount of labelled antigen bound

decreases as the concentration of unlabelled antigen increases, and vice versa. Bound and free antigen are separated by centrifugation. Activity of labelled antigen bound to the antibody was carried out by counting the resulting pellet in an automatic gamma counter (Model 1085, Nuclear Chicago). The plasma concentration of cortisol was calculated by extrapolating the sample concentration from a standard curve constructed using a series of cortisol standards of known concentrations.

3 Effects of cortisol on the levels of MFO enzymes

In this experiment, the levels of AHH and cytochrome P-450 were examined after an intraperitoneal injection of exogenous cortisol into non-oil-exposed fish. Enzyme assays were carried out simultaneously in control and experimental fish placed under similar environmental and handling conditions. Cortisol-treated fish were given daily doses of 0.01 mg of cortisol (Sigma Chemical Company) dissolved in 0.1 ml of corn oil for 5 consecutive days, while control fish were given the corn oil carrier only. This cortisol dose was calculated such that it would yield plasma cortisol levels similar to those observed in fish exposed to 100 µl/kg of crude oil for 1 day (refer to Fig. 10). Four cortisol-treated and four control fish were sampled at each time period of 1,2,3, and 4 days after initiation of treatment. Animals were rapidly decapitated and blood samples from the

ventral aorta were collected in heparinized centrifuge tubes. Blood sampling was carried out between 09.00 and 10.00 hours. Blood was then centrifuged for 10 minutes and cortisol levels were quantified in plasma by RIA as described previously.

After blood sampling, an abdominal incision was made to expose the liver which was then flushed *in situ* to remove blood cells. Preparation of subcellular fractions and assays for AHH and cytochrome P-450 were carried out following the procedure detailed in chapter II.

4 MFO induction and cortisol breakdown rate

In this experiment, the clearance of radioactive cortisol from the plasma of eels showing induced AHH activity was compared to the clearance of cortisol from non-induced fish.

In order to induce AHH levels in eels, an experimental group was force-fed daily for 3 days with 100 µl/kg of Norman Wells crude oil mixed with beef-liver homogenate (animals were fed using the same technique described in chapter I). Control fish were fed beef-liver homogenate only.

At the end of this period, feeding was stopped and both groups (oil-treated and control fish) were injected intraperitoneally with a single dose of 0.1 µCi ^{3}H-labelled cortisol (specific activity: 209 mCi/mg; Amersham/Searle, Arlington Heights, Ill.) dissolved in 0.1 ml of corn oil.

The amount of labelled cortisol used was previously calculated so that it would not alter the plasma cortsiol levels of the injected fish.

Five fish were sampled at each time period of 30 minutes, 2,4,6,9,12,24,36,48,60, and 72 hours after administration of labelled cortisol. At those times animals were rapidly decapitated and blood samples from the ventral aorta were obtained in heparinized tubes. Plasma was then separated by centrifugation and frozen at -70°C for radioactivity analysis. Livers were flushed *in situ* as previously described and removed for preparation of subcellular fractions and measurement of AHH levels.

Aliquots of plasma of 100 µl were mixed with 10 ml of Aquasol-2 (NEN, Canada) and were assayed for total ^{3}H-activity by liquid scintillation counting (LKB 1215, Rackbeta, Wallac.).

