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BIOACTIVATION OF POLYNUCLEAR AROMATIC HYDROCARBONS TO CYTOTOXIC AND MUTAGENIC PRODUCTS BY MARINE FISH

by

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ABSTRACT

Levels of hepatic cytochrome P-450 and mixed-function oxygenase activity differed markedly between marine fish species scup, Stenotomus versicolor, and winter flounder, Pseudopleuronectes americanus, and between male and female winter flounder. Hepatic preparations from all these fishes, however, were capable of efficiently activating carcinogenic polynuclear aromatic hydrocarbons to mutagenic derivatives. The results indicate that coastal marine fishes may be at a risk to carcinogenic aromatic hydrocarbons in marine sediments.

INTRODUCTION

Polynuclear aromatic hydrocarbons are metabolized or biotransformed by microsomal cytochrome P-450 dependent mixed-function oxygenases in tissues of diverse species (Walker, 1978). The metabolism of carcingogenic polynuclear aromatic hydrocarbons by some species is known to result in formation of mutagenic derivatives (Wislocki et al., 1976). Studies have indicated that certain metabolites are responsible for mediating the carcinogenic activity of these compounds (Levin et al., 1977, 1978; Slaga et al., 1977; Hecht et al., 1978). However, microsomal cytochromes P-450 from different mammalian tissues, different species, or animals subjected to different treatment appear to vary in their ability to metabolize and activate polynuclear aromatic hydrocarbons to mutagens in vitro (Ames et al., 1975; Levin et al., 1976). Such variation may indicate differences

in susceptibility to mutagenic or carcinogenic potential of polynuclear aromatic hydrocarbons.

Microsomal electron transport systems in fish tissues are qualitatively similar to those in mammals (Pohl et al., 1974; Chevion et al., 1977; Stegeman and Binder, 1979). The levels of components of these systems and the mixed-function oxygenase reactions carried out, however, are generally lower in fish (Pohl et al., 1974; Bend et al., 1977). Yet, in some species the activity of hepatic aryl hydrocarbon [benzo(a)pyrene] hydroxylase is normally found to be higher than seen in mammals (Ahokas et al., 1975; Stegeman and Binder, 1979). Data suggest these fish may have cytochrome(s) P-450 that catalytically resemble(s) 3-methylcholanthrene-induced cytochrome P-448 in mammals, whereas others may not (Bend et al., 1977). We do not know whether such apparent functional differences in cytochromes P-450 within or between fish species might be associated with varied capacity to activate polynuclear aromatic hydrocarbons. The present contribution describes aspects of hepatic cytochrome P-450 systems and the in vitro activation of selected polynuclear aromatic hydrocarbons by two species of marine fish, scup (porgy), and winter flounder.

MATERIAL AND METHODS

Chemicals--Benzo(a)pyrene used in enzyme assays was obtained from Aldrich Chemical Company (Milwaukee, WI). Benzo(a)pyrene, 7,12-dimethylbenzanthracene and 1,2,3,4-dibenzanthracene used in mutation assays were obtained from Sigma Chemical Co. (St. Louis, MO). NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, aminopyrine, Tris, and HEPES were obtained from Sigma.

Animals--Adult male and female scup, Stenotomus versicolor, about 100 to 200 g, were collected by angling in Great Harbor, Woods Hole, MA, in August 1977; winter flounder, Pseudopleuronectes americanus, were obtained in outer Narragansett Bay, RI, by otter trawl in December 1977. Males were 270 to 350 g and females 480 to 520 g. Scup were maintained for four months in 800 gallon tanks at 19° ± 1° C in flowing water filtered through gravel and sand at the National Marine Fisheries Service, Woods Hole, MA. Fish were fed a diet of chopped smelt and clams ad libitum every two days.

Flounder were maintained at ambient temperatures at the U.S. Environmental Protection Agency, Environmental Research Laboratory, Narragansett, RI, prior to transport to Woods Hole. At the time of use, the scup were sexually quiescent, and the winter flounder were fully hydrated, ready to spawn. No fish used in these studies received any experimental treatment.

Tissue preparations--Animals were killed by decapitation. Excised livers were placed immediately in ice-cold 0.1 M phosphate buffer, pH 7.3. Tissues were minced and homogenized in 4 volumes of 0.1 M PO_4 buffer pH 7.3, containing 1.15% KCl and 3 mM $MgCl_2$ by a Potter-Elvehjem tissue grinder with 4 passes of the pestle at 1350 and 4 at 1900 rpm. Post-mitochondrial supernatant (PMS) preparations for use in mutation assays were

collected after centrifuging at 9000 xg for 10 min. Microsomal fractions for enzyme assays were isolated from the 9000 xg supernatant as previously described (Stegeman and Binder, 1979). PMS preparations were frozen in liquid N_2 and then held at -80° C until use (up to two months). Enzyme assays on either PMS or microsomes were done immediately.

