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METABOLIC ACTIVATION AND COVALENT BINDING OF BENZO[*a*]PYRENE TO DEOXYRIBONUCLEIC ACID CATALYZED BY LIVER ENZYMES OF MARINE FISH*

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Abstract—Metabolic activation and covalent binding of benzo[*a*]pyrene (BP) to deproteinized salmon sperm DNA by supernatant fractions (10,000 *g*) of liver homogenates isolated from untreated, 3-methylcholanthrene (3-MC) or BP-treated starry flounder (*Platichthys stellatus*) and coho salmon (*Oncorhynchus kisutch*) were investigated. The influence of temperature, pH, time and concentrations of protein, BP, NADPH and DNA on covalent binding was investigated to obtain optimum conditions for *in vitro* binding (pmoles of BP equivalents bound/mg DNA/mg protein) of [^{3}H]BP to DNA for each of the two fish species. When the supernatant fractions from untreated starry flounder were used, the covalent binding of BP to DNA was 7.5 and 2.5 times greater than the values obtained with the supernatant fractions from untreated coho salmon or rat respectively. Treatment of both fish species with 3-MC or BP resulted in a marked (10- to 53-fold) increase in the binding. Ethyl acetate-extractable metabolites formed by fish liver supernatant fractions consisted of BP dihydrodiols (4,5-, 7,8-, and 9,10-dihydrodiols), phenols (3-OH, 9-OH, and 7-OH), quinones (3,6-, 1,6- and 6,12-Q) and BP 4,5-oxide. For both fish species, BP 9,10-dihydrodiol and BP 7,8-dihydrodiol were the major metabolites comprising as much as 48–72 per cent of the total ethyl acetate-extractable metabolites; 3-hydroxy BP was also present in significant amounts. The ratio of the non-K region dihydrodiols to phenols was significantly greater for both fish species compared to rat.

There is increasing evidence [1–8] that covalent interactions of metabolically activated polynuclear aromatic hydrocarbons (PAH) with cellular macromolecules (protein, RNA, DNA) are critical events leading to PAH-induced carcinogenesis in mammals. During metabolic activation of carcinogenic PAH, such as benzo[*a*]pyrene (BP), reactive electrophilic intermediates are formed; some of these metabolites are toxic [2,3], mutagenic [9–12] and form covalent bonds with nucleophilic residues of macromolecules.

The occurence of a high incidence of hepatomas has been reported in certain species of demersal fish belonging to the family Pleuronectidae [13, 14]. Moreover, some chemicals which are known carcinogens in mammals also cause liver tumors in several fish species including salmonids [15–17]. Thus, it appears that fish liver is a target organ for carcinogenesis. Although PAH-induced carcinogenesis has not been demonstrated yet in fish, the possibility that concentrations of PAH are increasing in the marine environment raises a question of the potentially toxic interactions of activated PAH with critical cellular constituents in fish.

Marine fish are able to accumulate significant concentrations of PAH when exposed either to sediments contaminated with petroleum [18] or to waterborne PAH [19–21]. Moreover, when exposed to PAH, such as napthalene, in flowing water [20] or in the diet [22–25], Pleuronectidae and salmonid fish readily metabolized this PAH into a variety of electrophilic metabolites, including 1,2-dihydro-1,2-dihydroxynapthalene, obtained from the corresponding epoxide; no such information is available for BP metabolism in these fish. Recent studies with freshwater trout (*Salmo trutta lacustris*) [26, 27] demonstrated that liver microsomes from these animals metabolized BP into dihydrodiols, phenols and quinones and catalyzed binding of activated BP to DNA. However, except for preliminary report from our laboratory [28] no data are available on the binding of metabolically activated PAH to DNA in marine organisms.

We report that liver enzymes from a salmonid (coho salmon, *Oncorhynchus kisutch*) and a Pleuronectidae (starry flounder, *Platichthys stellatus*) fish extensively metabolized BP into reactive intermediates which bind to DNA. Metabolites formed by liver extracts from these marine fish were characterized by high proportions of non-K region dihydrodiols, such as 9,10-dihydro-9,10-dihydroxy benzo[*a*]pyrene (BP 9,10-dihydrodiol) and 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene (BP 7,8-dihydrodiol).

MATERIALS AND METHODS

Chemicals. Deproteinized salmon sperm DNA, NADPH, sucrose, EDTA, 3-MC and non-radio-

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active BP were obtained from the Sigma Chemical Co. St. Louis, MO*. Generally labeled [^{3}H]BP was purchased from New England Nuclear, Boston, MA. The standards for the oxygenated metabolites of BP were provided by the courtesy of Dr. David G. Longfellow, NCI Carcinogenesis Research Program, Bethesda, MD.