RESULTS

1. Effects of crude oil ingestion on plasma cortisol levels

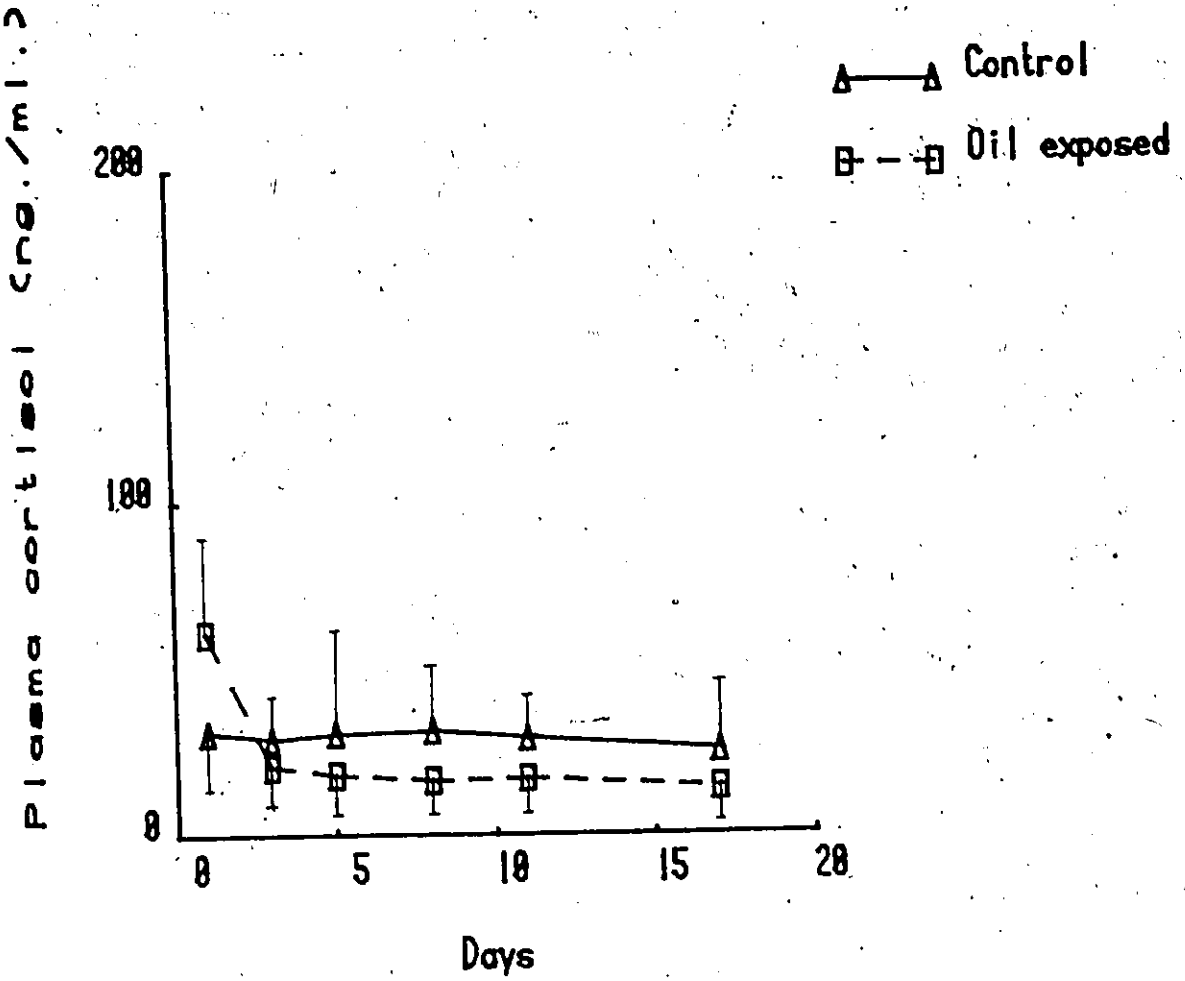
The cortisol concentrations in the plasma of A. rostrata during and following ingestion of crude oil at doses of 10 and 100 µl/kg are presented in Fig. 9 and 10 respectively. Statistical differences among and within treatments were established using a two-factor analysis of variance ($P < 0.05$).

In the 10 µl/kg oil-dosed fish, a significant rise in cortisol titers was observed on the first day of dosing. The plasma cortisol levels returned to control values on day 3 and remained unchanged for the rest of the experimental period. No changes in cortisol titers were observed for control fish throughout the experiment.

Ingestion of crude oil at a dose of 100 µl/kg resulted in elevated cortisol levels on days 1 and 3. This increase was followed by a drop to control levels by day 5 which was maintained throughout the experimental period. No significant differences in cortisol titers were found between control and oil-exposed fish from day 5 to 17, although the trend seems to indicate that dosed fish show lower levels than controls. No changes in cortisol titers were observed for control fish.