Enzyme assays--Benzo(a)pyrene hydroxylase and aminopyrine demethylase in hepatic preparations were assayed as previously described (Stegeman and Binder, 1979) by measuring fluorescent product formation and formaldehyde generation, respectively. NADPH-cytochrome c (P-450) reduction and cytochrome P-450 were measured as previously described (Stegeman and Binder, 1979). Protein was determined according to Lowry et al. (1951).

Bacterial mutation assays--Reverse mutation assays to histidine prototrophy were carried out with Salmonella typhimurium strain TA-98; forward mutation assays to 8-azaguanine resistance (Skopek et al., 1978a) employed S. typhimurium strain TM-677. The sources and storage conditions for these strains have been indicated (Skopek et al., 1978b).

Basic protocols for both the reverse and forward mutation assays have been described (Skopek et al., 1978a; 1978b). Exposure to promutagen was in liquid culture in 25 mℓ plastic tissue flasks and 5.0 mℓ volumes that contained an appropriate concentration of bacterial cells, 0.5 mℓ sterile PMS, and 6.5 μmoles NADPH. Hydrocarbons were added to duplicate flasks in 50 μℓ of dimethyl sulfoxide. Flasks were incubated without shaking for 2 hr at 29° C when scup PMS was employed and 25° C when winter flounder PMS was employed. The temperatures selected were near optimal temperatures for benzo(a)pyrene hydroxylase in these two species when assayed over a 2-hr period. Harvest of cells and plating procedures for estimating both bacterial survival and mutation are described elsewhere (Skopek, 1978b). Mutant fractions in both reverse and forward assays are presented as number of mutant clones (x factor)/number of survivor clones plated.

RESULTS

Levels of microsomal electron transport components and mixed-function oxygenase activities in hepatic microsomes were compared in scup, winter flounder, and mice. Results are presented in Table 1. The levels of cytochrome b_5, NADPH- and NADH-cytochrome c reductases in scup were about 20% of those measured in mice. Cytochrome P-450 present in scup was of comparatively greater amounts (about 50% more than observed in mice). However, the Soret absorption maximum of reduced, CO-treated microsomes was about 450 nm in both species. Aminopyrine demethylase activity was also much lower in scup than in mice, but benzo(a)pyrene hydroxylase activity was almost ten-fold greater in the scup.

TABLE 1. HEPATIC MICROSOMAL ELECTRON TRANSPORT COMPONENTS AND MIXED-FUNCTION OXYGENASES IN SCUP, WINTER FLOUNDER, AND MICE

Component	Scup[a]	Winter Flounder		Mouse[a]
		male	female	
	(N ≥ 10)	(N ≥ 3)	(N ≥ 3)	(N ≥ 3)
Liver wt./body wt. %	1.01±0.10[b]	1.02±0.05	2.43±1.7	5.24±0.29
mg mic. protein/g liver	12.4 ±0.5	17.5 ±0.7	23.0 ±2.9	20.0 ±0.32
Cytochrome P-450 nmoles·mg prot.$^{-1}$	0.62±0.08	0.90±0.21	0.19±0.2	1.14±0.12
Cytochrome b_5 nmoles·mg prot.$^{-1}$	0.06±0.02	-	-	0.33±0.03
NADPH-cytochrome c reductase units·mg prot.$^{-1}$ [c]	107± 5	48± 5	38± 5	510± 9
NAPH-cytochrome c reductase units·mg prot. $^{-1}$	183± 6	-	-	913± 59
Aminopyrine demethylase units·mg prot.$^{-1}$	206±34	84±13	51±19	800±151
Benzo(a)pyrene hydroxylase units·mg prot.$^{-1}$	693±40	213±15	77± 5	72± 14

[a] Data from Stegeman and Binder (1979). Mice were adult Charles River CD-1 females.

[b] All values are ± S.E.M.

[c] Units are nanomoles cytochrome c reduced·min^{-1} (reductases), nanomoles HCHO produced normalized to 1 hour (aminopyrine demethylase) and picomoles 3-OH-benzo(a)pyrene equivalents produced·min^{-1} [benzo(a)pyrene hydroxylase].

