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# Chemical Carcinogenesis in Feral Fish: Uptake, Activation, and Detoxication of Organic Xenobiotics

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**The high prevalence of liver neoplasms in English sole (*Parophrys vetulus*) and substantially lower prevalence of neoplasms in a closely related species, starry flounder (*Platichthys stellatus*) captured from industrialized waterways, provide a unique opportunity to compare biochemical processes involved in chemical carcinogenesis in feral fish species. Because levels of aromatic hydrocarbons (AHs) in urban sediments are correlated with prevalences of liver neoplasms in English sole, we have initiated detailed studies to evaluate the effects of endogenous and exogenous factors on uptake, activation and detoxication of carcinogenic AHs, such as benzo[a]pyrene (BaP), using spectroscopic, chromatographic, and radiometric techniques. The results obtained thus far show that sole readily takes up AHs associated with sediment from urban areas and that the presence of other xenobiotics, such as PCBs, in sediment increases tissue concentrations of BaP metabolites. Extensive metabolism of BaP occurred whether sole was exposed to this AH via sediment, per os, or intraperitoneally. Substantial modification of hepatic DNA occurred and persisted for a period of 2–4 weeks after a single exposure to BaP. The level of covalent binding of BaP intermediates to hepatic DNA was 10-fold higher in juvenile than adult sole and 90-fold higher in juvenile sole than in Sprague-Dawley rat, a species which is resistant to BaP-induced hepatocarcinogenesis. The level of chemical modification of hepatic DNA in juvenile flounder was 2-4 fold lower than that for juvenile sole and concentration of BaP 7,8-diol glucuronide in bile of sole was significantly higher than that in flounder bile, although the rate of formation of BaP 7,8-diol by hepatic microsomes was comparable for both species. Moreover, liver microsomes from both species, in the presence of exogenous DNA, metabolized BaP into essentially a single adduct, identified as (+)*anti*-7,8-diol-9,10-epoxy-7,8,9,10-tetrahydroBaP-dG. These results, along with our findings that hepatic GST activity in flounder was two times higher than in sole, demonstrate that microsomal metabolism of BaP does not accurately reflect the differences in the ability of these fish to form BaP-DNA adducts *in vivo* and also suggest that detoxication of reactive intermediates is an important factor in determining the levels of DNA modification by AHs and resulting toxic effects in feral fish.**

## Introduction

Certain fish species (e.g., rainbow trout and medaka), bred and reared in the laboratory, have been well established as sensitive models to evaluate the effects of exogenous and endogenous factors on chemical carcinogenesis (*1, 2*). In contrast, at present virtually no information is available on the suitability of feral fish species in studies of chemically induced carcinogenesis. Development of feral fish species as models poses a number of problems, including maintaining these fish in the laboratory over long periods, obtaining sufficient quanities of fish of the required age and sex from relatively uncontaminated areas, and working with genetically diverse populations. Additionally, very little is known about the nutritional requirements for these fish and about culturing them in the laboratory so that the fish are available throughout their entire life cycle. Nevertheless, most of these problems can be minimized or overcome by careful planning and conduct of experiments. The advantages of using feral fish species to study processes involved in chemical carcinogenesis far outweigh the above-mentioned difficulties because of the availability of valuable epizootological information on the same species with regard to the prevalence of cancer in chemically contaminated environments. For example, in Puget Sound, WA, data have been obtained over the last five years on the prevalence of diseases, including liver neoplasia, in several benthic fish species and on the degree of xenobiotic contamination in the sediments where the fish are found (*3–5*). The results show a strong positive correlation between the concentrations of aromatic hydrocarbons (AHs) in sediment and the prevalence of liver neoplasms in a benthic fish,

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the English sole (*Parophrys vetulus*). Moreover, a positive correlation has been demonstrated between levels of aromatic compounds fluorescing at wavelengths appropriate for benzo[a]pyrene (BaP) in bile and the prevalences of liver neoplasms in English sole sampled from contaminated estuaries (*6,7*). As described elsewhere in this volume (*8*), polychlorinated biphenyls (PCBs) and other chlorinated hydrocarbons, such as hexachlorobenzene and hexachlorobutadiene, are also detected in urban sediments where English sole exhibits high prevalences of liver lesions; however, the concentrations of these xenobiotics were not strongly correlated with the prevalences of liver lesions (see Table 1 for representative data from the above studies).