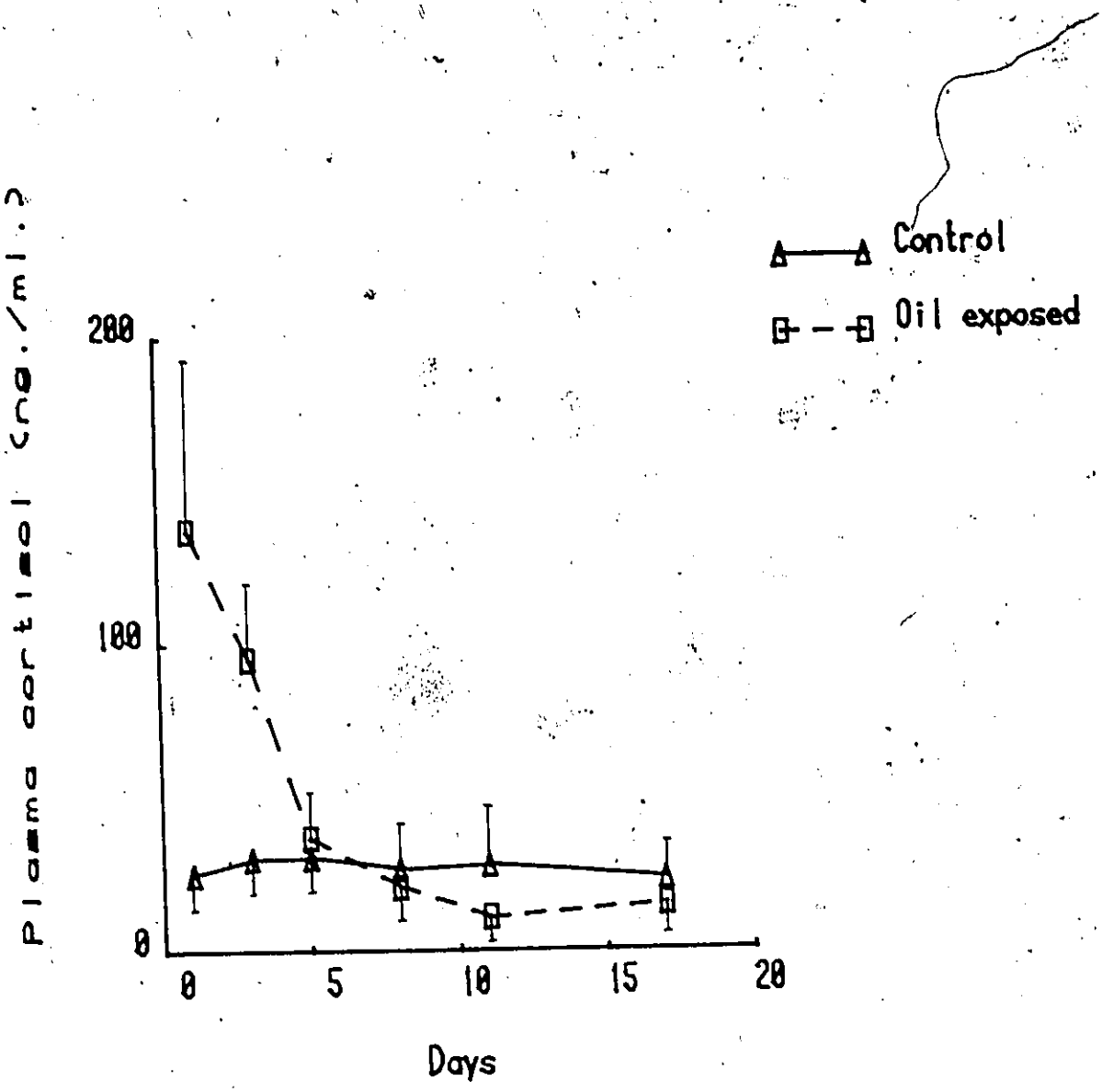
Cortisol concentrations in the plasma of the American eel, Anguilla rostrata, during and following ingestion of crude oil at a dose of 10 µl/kg, given for five consecutive days. Each point represents the mean of four control and five experimental fish, ± standard deviation.

Figure 9: Plasma cortisol following 10 µl/kg oil dose


Plasma cortisol (ng./ml.)
200
100
0
0
5
10
15
20
Days
Control
Oil exposed

Cortisol concentrations in the plasma of the American eel, Anguilla rostrata, during and following ingestion of crude oil at a dose of 100 µl/kg, given for five consecutive days. Each point represents the mean of four control and five experimental fish ± standard deviation.

Figure 10: Plasma cortisol following 100 µl/kg oil dose


Control
Oil exposed
Plasma cortisol (ng./ml.)
200
100
0
0
5
10
15
20
Days

The initial elevation in plasma cortisol levels appears to be dose dependent as indicated by the significantly higher cortisol titers observed at days 1 and 3 for 100 µl/kg dosed fish as compared to the 10 µl/kg oil-treated animals. In both cases, however, the increase in cortisol levels is followed by a significant drop to control levels.