During spawning, pronounced sex differences in winter flounder were observed in microsomal cytochromes P-450. The Soret absorption maximum of reduced, CO-treated microsomes from male fish was quite clearly at 448 nm rather than 450 nm seen in females, or in scup or mice. The levels of cytochrome P-450 seen in males were more than five times those of females and were greater than those observed in scup. Unlike cytochrome P-450, NADPH-cytochrome c reductase activity was quite similar in male and female flounder, and the levels were lower than in scup. The levels of both

aminopyrine demethylase and benzo(a)pyrene hydroxylase activity were also lower in male and female winter flounder than in scup. However, a sexual difference was apparent in the levels of these activities (levels in females were lower than those in males). Benzo(a)pyrene hydroxylase activity in female flounder was almost as low as that seen in mice, while this activity in male flounder was several times greater.

The metabolic activation of benzo(a)pyrene to toxic and mutagenic derivatives by fish liver preparations like those described in Table 1 was initially determined by a reverse mutation assay. Results indicate that untreated scup liver PMS when incubated with NADPH and benzo(a)pyrene was capable of stimulating almost an 85-fold increase in the his$^+$ revertant fraction in S. typhimurium strain TA-98. At the same time, there was a 20-fold reduction in survival of S. typhimurium in the complete incubation with 50 μM benzo(a)pyrene. The activation indicated in Table 2 was dependent on the presence of PMS, as well as benzo(a)pyrene and NADPH; a linear dose-dependent increase was observed in both the mutant fraction and the toxicity up to a peak at benzo(a)pyrene concentrations between 40 to 60 μM.

TABLE 2. REQUIREMENTS FOR ACTIVATION OF BENZO(a)PYRENE TO TOXIC AND MUTAGENIC DERIVATIVES IN S. TYPHIMURIUM STRAIN TA-98 BY SCUP LIVER PMS*

Incubation Conditions	Relative Survival	Observed Mutant$^+$ Fraction x 10^8
Complete [50 μM B(a)P]	0.05	47.2
Minus B(a)P	1.00	0.55
Minus NADPH	0.91	0.21

*From Stegeman (1977)

$^+$Mutant Fraction refers to the number of his$^+$ revertant clones x 10^{-6} per number of survivor clones. Incubation conditions are as described in Materials and Methods.

TABLE 3. ACTIVATION OF POLYNUCLEAR AROMATIC HYDROCARBONS TO MUTAGENIC DERIVATIVES IN S. TYPHIMURIUM STRAIN TM-677 BY SCUP AND WINTER FLOUNDER LIVER PMS

PMS Source (N)[a]	Compound[b]	Concentration[c] (μM)	Relative Survival	Induced Mutant[d] Fraction x 10^5
Scup (9)	B(a)P	40	0.48	85.5
	DBA	36	0.70	32.6
	DMBA	20	0.85	14.6
Winter Flounder				
Male (2)	B(a)P	40	0.73	144.5
Female (2)	B(a)P	40	0.84	79.0

[a] Livers from N fish pooled for PMS preparation.

[b] Abbreviations are B(a)P, benzo(a)pyrene; DBA, 1,2,3,4-dibenzanthracene; DMBA, 7,12-dimethylbenzanthracene.

[c] Data presented have been selected from dose-response curves and represent concentrations at which maximal response was detected, except for female winter flounder. Here the data at 40 μM were selected for comparison with both male winter flounder and scup.

[d] Mutant fraction refers to the number of 8-azaguanine resistant clones x 10^{-4} per number of survivor clones. Background mutant fractions (0 compound) were 4-6(x10^5) within the range previously reported (Shopek et al., 1978b) and have been subtracted.

Activation of polynuclear aromatic hydrocarbons by scup and winter flounder liver PMS was compared, using the forward mutation assay to 8-azaguanine resistance in S. typhimurium strain TM-677. The levels of benzo(a)pyrene hydroxylase activity per mℓ of hepatic PMS in the specific preparations used were 4000 pmol 3-OH-benzo(a)pyrene equivalents/min/mℓ for scup, which was twice that in the male winter flounder, 2018 pmol/min/mℓ, and more than six times that in the female, 632 pmol/min/mℓ. The results are presented in Table 3. The mutant fraction resulting from activation of benzo(a)pyrene by scup liver PMS in this assaay was high, as was observed in the his^+ reversion assay. Scup liver PMS also readily activated 1,2,3,4-dibenzanthracene and 7,12-dimethylbenzanthracene, but the mutant fractions observed were lower than with benzo(a)pyrene.

The mutant fraction induced by activation of 40 μM benzo(a)pyrene by female winter flounder PMS was comparable to that seen with scup. Male winter flounder PMS, on the other hand, was able to induce a somewhat higher mutant fraction than scup with 40 μM benzo(a)pyrene. It is noteworthy that these results are quite different from that seen in levels of benzo(a)pyrene hydroxylase activity in PMS preparations, or found in hepatic microsomal preparations of scup and winter flounder. This suggests that the level of benzo(a)pyrene hydroxylase activity in fish is not a suitable indicator of the capacity to form mutagenic derivatives from a polynuclear aromatic hydrocarbon such as benzo(a)pyrene.