[^{3}H]BP and non-radioactive BP were both purified by elution from a silica gel column by the method of Yang *et al.* [29] using nitrogen saturated solvents. Benzene was removed by evaporation under a nitrogen stream, and the [^{3}H]BP and non-radioactive BP were dissolved together in absolute ethanol to yield BP having a sp. act. of 500 mCi/mmole.

Animals. Sexually immature starry flounder (131 ± 42 g) from the estuary of the Columbia River and coho salmon (93 ± 11 g) from Manchester, WA, were acclimatized to flowing unfiltered seawater for a minimum of 2 weeks. Some of the fish were injected intraperitoneally with 10 mg/kg of BP or 3-methylcholanthrene (3-MC) in corn oil. There was no detectable difference in the binding of metabolically activated [^{3}H]BP to DNA when liver enzymes from untreated or corn oil-treated fish were used. Accordingly, most of the data for control fish in these studies were from untreated fish.

The fish were killed 24 hr after the injection, and supernatant fractions (10,000 *g*) of liver homogenates were prepared according to procedures described previously [30]. Liver supernatant fractions (10,000 *g*) from control and 3-MC-pretreated male Sprague–Dawley rats (≈125 g) were also prepared. Protein concentrations in the 10,000 *g* supernatant fractions were determined by the method of Lowry *et al.* [31].

Determination of covalent binding of activated BP to DNA. The basic incubation protocol was derived from the method of Gelboin [32]. Determination of the optimum temperature for incubation using fish liver extracts was carried out before determining the influence of other parameters (e.g. concentration of NADPH, protein, DNA and BP, as well as pH and time) on covalent binding of metabolically activated BP to DNA. The standard reaction mixture after optimization experiments contained 2 mg of DNA added in 2.5 ml of 0.02 M phosphate buffer (pH 7.4), 0.75 mg NADPH added in 0.1 ml of 0.1 M EDTA (pH 7.4) and 0.2 ml of the 10,000 *g* supernatant fraction (5 mg protein). The reaction was started by adding 5 nmoles BP in 50 μl ethanol. The mixture was incubated in the dark for 15 min at 25° when the fish liver supernatant fraction was used, and at 37° when the rat liver supernatant fraction was used. The reaction mixture was then treated according to the procedure described by Buty *et al.* [8] with the following modification: no $MgCl_2$ was added to the first redissolution of DNA in water. Radioactivity was determined by liquid scintillation spectrometry and DNA was measured by the diphenylamine reaction [8]. All optimization studies were carried out using 10,000 *g* supernatants fractions isolated from livers of 3-MC-treated fish when the temperature of the surrounding water was 8 ± 1°. When studies on the influence of 3-MC or BP treatment of both fish species on BP metabolism and binding of BP to DNA were carried out, the water temperature was 13 ± 1°.

The binding for incubations without NADPH was less than 5 per cent of the value obtained for the same incubation with NADPH. Incubations minus NADPH were used as blanks in each experiment and blank values were subtracted from each test value. In most cases, binding at zero time was also recorded. For each experiment, 10,000 *g* supernatant fractions pooled from the livers of five animals were used and incubations were carried out in triplicate. In most cases each value of binding represents an average of two experiments. The maximum standard deviation between the values obtained in the two separate experiments was 14 per cent.

Metabolism of BP and characterization of ethyl acetate-extractable metabolites. Metabolites were formed by incubating liver supernatant fractions with [^{3}H]BP under the conditions described above, without the addition of DNA. The mixture was extracted with ethyl acetate (2 × 6 ml) to remove unreacted BP and ethyl acetate-extractable metabolites from the aqueous phase as described previously [33]. Radioactivity in both aqueous and organic phases was determined. Ethyl acetate extracts were spotted on silica gel plates and developed in benzene–ethanol (9:1, v/v) as described previously [33]; the extracts were cochromatographed with known standards of BP and its metabolites. Separation and quantification of the metabolites were carried out according to procedures described previously [33]. All operations were carried out under dim light to reduce possible photo-oxidation.