2 Effect of cortisol administration on hepatic mixed function oxidases

The effect of intraperitoneal administration of cortisol on hepatic benzo[a]pyrene hydroxylase activity and cytochrome P-450 content are presented in Table 5. When studying the time course of the circulating cortisol levels, no change in plasma cortisol could be observed in cortisol-treated fish on the first day of administration. Subsequent injections, however, resulted in elevated plasma titers which remained high throughout the experimental period. No changes in cortisol levels were observed for control fish.

The cortisol treatment did not significantly affect either the cytochrome P-450 content or the enzyme activity of BaP hydroxylase in the liver.

3 Effect of induced MFO levels on the breakdown of circulating cortisol

The clearance of radioactivity from the plasma of *A. rostrata* injected intraperitoneally with 0.1 µCi of ^{3}H-labelled cortisol, comparing control to oil-exposed fish

TABLE 5

MFO response to cortisol injections

Responses in hepatic BaPH and Cytochrome P-450 to five doses of cortisol, given intraperitoneally at 10µg/day, in the American eel, Anguilla rostrata, compared to control fish. Values are the means of four fish ± standard deviation.

Treatment Day	Plasma Cortisol(a)	BaPH(b)	P-450(c)
Experimental			
1	30.75	277.90 ± 63.62	0.15 ± 0.05
2	250.00	209.04 ± 66.59	0.20 ± 0.09
3	260.00	199.29 ± 59.32	0.19 ± 0.07
4	285.00	168.63 ± 61.28	0.21 ± 0.08
5	270.00	200.00 ± 31.07	0.22 ± 0.06
Control			
1	23.66	259.82 ± 23.61	0.17 ± 0.05
2	29.66	183.12 ± 35.86	0.14 ± 0.04
3	28.33	214.54 ± 34.59	0.16 ± 0.06
4	31.66	187.01 ± 50.18	0.18 ± 0.09
5	26.33	195.17 ± 31.12	0.15 ± 0.07

a = ng/ml
b = benzo[a]pyrene hydroxylase activity expressed as nmoles of hydroxylated BaP/mg supernatant protein/30 min.
c = P-450 concentrations in nmoles/mg microsomal protein

is presented in Fig. 11. Statistical differences between treatments were established using a one-way analysis of covariance at $P < 0.05$.

Overall, the oil-treated fish showed an increased clearance rate of plasma cortisol when compared to control eels. Biological half-life for cortisol, calculated from the clearance curves of Fig. 11, were found to be 11 hours for oil-exposed and 19 hours for non-exposed fish.

Levels of BaPH activity and cytochrome P-450 content in the liver of control and oil-exposed fish were measured at 24 and 72 hours as shown in Table 6. Significant differences between groups were established using the Student's 't' test at $P < 0.05$. At both times, oil-exposed fish showed a major induction in BaPH, 7-8 times control levels. Cytochrome P-450 was also induced, showing an increase of over 2 times when compared to hepatic levels in control fish.

Clearance of radioactivity from the plasma of A. rostrata injected intraperitoneally with 0.1 µCi of ^{3}H-labelled cortisol, comparing six control to six experimental fish exposed for three consecutive days at a dose level of 100 ul/kg.

