Induction of Hepatic Aryl Hydrocarbon Hydroxylase in Salmon Exposed to Petroleum Dissolved in Seawater and to Petroleum and Polychlorinated Biphenyls, Separate and Together, in Food

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Aryl hydrocarbon hydroxylases (AHH) are among several xenobiotic metabolizing enzymes known to occur in hepatic microsomes of aquatic animals (ADAMSON, 1967; BUHLER and RASMUSSON, 1968; CREAVEN, 1965; DEWAIDE, 1970; PEDERSEN, et. al., 1974). These enzymes mediate chemical transformations of compounds, which are foreign to the body, thus facilitating their depuration and excretion. Recent studies have demonstrated that certain chemicals can either stimulate (induce) or inhibit the activities of AHH, or they may have little or no effect, depending on the molecules involved (DAO and YOGO, 1964; IKEDA and OHTSUJI, 1971; FOUTS, 1973).

In marine waters, where a host of contaminants exist together, petroleum oils are sources of numerous xenocompounds and polychlorinated biphenyls (PCB's) are generally ubiquitous. Accordingly, it is important to determine how petroleum hydrocarbons and PCB's, separately and together, affect biochemical systems of marine organisms.

This paper describes studies of *in vivo* inductions of AHH, as benzo[a]pyrene hydroxylase, in hepatic microsomes of young salmon. Fish were exposed to components of Prudhoe Bay crude oil (PBCO) dissolved or dispersed in seawater and to PBCO impregnated in food with and without PCB's.

MATERIALS AND METHODS

Two groups of young coho salmon (*Onchorhynchus kisutch*) were used. One group, which was used for exposures to a water-soluble fraction (WSF) of PBCO, was obtained from the Leavenworth National Fish Hatchery of the U.S. Fish & Wildlife Service. The other group, which was used for exposures to PBCO and PCB's in food, was obtained from among cohorts reared in our laboratory from eggs of two or three spawning salmon. All fish were acclimatized to their respective environments prior to the initiation of exposures to contaminants. Fish

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exposed to the WSF were held for at least two weeks in a large outdoor holding tank before being placed for exposures in aquaria containing seawater maintained at 8.5°-9.0°C. Fish for the feeding experiments were held in flow-through tanks containing fresh water maintained at 11°-12°C for about two weeks before the start of the contaminant feeding.

A method similar to that of ANDERSON, et. al. (1974) was employed to produce a WSF of PBCO. Approximately 20 liters of stock solution of WSF was made by mixing 10 ml PBCO/liter of filtered seawater in a closed container at room temperature for 18-20 hr with stirring such that the vortex of the mixture remained at 15% to 20% of the distance from the bottom of the mixture. Then, the mixture was allowed to stand undisturbed for 4 hr, and the water was siphoned from beneath the oil layer. Dilute solutions of 5% and 0.5% (v/v) of the stock WSF were made up with filtered seawater and used to fill several 20-liter cylindrical jars, which served as aquaria. Ten fish were placed in each jar, while filtered air was bubbled at 50 ml/min through aeration stones. The exposures of fish to WSF was a static test that lasted for 6 days. A parallel control experiment without the WSF was employed. On the seventh day, the fish were killed, and weights, lengths, and sex were determined. Finally, livers were removed, rinsed with cold 1.15% KCl solution, immediately frozen at -50°C, and held at that temperature overnight for enzyme assays.