Ethyl acetate extracts from the incubations containing liver supernatant fractions (10,000 *g*) from 3-MC treated fish species and rats were also analyzed using a high pressure liquid chromatograph (Hewlett Packard 4485B) fitted with a Perkin–Elmer HC-ODS reversed phase column (0.26 × 25 cm) with simultaneous detection by u.v. spectrometry and fluorescence spectrometry. Before and after the analysis of each metabolite extract, the metabolite standards were chromatographed and detected by both u.v. and fluorescence spectrometry. Retention times of metabolites were matched against those of the standards. Further characterization of metabolites present in the ethyl acetate extract was carried out by radiometry. Elution was carried out at 50° using a nonlinear methanol–water gradient from 10 to 100% methanol. Fractions were collected at 15-sec intervals, and radioactivity was determined as described previously [34]. From the retention times of metabolites present in each ethyl acetate extract, most radioactive peaks were characterized and quantified.

RESULTS

Influence of several variables on in vitro *binding of BP to DNA.* The radioactivity bound to DNA was not removable by repetitive extractions with organic solvents such as ethanol and ethyl ether, and, therefore, it was assumed that activated BP was

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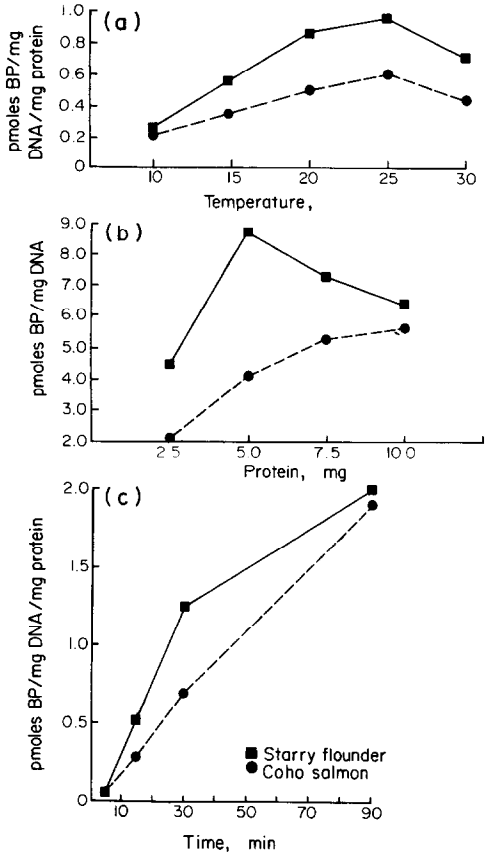

Fig. 1. *In vitro* covalent binding of [^{3}H]BP to DNA by liver supernatant fractions (10,000 *g*) of fish as a function of (A) temperature, (B) protein/reaction mixture and (C) time. Data are from single experiments which gave similar results when repeated. Measurements were made in triplicate. See Materials and Methods for further details.

covalently bound to DNA [32, 34]. For both fish species, the optimum temperature for the *in vitro* binding was close to 25° (Fig. 1a). A noticeable decline in the binding (pmoles of BP equivalents bound/mg DNA/mg protein) occurred when the incubation temperature was increased to 30°. Figure 1b shows that *in vitro* binding of [^{3}H]BP to DNA for both species increased with increasing amounts of protein from 2.5 to 5 mg/reaction mixture; a further increase in protein concentration resulted in a decline in the binding when the starry flounder liver supernatant fraction was used. The NADPH concentration was not varied with the protein concentration (Fig. 1b). Figure 1c shows that for both fish species the binding increased linearly with time for the first 30 min. Figure 2a shows that for both fish species the covalent binding of [^{3}H]BP to DNA declined with increasing DNA concentration from 1 to 4 mg/reaction mixture. Figure 2b depicts the steep increase in binding with increase in NADPH concentration up to 0.5 mg NADPH/reaction mixture. Moreover, binding in the absence of NADPH was less than 5 per cent of the lowest reported value (Fig. 2b). Compared to coho salmon, the starry flounder preparation was more sensitive to the increase in pH of reaction mixture beyond 7.4 (Fig. 2c). Increase in BP concentration from 1 to 4 nmoles/reaction mixture resulted in concurrent increases in the binding of BP to DNA for both coho salmon and starry flounder. A further increase in BP concentration caused no change in the binding for starry flounder and a small decline for coho salmon (Fig. 2d).