◇- - -◇ , oil-exposed eels

△——△ , control eels

Figure 11: Clearance of injected ^{3}H-labelled cortisol

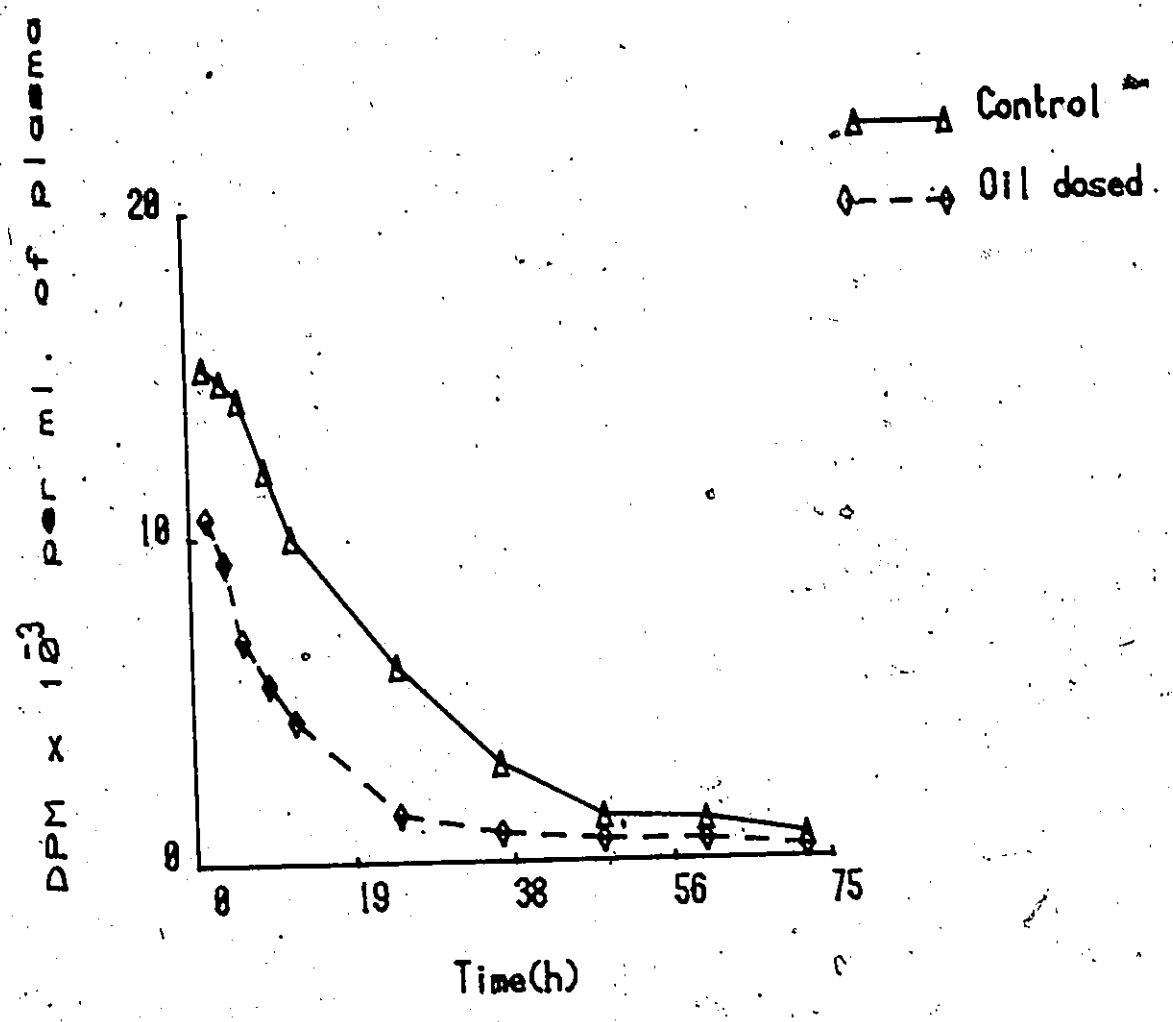

Control
Oil dosed
DPM x 10^-3 per ml. of plasma
20
10
0
0
19
38
56
75
Time(h)

TABLE 6

Effect of oil ingestion on hepatic BaPH and cytochrome P-450

Responses in hepatic BaPH and cytochrome P-450 following ingestion of crude oil at a dose of 100 µl/kg for 3 consecutive days. Enzyme levels were measured on the first and third day after dosing. Each value represents the mean of 6 fish ± standard deviation.

	Controls		Oil-dosed	
	day 1	day 3	day 1	day 3
BaPH[a]	184 ± 33	198 ± 32	1,489 ± 410	1,320 ± 398
P-450[b]	0.18 ± 0.09	0.19 ± 0.06	0.45 ± 0.11	0.38 ± 0.12

a - benzo[a]pyrene hydroxylase activity expressed as nmoles of hydroxylated BaP/mg supernatant protein/30 min.
b - cytochrome P-450 levels in nmoles per mg microsomal protein

DISCUSSION

Cortisol is generally recognized as the major circulating glucocorticoid in teleost fish (Idler and Truscott, 1972). It is known to promote gluconeogenesis and this process underlies its major role in intermediary metabolism (Chester Jones et al., 1972). In addition, cortisol is also involved in ion regulation and although its precise role in osmoregulation in fish remains obscure, it has been shown to affect ion-transport mechanisms at the gills (both influx and outflux), the kidney of freshwater-adapted fish and the cell membranes throughout the animal (Chester Jones et al., 1972).