The concentration of WSF of PBCO in the dilute solutions was determined by spectrophotofluorometry (KEIZER and GORDON, 1973), as follows: A one liter sample of solution to be tested was passed through a 2 cm diameter x 12 cm high bed of Amberlite XAD-2 resin (Rohm & Haas Co.)[a] to extract the soluble organics from the water (BURNHAM et. al., 1972). Prior to use, the XAD-2 resin was cleaned by successive Soxhlet extractions with acetonitrile, diethyl ether, and methanol, and packed columns were sucessively eluted with methanol and distilled water. The distilled water was also treated to remove dissolved organics by passing it through a separate clean XAD-2 column. Solvents were pesticide grades or distilled-in-glass grades. The absorbed organics were recovered from the resin by eluting with diethyl ether. Water was removed from a concentrated ether extract (10-12 ml) by a brief (ca. 10 sec) freezing with a dry ice-acetone mixture and

[a]Mention of commercial products is for information only and does not constitute endorsement by the U.S. Department of Commerce.

quickly decanting into a graduated tube. The ether was evaporated and replaced with cyclohexane. The extracts in cyclohexane were assayed with an Aminco-Bowman spectrophotofluorometer (SPF), using slit arrangement number 4 suggested by the manufacturer, American Instrument Co. Quinine sulfate(1µg/ml in 0.1 N-H_2SO_4) was used to standardize the SPF. A quantity of WSF was extracted from a separate stock solution with trifluorotrichloroethane (Freon 113, E.I. Du Pont De Nemours & Co.) and recovered gravimetrically. Standard solutions were prepared from the WSF gravimetric extract to establish a standard curve for the SPF analyses. The stock solution of WSF used in the aquaria assayed at 3.0 mg/liter before the dilutions.

Oregon moist fish pellets (3/32-inch size, Formula II, Moore-Clark Co., LaConner, WA) served as a basal diet for exposures via the diet. The pellets were impregnated by a method similar to that reported by GRUGER et. al. (1975) to give three diets containing 1 ppm (w/w) PCB's (as Aroclor 1242), 1 ppm PBCO, and a 50-50 mixture of 1 ppm PCB's and 1 ppm PBCO. A flowing freshwater fish-holding system was employed for fish receiving the various diets (GRUGER et. al., 1975). The fish were fed once each day, 5 days a week, at a rate of 3% of their initial body weights per day. Ten animals were used at each exposure concentration. After 60 or 68 days of feeding, the fish were killed and treated as described for the WSF-exposed fish.

The AHH activities were measured by adaptations of (1) a fluorometric method of DEHNEN et. al. (1973), and (2) a ^{14}C-labeled substrate method of DEPIERRE et. al. (1975). The fluorometric method was performed on 15,000 x g (15 min) supernatant fractions of livers from fish exposed to the WSF. The supernatant fraction was obtained from homogenates of 1 part liver and 9 volumes of ice-cold 0.25 molar sucrose solution. Homogenization was performed with a teflon-coated pestle in a Potter-Elvehjem grinder held in an ice-water bath. The ^{14}C-labelling method was performed on 0.25 M-sucrose suspensions of microsomes from livers of fish fed PCB's and PBCO in diets. The labelled substrate, [7,10-^{14}C]-benzo[a]pyrene (21 mCi/mmole, Amersham/Searle, Arlington Heights, Ill.), was extracted with 0.25 M-NaOH in 40% (v/v) ethanol to lower the background of assay blanks. Preliminary AHH assays established optimum conditions of 25°C and pH 7.4 for coho salmon hepatic microsomes. These conditions agree with findings of BUHLER and RASMUSSON (1968) and PEDERSEN et. al. (1974) for salmonids. The microsomes were prepared by homogenizing 1 part liver and 4 volumes of ice-cold 0.25 M-sucrose, centrifuging at 9,000 x g (20 min), and sedimenting

microsomal pellets by centrifuging the 9,000 x g supernatant fractions at 100,000 x g (60 min). All centrifugations were done at 3°-5°C.

RESULTS AND DISCUSSION

Analyses indicated that the experimental fish were exposed initially to a maximum of WSF compounds at 150 parts-per-billion (ppb, 10^{-9} g/ml) and 15 ppb, respectively, in 5% and 0.5% dilutions of the WSF stock solution; however, the aeration of these dilute solutions resulted in losses of the volatile components. In order to estimate these losses, an experiment without fish was carried out on a series of 20-liter quantities of the 5% dilution of WSF stock solution. Spectrophotofluorometric analyses of extracts for various time periods indicated that 60-65% of the dissolved components were lost during the first 3-5 hr of aeration; the concentration of WSF remained constant between 5 and 71 hr.