DISCUSSION

Both fish species examined possessed marked ability to metabolically activate polynuclear aromatic hydrocarbons to mutagenic products in vitro. The mutant fractions induced with 40 μM benzo(a)pyrene in fact exceeded that [72 ($\times 10^5$)] obtained with 40 μM benzo(a)pyrene and hepatic preparations from Aroclor 1254-induced rats in the forward mutation assay with S. typhimurium TM-677 (Skopek et al., 1978b). This is particularly interesting because fish used in these studies had received no experimental treatment.

Hepatic preparations from rats uninduced or induced with phenobarbital usually have much lower capacity for activation of benzo(a)pyrene than those from animals treated with 3-methylcholanthrene or mixed inducers such as Aroclor 1254 (Ames et al., 1975). This can be attributed to differences in the ability of cytochromes P-450 in the variously treated animals to produce certain mutagenic derivatives of benzo(a)pyrene (Levin et al., 1976). By analogy, the ready formation of mutagenic derivates of benzo(a)pyrene by the fish here suggests that cytochromes P-450 in these animals may in some way be catalytically similar to cytochromes P-448 in some mammals. This is consistent with the observation that hepatic benzo(a)pyrene hydroxylase in untreated scup at least is strongly inhibited by 10^{-4} M 7,8-benzoflavone (Stegeman and Binder, 1979), a characteristic of 3-methylcholanthrene-induced cytochrome P-448 in some mammals (Weibel et al., 1971). However, it is questionable whether these are features of constitutive cytochromes P-450 in scup (Stegeman and Binder, 1979). Possibly, the fish used in our study had been exposed incidentally to aromatic hydrocarbons in the environment, and thus the extent of

benzo(a)pyrene activation, as with the reported inhibition by 7,8-benzoflavone, may not be characteristic of an uninduced state.

A variety of primary and secondary metabolites of benzo(a)pyrene are formed by mammalian liver enzymes. Among the primary metabolites, benzo(a)pyrene-4,5-epoxide is the most potent mutagen, but this arene oxide is readily inactivated by epoxide hydrase, yielding benzo(a)-pyrene-4,5,-dihydrodiol (Levin et al., 1976). Benzo(a)pyrene-7,8-dihydrodiol is formed in a similar manner but, after further metabolism by cytochrome P-450, can yield benzo(a)pyrene-7,8-diol-9,10-epoxides--highly mutagenic secondary metabolites (Wislocki et al., 1976) that are not good substrates for, and thus not readily inactivated by, epoxide hydrase (Wood et al., 1976). An isomer of benzo(a)pyrene-7,8-diol-9,10-epoxide, more readily formed by 3-methylcholanthrene-induced cytochrome P-448 (Huberman et al., 1976; Yang et al., 1976), is believed to be the ultimate carcinogenic form of benzo(a)pyrene (Levin et al., 1977).

The patterns of metabolites of benzo(a)pyrene produced by scup and winter flounder have not been established. However, preliminary studies (Stegeman and Tjessem, unpublished) have indicated that little or no benzo(a)pyrene-4,5-dihydrodiol is formed in vitro by scup liver microsomes, whereas substantial amounts of 7,8-dihydrodiol and 9,10-dihydrodiol are formed. At the same time these studies confirm that epoxide hydrase is present in fish liver (Bend et al., 1977) and indicate that benzo(a)pyrene-4,5-epoxide is not responsible for mutation induced at least by the scup preparations. Further, formation of isomeric benzo(a)pyrene-7,8-diol-9,10-epoxide would be possible, although it is not known if any isomers of this metabolite would be preferentially formed.

The discrepancy between levels of benzo(a)pyrene hydroxylase activity and the activation of benzo(a)pyrene by the fish we studied indicates that metabolite patterns formed by these animals probably differ, although it is recognized that factors other than catalytic function of cytochrome P-450 may influence mutagenic activity detected using a PMS preparation (Ames et al., 1975). Yet the formation of mutagenic and carcinogenic diol-epoxides of polynuclear aromatic hydrocarbons by both species and sexes is a distinct possibility. It is known that patterns of metabolites formed by microsomes and intact cells can differ (Selkirk, 1977), but it is likely that toxic and mutagenic derivatives similar to those formed in vitro can result from metabolism in vivo in these and other (Ahokas et al., 1977) fish. Thus, the results clearly suggest that marine fish may be at risk to carcinogenic activity of polynuclear aromatic hydrocarbons known to be present in recent coastal marine sediments (Laflamme et al., 1978) and presumably coastal waters.

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