Table 1 shows that the covalent binding of [^{3}H]BP to DNA was, respectively, 7.5 and 2.5 times greater when liver supernatant fraction (10,000 *g*) from untreated starry flounder were used compared to the values obtained with the liver supernatant fractions

Table 1. *In vitro* binding of activated BP to DNA catalyzed by 10,000 g liver supernatants fractions from control and PAH-treated fish and rats*

Species	Treatment†	Pmoles BP equivalents /mg DNA/mg protein	% of control value
Starry flounder	Control§	0.15	100
	3-MC§	1.62	1080
	BP§	1.70	1130
	3-MC‖	0.53	
Coho salmon	Control§	0.02	100
	3-MC§	0.97	4850
	BP§	1.06	5300
	3-MC‖	0.30	
Rat	Control	0.06	100
	3-MC	0.69	1150

* Liver supernatant fractions (5 mg protein) from different animals were incubated in the dark with 5 nmoles BP, 2 mg salmon sperm DNA and cofactors for 15 min at 25° (for fish) and 37° (for rat).

† Experimental animals were injected intraperitoneally with either 10 mg/kg of 3-methylcholanthrene (3-MC) or benzo[*a*]pyrene (BP) dissolved in corn oil. There was no difference in binding whether controls were injected with corn oil or not; therefore, untreated animals were used as controls.

‡ Each value is an average of two experiments and three replicate measurements using pooled liver extracts from five animals. Incubations without NADPH were used as blanks in each experiment and the blank values (≤ 0.001) were subtracted from the test values.

§ Livers were obtained from fish when the water temperature was 13°.

‖ Livers were obtained from fish when the water temperature was 8°.

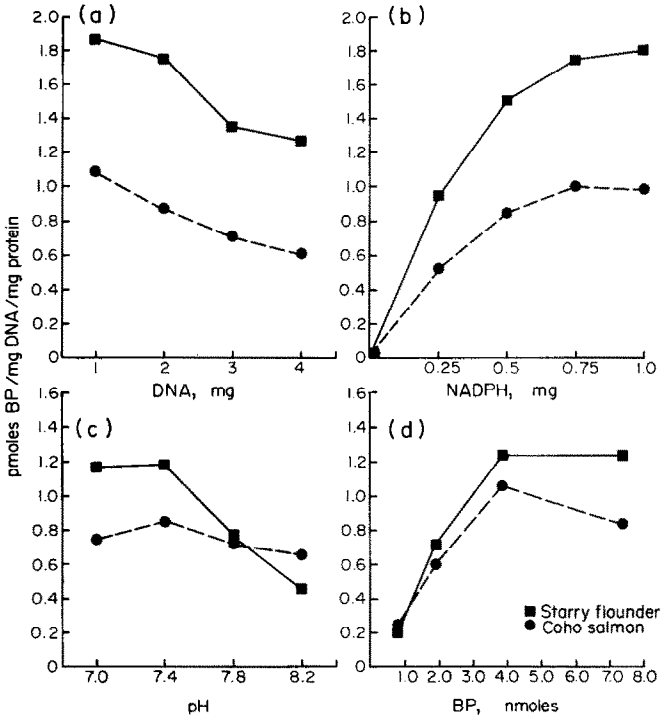

Fig. 2. *In vitro* covalent binding of [^{3}H]BP to DNA by liver supernatant fractions (10,000 *g*) of fish as a function of (a) DNA/reaction mixture, (b) NADPH/reaction mixture, (c) pH, and (d) substrate/reaction mixture. Data are from single experiments which gave similar results when repeated. Measurements were made in triplicate. See Materials and Methods for further details.

from untreated coho salmon or rat. The binding values obtained with the liver supernatant fractions from 3-MC- and BP-treated starry flounder were about 10 times greater than that obtained with the untreated fish; the increase in the value of binding was, respectively, 48- and 12-fold for coho salmon and rat after treatment with 3-MC (Table 1). For both species of fish, the binding of BP to DNA was slightly greater when fish were treated with BP than when they were treated with 3-MC.

The values of binding for both fish species treated with 3-MC were about 3 times lower when liver supernatant fractions were isolated from the fish held at 8°, compared to the respective values obtained when fish were held at 13°.

Metabolism of BP. Benzo[*a*]pyrene was more extensively biotransformed by liver supernatant fractions (10,000 *g*) from control starry flounder than by liver supernatant fractions from either coho salmon or rat (Table 2). Liver supernatant fractions from starry flounder produced 2–6 times as much ethyl acetate-extractable metabolites as did the liver preparations from either coho salmon or rat (Table 2). Less than 3 per cent of BP was biotransformed when incubations contained liver enzymes but no NADPH. Hardly any BP was biotransformed when boiled liver extracts were used.