Changes in plasma glucocorticoid concentration appears to be a generalized adaptive response to a variety of disturbances in many vertebrates (Selye, 1976). Circulating corticoid levels in fish have been shown to increase in response to pollutant exposure such as to heavy metals (Schreck and Lorz, 1978), pesticides (Grant and Mehrle, 1973) and naphthalene (Di Michele and Taylor, 1978). In the present study ingestion of petroleum hydrocarbons by the American eel at doses of 10 and 100 µl/kg resulted in marked elevations of plasma cortisol titers during treatment independent of the oil exposure levels. The initial rise in plasma cortisol levels appears to be dose-dependent as indicated by the lower plasma cortisol levels observed in the 10 µl/kg oil-exposed fish when compared to the 100 µl/kg treated

fish. Exposure of F. heteroclitus to varying concentrations of naphthalene in the water also resulted in a dose dependent elevation in plasma cortisol titers (Di Michele and Taylor, 1978).

The stress response to environmental agents has been previously defined as a multiphasic process whose stages are marked by dynamic changes in circulating glucocorticoid levels (Pines, 1966, cited in De Bruin, 1976; Selye, 1976). In the present study, the different phases in the stress response of eels exposed to petroleum hydrocarbons could not be distinguished because of the discontinuous sampling times. No major changes in the dynamics of circulating cortisol levels were observed between the 10 and the 100 µl/kg crude oil-exposed fish. In both cases an increase in plasma cortisol titers was observed. Similar responses have been found in coho and sockeye salmon after exposure to heavy metals (Donaldson and Dye, 1975; Schreck and Lorz, 1978). The initial elevation in cortisol titers observed on day 1 in eels exposed to crude oil, is followed by a drop to control levels regardless of the dose employed. No further increases were observed for the oil-treated fish which probably indicates that the exposure levels were of a sublethal type and, as a result, the animal is able to compensate and return to pre-stress condition.

There is some evidence indicating that exposure of rainbow trout and carp to PCB's for 4 weeks results in a

significant decrease in plasma concentration of corticoids (Sivarajah et al.,1978). This is interesting in relation to this study because those low levels in plasma corticoid were accompanied by induction of hepatic microsomal enzymes such as aminopyrene demethylase and cytochrome P-450, suggesting a relationship between enhanced hepatic microsomal enzyme activity and decrease in steroid levels. In the American eel, once the cortisol levels returned to control levels, no significant differences were found between oil-exposed and control eels. It should be mentioned, however, that there seems to be a tendency towards lower cortisol levels in the oil-treated fish when compared to control fish. This may be important because, as shown in Chapter II, exposure to petroleum hydrocarbons in A. rostrata resulted in a marked induction of microsomal enzymes measured as BaPH and cytochrome P-450.

Early studies have indicated a striking similarity in the microsomal enzymatic activity with respect to steroid hydroxylation and the oxidative metabolisms of lipid soluble foreign compounds (Conney, 1967; Kuntzman, 1969). Furthermore, different investigations have suggested that administration of a variety of MFO inducers in mammals causes an increase in the metabolism of steroids by microsomal enzymes (Conney et al., 1965; Conney and Burns, 1972).

In this investigation attempts were made to establish a correlation between induction of MFO enzymes and changes

in the breakdown rate of cortisol. The results obtained indicated an increased clearance rate in circulating plasma cortisol levels for the oil-treated eels when compared to control fish. The significant elevations in the levels of hepatic microsomal BaPH and cytochrome P-450 indicated an enhanced MFO capacity, which is perhaps responsible for the more rapid rate of breakdown observed for cortisol. Since cortisol has an important role in osmoregulation and ion homeostasis, as well as in the intermediary metabolism in eels (Lidman et al., 1979), physiological consequences are suggested to result from an interaction between petroleum-induced microsomal enzmes and steroid hormones in fish.