ANDERSON et. al. (1974) reported 80-90% loss of the hydrocarbons from a WSF of petroleum in 24 hr by gentle aeration. The components of WSF which remained after 24 hr included nonvolatile aliphatic and aromatic hydrocarbons, which are "accommodated" perhaps in colloidal form rather than solubilized (BOEHM and QUINN, 1974; LEE et. al., 1974; GORDON et. al., 1973). BOYLAN and TRIPP (1971) indicated that a variety of methylated naphthalenes and polyalkylated benzenes are found in seawater extracts of petroleum products. These facts are important when considering the nature of the WSF used in the present experiments, since no qualitative information was obtained. Accordingly, in essence, the fish were exposed to the remaining nonvolatile hydrocarbons for the 6-day exposure period.

The hepatic AHH activities of the fish exposed to WSF are given in Table 1. The AHH activities are reported (separately and combined) according to the sex of the fish and for the total group. There was no significant difference found from the AHH data relative to the sex of fish. No significant difference was found when comparing AHH activities in 15 ppb-WSF-exposed fish and control fish. However, there was significant ($0.01<P<0.05$) enhancement of AHH activity with fish exposed to 150 ppb WSF relative to that for control fish. The data on weights and lengths of fish (Table 1) indicate that the three groups are comparable with respect to these parameters

PAYNE and PENROSE (1975) found that petroleum induces the AHH in tissue homogenates from liver and

TABLE 1

Hepatic Aryl Hydrocarbon Hydroxylase (AHH) Activities in Coho Salmon Exposed for Six Days to a Diluted Water-Soluble Fraction (WSF) of Prudhoe Bay Crude Oil

Parameter	No. of Fish	Wt. of Fish (g)	Length of Fish (cm)	AHH Activity*		
				Females	Males	Combined
150 ppb-WSF treated	10	20.6 ± 5.1	11.8 ± 0.9	0.85 ± 0.27 (4df)	0.71 ± 0.22 (4df)	0.78 ± 0.24 (9df)**
15 ppb-WSF treated	9	22.4 ± 4.7	12.1 ± 0.9	0.78 ± 0.37 (1df)	0.47 ± 0.15 (6df)	0.54 ± 0.23 (8df)
Control	9	22.2 ± 5.6	12.1 ± 1.1	0.41 ± 0.06 (3df)	0.50 ± 0.23 (4df)	0.46 ± 0.17 (8df)

* Activity = Percent relative fluorescence of phenolic or hydroxylated products formed / mg 15,000 x g supernate protein / 30 min. incubation. Standard deviations given with degrees of freedom (df).

** Significant ($0.01 < P < 0.05$) difference relative to controls. Student's t test, $t = 2.608$ (17 df).

gills of brown trout (Salmo trutta) and capelin (Mallotus villosus). Hepatic AHH activity has been shown to be higher in fish taken from petroleum-contaminated sites than in fish taken from control sites (PAYNE, 1975).

Two separate experiments involving PCB's with PBCO in the diet were carried out with young coho in fresh water. The results are reported in Table 2. The sizes of the fish are comparable to those used in the exposures to the WSF. In Experiment 1, two livers of the corresponding sex were combined for assays where possible. Individual livers were assayed in Experiment 2. Activities of the AHH enzyme system in livers of males and females were compared, and no significant difference between sexes could be established. As with the seawater exposures, comparisons were made among the combined female-male exposure groups. Statistical analysis of variance (SOKAL and ROHLF, 1969) showed that there were differences among the contaminant dietary exposures, which had a significant ($P<0.05$) effect on the hepatic AHH activity. Statistical t-test analyses showed significant differences in hepatic AHH activities for PBCO-fed fish compared to fish fed the mixture of PCB's and PBCO in both Experiment 1 ($P<0.05$) and Experiment 2 ($P<0.10$) (Table 2). Also, in Experiment 2, there were significant differences ($P<0.05$) comparing PCB-fed fish to control fish, as well as differences ($P<0.01$) comparing mixed PCB/PBCO-fed with control fish. All other comparisons did not show statistically significant differences in AHH activities. The wide range of values for AHH activities reported in Table 2, and by others (PEDERSEN et. al., 1976; PAYNE and PENROSE, 1975), may simply reflect natural biological variabilities among individual fish.