Treatment of both species of fish with either 3-MC or BP resulted in a considerable increase in the extent of *in vitro* metabolism of BP; large increases in radioactivity in the organic and aqueous phases were observed (Table 2). Treatment of rats with 3-MC also resulted in an increase in the *in vitro* biotransformation of BP; however, this increase was reflected only in the increase in the amounts of ethyl acetate-extractable metabolites.

Thin-layer chromatography (t.l.c.) revealed that phenols and quinones of BP were the major metabolite classes (40–60 per cent) of the ethyl acetate-extractable metabolites produced by rat liver extracts, whereas metabolites produced by the fish liver extracts were characterized by large amounts (40–70 per cent) of the ethyl acetate-extractable metabolites, BP 7,8-dihydrodiol and BP 9,10-dihydrodiol (Table 2). BP 4,5-dihydrodiol was a minor component of the ethyl acetate-extractable metabolites for all three species, regardless of treatment.

The treatment of rats with 3-MC resulted in large increases in each class of metabolites (phenols, quinones, diols and 'prediol' metabolites having an R_f lower than BP 9,10-dihydriol). For both starry flounder and coho salmon, treatment with BP or 3-MC resulted in obvious increases in the amounts of 4,5- and 9,10-dihydrodiols, and 'prediol' metabolites. For both fish species, there was a small decline in the amounts of phenols in incubations from 3-MC or BP-treated fish compared to that from the respective control fish.

The dihydrodiols were not well resolved via t.l.c. Therefore, ethylacetate-extractable metabolites obtained from the 3-MC treated fish species and rat

Table 2. Metabolism of [^{3}H]BP catalyzed by 10,000 *g* liver supernatant fractions from starry flounder, coho salmon and rat

	Starry flounder			Coho salmon			Rat	
	Control	3-MC-treated	BP-treated	Control	3-MC-treated	BP-treated	Control	3-MC-treated
	(% of total radioactivity)*							
Untreated BP	40 (2000)†	13 (650)	8 (400)	59 (2950)	5 (250)	5 (250)	62 (3100)	35 (1750)
Organic-soluble metabolites	39 (1950)	52 (2600)	59 (2950)	20 (1000)	40 (2000)	51 (2550)	6 (300)	38 (1900)
Aqueous phase	21	35	33	21	55	44	32	27
	Ethyl acetate-extractable metabolites separated by thin-layer chromatography							
Quinones	6.8 (130)	4.2 (110)	4.9 (140)	7.8 (80)	8.9 (180)	10.5 (270)	23.9 (70)	26.7 (510)
Phenols	15.3 (300)	7.6 (200)	3.8 (110)	30.6 (310)	13.8 (280)	10.7 (270)	24.6 (70)	30.8 (580)
4,5 Dihydrodiol	0.6§ (10)	2.2§ (60)	1.6§ (50)	2.7§ (30)	6.6§ (130)	2.9§ (70)	3.2 (10)	5.1 (100)
7,8-Dihydrodiol	47.3§ (920)	38.3§ (1000)	34.9§ (1030)	31.8§ (320)	24.9§ (500)	37.4§ (950)	14.4 (40)	13.8 (260)
9,10-Dihydrodiol	24.3§ (470)	25.9§ (670)	36.4§ (1070)	16.5§ (160)	22.0§ (440)	17.0§ (430)	13.1 (40)	14.0 (270)
Unclassified‖	3.8 (80)	17.1 (440)	15.0 (440)	5.9 (60)	15.0 (300)	16.1 (410)	13.3 (40)	8.0 (150)
At origin	1.8 (40)	4.7 (120)	3.4 (100)	4.7 (50)	8.8 (180)	5.5 (140)	7.6 (20)	1.6 (30)

* Incubations were conducted as described in the text. Total radioactivity is due to 5 nmoles of [^{3}H]BP (500 mCi/mmole) added to the reaction mixture.
† Values in parentheses are expressed in pmoles.
§ Not well resolved.
‖ Primarily 'prediol' components having lower R_f values than BP 9, 10-dihydrodiol.
‡ Percentage based on total metabolite in ethyl acetate. Values in parentheses are expressed in pmoles.