From studies carried out on mammals, it is well established that administration of exogenous steroid hormones of all kinds including corticosteroids, androgens, and estrogens could result in the stimulation of hepatic microsomal enzymes (Conney, 1967). In the present study, calculated dose administrations of cortisol resulted in plasma cortisol titers similar to those previously observed in the plasma of eels exposed to crude oil at a dose of 100 µl/kg (refer to Fig.10). The range of the measured cortisol levels in the control fish was within the normal range previously reported for eels (Lewander et al., 1976). In this investigation, intraperitoneal administration of cortisol and consequent elevations of plasma cortisol for 4 days to stress levels did not significantly affect the levels of he-

patic BaPH and cytochrome P-450 in A. rostrata. This finding is supported by the results reported by Hansson and Lidman (1978). In that study, chronic administration of cortisol for 21 days into rainbow trout also failed to induce the levels of cytochrome P-450. A significant increase in the activity of NADPH cytochrome c reductase in cortisol-treated rainbow trout, however, was found at all sampling events, suggesting that cortisol may activate the in vivo metabolism of endogenous substrates. This was further substantiated by a decline in plasma cortisol levels in the cortisol-treated trout despite continuous administration of exogenous cortisol.

From the previous discussion, it appears that the induction levels of hepatic microsomal enzymes observed in eels after ingestion of petroleum hydrocarbons are then not a response to the high cortisol levels observed in oil-treated eels. Additional work is of interest, however, in order to establish the effect of elevated cortisol on the induction of other MFO's, and a possible change in the biotransformation of other steroid hormones, as well as foreign compounds.

The results presented in this study indicated that ingestion of crude oil by the American eel, A. rostrata, resulted in a generalized corticoid stress response which appears to be dose-dependent. In addition, exposure to petroleum in eels caused an increase in the rate of breakdown of

circulating cortisol which is probably due to enhanced hepatic MFO enzyme activity.

SUMMARY AND CONCLUSION

Ingestion of crude oil by the American eel, Anguilla rostrata, results in a rapid uptake and accumulation of hydrocarbons in key organs, particularly those of recognized high lipid content or those that represent sites of metabolic breakdown and excretion. Liver exhibited the highest hydrocarbon concentration; other tissues had markedly lower hydrocarbon concentrations.

An efficient system for the removal of hydrocarbons is suggested by a rapid decrease, by a factor of 20, in the total hydrocarbon concentration at the end of the depuration period when compared to the total maximum hydrocarbon concentration during the uptake phase.

Maximum hydrocarbon activity was found in bile which showed 45 times higher levels than liver. This suggests a biliary route of hydrocarbon excretion.

Most of the tracer activity found in bile was associated with the water-soluble fraction. This indicates that the hepatic enzyme system of eels is capable of transforming

non-polar foreign hydrocarbons into polar metabolites which can then be readily eliminated from the organism.

Additional support for the existence of a detoxification mechanism is given by the findings of a marked activation of hepatic microsomal enzymes measured as BaPH and cytochrome P-450. This induction in MFO enzymes likely results in an enhanced hepatic biotransformation of petroleum hydrocarbons which may then account for the high levels of hydrocarbon metabolites found in the bile of oil-exposed fish. It appears therefore, that although short term exposure to crude oil results in a rapid accumulation of petroleum hydrocarbons in eel tissues and fluids, hepatic enzyme systems are simultaneously induced by the oil exposure allowing an effective removal of petroleum hydrocarbons from the fish system.

Oil exposure in A. rostrata results in a generalized adrenal corticoid stress response indicated by the increase in cortisol levels observed in oil-treated fish. Elevations in plasma cortisol titers appeared to be dose-dependent and increasing from 25 ng/ml in the control fish to 60 ng/ml for the 10 µl/kg oil-exposed fish, whereas the 100 µl/kg treated eels showed an increase to 120 ng/ml.

The adrenal corticoid stress reaction is known to be an important adaptive endocrine response, but exposure to the petroleum hydrocarbon stressor is known from this study to result in an enhanced hepatic microsomal enzyme activity

in the eel. This enzyme induction appears to be responsible for the higher breakdown rate of circulating cortisol observed in the oil-exposed eels. The increase in the breakdown rate was reflected in a significantly shorter cortisol half-life for the oil-exposed fish when compared to control eels. An extrapolation of these findings is that long-term exposures to petroleum hydrocarbons in eels may have a deleterious effect in accelerating the metabolism of cortisol, which could result in the breakdown of the adrenal corticoid stress-response and, as a consequence, in a long-lasting negative effect on cortisol balance in fish.

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