LITTERST and VAN LOON (1974), TURNER and GREEN (1974), and others found that PCB's are inducers of xenobiotic enzymes in animals. In some cases, PCB's are more powerful enzyme inducers than DDT and phenobarbital (LITTERST and VAN LOON, 1972); the latter compounds have been widely studied in the inductions of drug-metabolizing enzymes (CONNEY et. al., 1973). GROTE et. al. (1975) showed that metabolism of foreign compounds is altered in animals after treatment by PCB's. PAYNE (1975) reported no induction of benzo[a]pyrene hydroxylase in rainbow trout exposed to chlorobiphenyls (Aroclor 1016). GRUGER et. al. (1976) report the induction of an AHH system in coho salmon by a pentachlorobiphenyl.

TABLE 2

Hepatic Aryl Hydrocarbon Hydroxylases (AHH) Activities in Coho Salmon Fed 1 Ppm of PCB's and PBCO, Separately and Together, in Food Pellets

Experiment No. *	Diet	No. of Fish	Wt. of Fish (g)	Length of Fish (cm)	AHH Activity **		
					Females	Males	Combined
1	PCB/PBCO	8	21.4 ± 3.3	12.4 ± 0.6	2.41 ± 1.89	2.30 ± 0.01	2.36 ± 1.09 (3df)
1	PBCO	9	23.9 ± 3.2	11.9 ± 0.5	0.69 ± 0.36	1.28 ± 1.02	0.98 ± 0.66 (4df)
2	PCB/PBCO	10	21.8 ± 4.1	12.1 ± 0.8	4.01 ± 1.15	5.12 ± 1.83	4.57 ± 1.55 (9df)
2	PBCO	9	19.8 ± 3.7	11.7 ± 0.6	2.91 ± 1.94	3.54 ± 1.13	3.33 ± 1.35 (8df)
2	PCB	10	20.5 ± 4.0	11.8 ± 0.7	4.08 ± 3.19	6.23 ± 4.52	5.58 ± 4.10 (9df)
2	Control	10	21.5 ± 2.7	11.9 ± 0.5	2.50 ± 1.37	3.03 ± 0.96	2.68 ± 1.22 (9df)

* Experiment No. 1: After 60 days feeding, fish were sacrificed and livers, except one PBCO sample, were combined in pairs by sex and assayed.

Experiment No. 2: After 68 days feeding, fish were sacrificed and individual livers were assayed.

** Activity = nanomoles of phenolic or hydroxylated products formed / mg microsomal protein / 20 min. incubation. Standard deviations given with degrees of freedom (df).

CONCLUSIONS

1. Hepatic AHH is induced in coho salmon exposed to 1 ppm PCB's in the diet; however, comparable experiments with 1 ppm of Prudhoe Bay crude oil did not significantly alter the AHH activities. These findings suggest that in aquatic food-chains containing both petroleum hydrocarbons and PCB's, the latter compounds may exert a predominant influence on the induction of the hepatic AHH enzyme system.

2. Despite the lack of induction of hepatic AHH with 1 ppm Prudhoe Bay crude oil in the diet, young coho salmon exposed to 150 ppb (0.15 ppm) of a seawater-soluble fraction for 6 days exhibited a significant induction of this enzyme system. Thus, crude oil components of relatively high water solubility (e.g., methylated naphthalenes and polyalkylated benzenes) may be more effective than high molecular weight insoluble components in inducing AHH.

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