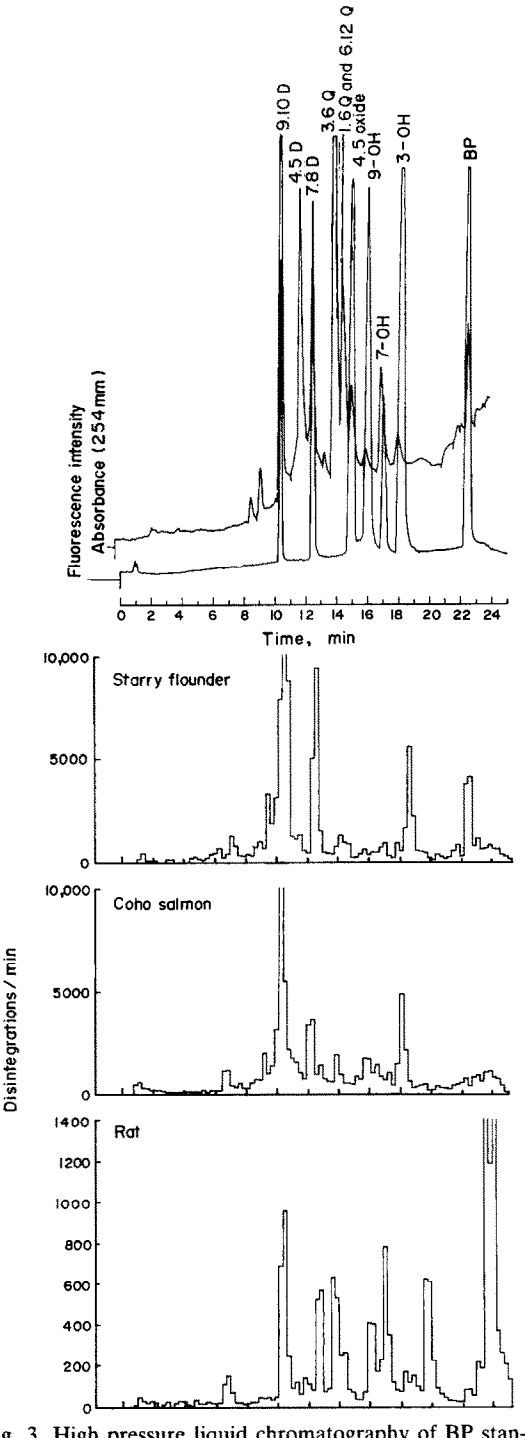

Fig. 3. High pressure liquid chromatography of BP standards and ^{3}H-metabolites: reference BP metabolites (top portion of panel) and ethyl acetate-extractable metabolites of [^{3}H]BP produced by liver supernatant fractions of 3-MC treated starry flounder, coho salmon, and rat. Reference compounds and metabolites were detected by both u.v. and fluorescence spectrometry. Metabolites formed by incubating 5 nmoles [^{3}H]BP with liver supernatants fractions (5 mg protein) of fish at (25°) and rat (at 37°) for 15 min were separated by fractions collected at 15-sec intervals. Abbreviations are: 9,10-D, BP 9,10-dihydrodiol; 4,5-D, BP 4,5-dihydrodiol; 7,8-D, BP 7,8-dihydrodiol; 3,6-Q, BP 3,6-quinone; 1,6-Q and 6,12-Q, 1,6-BP quinone and 6, 12-BP quinone; 9-OH, 9-hydroxy BP; 7-OH, 7-hydroxy BP; 3-OH, 3-hydroxy BP; and BP, benzo[*a*]pyrene.

were also analyzed via high pressure liquid chromatography (h.p.l.c.). The results (Table 3) show that the general pattern of metabolite classes obtained via h.p.l.c. was similar to that obtained via t.l.c. Analysis via h.p.l.c. revealed the presence of several phenolic derivatives of BP (7-OH, 3-OH, 9-OH, and presumably 1-OH) present in incubations containing fish liver enzymes (Fig. 3).

The concentrations of BP 9,10-dihydrodiol that were determined by h.p.l.c. were about 3 times greater than those of BP 7,8-dihydriol in incubations containing liver supernatant fractions from 3-MC-treated fish species (Fig. 3, Table 3), whereas in t.l.c. analyses of the same samples, the BP 7,8-dihydrodiol concentrations were greater than or equal to the concentrations of BP 9,10-dihydrodiol (Table 2). It is possible that because these two dihydrodiols had similar R_f values (t.l.c.) in our solvent system, when one dihydrodiol was present in a much greater amount than the other these compounds were not well resolved via t.l.c. It was also observed that the proportion of quinones was somewhate higher in t.l.c. analyses compared to h.p.l.c., indicating that some oxidation of phenols may have taken place during t.l.c. In this study, data from t.l.c. analyses were used to compare patterns of metabolite classes obtained with untreated and PAH-treated animals, and the information from h.p.l.c. was used for detailed examination of the types of individual metabolites present in fish and rats. The total amounts of dihydrodiols (4,5-, 7,8- and 9,10-) were calculated to be the same in any one sample by either method of analysis (Table 3). Moreover, analyses by both methods revealed the presence of high proportions of 'prediol' peaks (Tables 2 and 3) in metabolite extracts from the PAH-exposed fish. Results from both t.l.c and h.p.l.c. analyses show that ratios of dihydrodiols to phenols (D/P) and of diols to phenols plus quinones [D/(P + Q)], for the untreated and 3-MC treated fish, were greater ($P < 0.05$) than those obtained from rats treated similarly.

DISCUSSION

Covalent binding of metabolically activated BP to DNA. The optimum temperature for the *in vitro* binding of BP to DNA using fish liver enzymes was close to 25°, which is much higher than the actual temperatures at which these fish species live. However, while conducting comparisons of the extent of DNA binding *in vitro* using liver enzymes from different species of fish and from rats, it was considered necessary to carry out these reactions at an optimum temperature *in vitro* for each species.

For poikilothermic organisms the temperature of the surrounding water has a considerable influence on the activity of certain enzymes [35]. Dewaide [36] reported that rainbow trout (*Salmo gairdneri*) and roach (*Rutilus rutilus*), when acclimated at 5°, exhibited higher activity of hepatic mixed function oxidases (MFO) than when acclimated at 23°. Stegeman [37] reported that treatment of fish (*Fundulus heteroclitus*) with 3-MC at 6° resulted in hardly any induction in the activity of hepatic aryl hydrocarbon mono-oxygenases (AHM), whereas induction for the fish at 16° was substantial. These results [37] provide

Table 3. Comparison of high pressure liquid chromatography and thin-layer chromatography of ethyl acetate-extractable metabolites*

	% Ethyl acetate-extractable metabolites					
	Rat		Starry flounder‡		Coho salmon‡	
Metabolites†	h.p.l.c.	t.l.c.	h.p.l.c.	t.l.c.	h.p.l.c.	t.l.c.
'prediol'§	8.1	5.7	12.9	13.4	15.2	13.8
9,10-D	17.7		47.3		33.3	
7,8-D	9.8	32.9**	15.2	66.4**	9.0	53.5**
4,5-D	2.6		2.7		4.8	
3,6-Q	11.2	26.4**	3.5	4.2**	3.5	8.9**
1,6- and 6,12-Q	4.7		tr‖		5.0	
4,5-OX	8.9	—¶	tr	—	1.9	—
7-OH	2.4		2.5		3.1	
3-OH	14.1	30.8**	9.9	7.6**	10.7	13.8**
9-OH	12.8		1.8		4.8	
1-OH(?)	2.5		1.3		2.2	
Unclassified	5.3	2.3	2.9	3.8	6.4	1.2

* See Table 2 for details. Conditions for t.l.c. and h.p.l.c. are given under Materials and Methods.

† Abbreviations: 9,10-D, BP 9,10-dihydrodiol; 4,5-D, BP 4,5-dihydrodiol; 7,8-D BP 7,8-dihydrodiol; 3,6-Q, BP 3,6-quinone; 1,6-Q and 6,12-Q, 1,6-BP quinone and 6,12-BP quinone; 9-OH, 9-hydroxy BP; 7-OH, 7-hydroxy BP; 3-OH, 3-hydroxy BP; and BP, benzo[*a*]pyrene.

‡ Treated with 3-methylcholanthrene (10 mg/kg).

§ Components eluted before BP 9,10-dihydrodiol.

‖ tr, trace.

¶ —, not resolved by t.l.c.

** Values for total diols, quinones or phenols from t.l.c.

a possible explanation for the present findings showing that the values for binding of BP to DNA were much lower when liver supernatant fractions (10,000 *g*) from 3-MC-treated fish at 8° were used compared to the values obtained with the liver supernatant fractions from 3-MC-treated fish at 13° (Table 1). In a recent study [27] with a freshwater salmonid (*Salmo trutta lacustris*) held at 4–6°, binding of activated BP to DNA by fish liver microsomes was found to be 3–4 times greater than that by rat liver microsomes. Our preliminary results (U.Varanasi and E. Egaas, unpublished results) show that the binding of BP to DNA was very small when liver extracts of rainbow trout held at 16° were used. It appears, therefore, that in addition to species-specific differences, variations in water temperature contributed significantly to the differences in binding observed with these salmonid fish.

Many environmental and species-specific differences may influence the xenobiotic metabolizing capabilities of aquatic animals [21,38]; therefore, it would be imprudent to attribute the marked difference observed in values of binding for the two fish species to any particular factor. Moreover, wide variations in AHM activity have been reported to occur among different strains of a single species of fish [30] or mice [39]. However, it is possible that starry flounder, being a demersal fish, are habitually exposed to a multitude of xenobiotics (some of which are inducers of MFO) present in bottom sediments [18], and, therefore, may give rise to an initial high value for the *in vitro* covalent binding of BP to DNA. This would also explain why pre-exposure of starry flounder to 3-MC or BP did not result in an increase in binding as great as that for coho salmon.

The magnitude of the increase in the values of binding on pre-exposure of fish or rat to PAH (3-MC or BP) was far greater than the increase in the amounts of ethyl acetate-extractable metabolites. It has been proposed that the induction of hepatic AHM in fish can indicate the presence of certain toxic chemicals in their environment [40]. It appears from the present results that covalent binding of BP to DNA may prove to be a more sensitive index than AHM activity to indicate pre-exposure of fish to certain toxic chemicals.

Metabolism of BP. The profiles of the metabolites of BP produced by liver supernatant fractions (10,000 *g*) of both coho salmon and starry flounder were qualitatively similar to those produced by liver microsomes and supernatant fractions from rat and other animals [39, 41–45]. However, regardless of the treatment, the metabolites formed by fish liver supernatant fractions were characterized by a preponderance of non-K region dihydrodiols. Non-K region oxygenation, which is apparently very important for the covalent binding of metabolites of PAH to DNA, is favored under conditions of a high cytochrome P_1-450/P-450 ratio [46]. No information is available on characterization of liver cytochromes from coho salmon or starry flounder; however, it has been reported [27] that trout liver cytochrome is closely related to PAH-specific cytochrome P-448 of rodent liver microsomes. Note that profiles of BP metabolites formed by trout liver microsomes [27] closely resemble those formed by the liver supernatant fractions from untreated coho salmon in the present study.

Jernstrom *et al.* [47] reported that, although BP 7,8-dihydrodiol and BP 9,10-dihydrodiol were fur-

ther metabolized by liver nuclei from 3-MC treated rats, only BP 7,8-dihydrodiol gave rise to reactive intermediates that were bound to DNA. No detectable binding occurred with BP 9,10-dihydrodiol. Results of Sims *et al.* [48] suggest that BP 9,10-dihydrodiol is resistant to further activation. Booth and Sims [49] reported that the major metabolite of BP 9,10-dihydrodiol was 9,10-dihydroxy BP. It is possible that one of the 'prediol' peaks, observed via both t.l.c. and h.p.l.c. analyses of metabolites from fish, was this compound. In their studies of metabolism of BP by rat hepatocytes, Burke *et al.* [41] and Jones *et al* [42] found that the release of BP 9,10-dihydrodiol into extracellular fluid was much more pronounced than that of BP 7,8-dihydrodiol which was equally divided between the cell and extracellular medium; 3-hydroxy BP was preferentially retained within the cell over a long period. Thus, the production of large proportions of BP 9,10-dihydrodiol by fish liver enzymes may have important consequences in efficient removal of BP from the liver. Moreover, preferential production and removal of the BP 9,10-dihydrodiol may also have some importance in its transport from liver to other tissues (e.g. muscle) via the circulatory system.

Our results also show that the extent of BP metabolism and the proportion of 'prediol' components increased considerably in incubations containing liver supernatant fractions from PAH (3-MC or BP)-treated fish, indicating that extensive secondary metabolism of BP had occurred, some of which may have taken place via epoxides of previously formed dihydrodiols and phenols. Jones *et al.* [42] demonstrated that BP 7,8-dihydrodiol is further metabolized to an ethyl acetate-extractable metabolite, 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxy BP, which is formed via BP 7,8-dihydrodiol-9,10-epoxide, the postulated ultimate carcinogen of BP [48].

The accumulation of reactive metabolites and the extent of their interactions with cellular nucleophiles, such as DNA, will depend on their rates of formation and degradation, which may be quite different *in vivo* from *in vitro*. Accordingly, the present results of the *in vitro* studies served primarily as an indication that, in liver of marine fish, possibilities exist for extensive conversion of BP into reactive metabolites which bind covalently to DNA. However, in view of the possibility that concentrations of carcinogenic PAH will be increasing in the aquatic environment [50], together with the fact that fish are an important component of human diet, this ability of fish liver enzymes to activate BP to promutagens and procarcinogens is of concern and merits further investigation.

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