A major focus of our research effort has been the development of English sole as a laboratory model for studying the biochemical basis of chemical carcinogenesis in fish, with an emphasis on understanding the processes involved in the activation and detoxication of carcinogenic AHs. In addition, we are interested in evaluating factors which may affect the initiation of chemical carcinogenesis in benthic fish. Thus, we are currently evaluating the uptake and metabolism of sediment-associated pollutants (e.g., PCBs and AHs) by benthic fish, the effects of exogenous and endogenous factors on uptake, activation, and detoxication of AHs, and differences in the metabolism of AHs between closely related species of benthic flatfish (e.g., English sole and starry flounder [*Platichthys stellatus*]). The results from these types of studies should be useful in the planning of tumorigenesis studies, where the great expense, both in terms of time and money, requires that only those studies be done which have the best chance of yielding useful information.

**Table 1. Concentrations of selected organic xenobiotics in sediment and in bile of English sole and prevalences of hepatic neoplasms in English sole from Puget Sound, WA.[a]**

| Site | Xenobiotics in sediment, ng/g dry weight sediment: PCBs | 4- and 5-ring AHs[b] | Bile fluorescence response[c] | % of fish having hepatic neoplasms |
|---|---|---|---|---|
| Eagle Harbor | < 2.3 | 93,000 | 2,100 | 26.7 |
| Duwamish Waterway | 330 | 980 | 1,400 | 20.7 |
| Inner Everett Harbor | 29 | 1,600 | 520 | 5.0 |
| President Point | < 2.3 | 350 | 100 | 0 |
| Useless Bay | < 2.3 | 41 | 67 | 0 |

[a] Data adapted from the literature (*3,4,7*).
[b] Sum of concentrations in sediment of fluoranthene, pyrene, and benzo(a)pyrene. Metabolites of these compounds fluoresce at the BaP wavelength pair (*7*).
[c] Fluorescence response was measured at the BaP wavelength pair (380/430 nm) and converted to ng BaP equivalents/g wet weight bile (*7*).

## Uptake and Disposition of Sediment-Associated AHs and PCBs in English Sole

Information on bioavailability of sediment-associated pollutants to benthic fish is essential in our attempts to delineate cause and effect relationships between chemicals in the marine environment and observed biological abnormalities. In recent years, considerable indirect evidence has been obtained from field studies which suggests that sediment is a major source of contaminants accumulated by benthic fishes (*9–11*). However, factors such as fish migration (*12*) and heterogeneity of sediment contamination (*3,4*) often make it difficult to directly correlate the accumulation of chemicals in organisms to the presence of chemicals in the environment where they are caught.

Accordingly, we conducted several laboratory studies in which English sole was exposed to: (a) sediment from a reference (relatively uncontaminated) area to which $^{14}$C-naphthalene (NPH) and $^{3}$H-BaP (dissolved in 1% Prudhoe bay crude oil) were added (*13*); (b) sediment from a reference area to which environmentally realistic levels of BaP or PCBs were added (*14*); and (c) sediments from the Duwamish Waterway, Puget Sound, WA—an urban estuary where English sole exhibits a consistently high prevalence of hepatic neoplasms—and from a reference area virtually free of chemical contaminants (*15,16*). In studies (b) and (c), the radiotracers $^{3}$H-BaP and $^{14}$C-PCBs (Aroclor 1254) were also added. Results from all three studies showed that fish had measurable levels of radioactivity in most tissues and fluids, indicating that AHs and PCBs added to sediment were indeed bioavailable. More importantly, however, in study (c), bile of fish exposed to test sediment (containing 16 ppm of two- to six-ring AHs and 1.2 ppm PCBs, based on wet weight of sediment) and reference sediment was analyzed by HPLC-fluorimetric techniques to show that bile of sole exposed to Duwamish Waterway sediment contained significantly higher concentrations of compounds that fluoresce at the wavelength pairs specific for NPH (2- to 3-fold), phenanthrene (6- to 10-fold) and BaP (10- to 27-fold) (Fig. 1) than did bile of fish exposed to reference sediment (*15*). Moreover, analyses by gas chromatography (GC) showed that liver of the test fish contained significantly higher (ca. 10-fold) concentrations of PCBs than the reference fish. These results show conclusively that English sole is able to take up AHs and PCBs present in sediment from urban estuaries. Although the precise route of uptake [i.e., direct uptake of particle-bound contaminants or uptake of xenobiotics released into the sediment-associated water (SAW)] could not be determined, it should be noted (*15,16*) that SAW did not contain detectable levels of AHs (< 0.6 ppb) or PCBs (< 1 ppb) as analyzed by GC or by measurement of radioactivity by liquid scintillation spectrometry ($^{3}$H-BaP < 0.05 ppb). Recent work by Rubinstein et al. (*17*) has also shown that when fish are allowed direct contact with contaminated sediment, they show increased accumulation of xenobiotics compared to fish which are

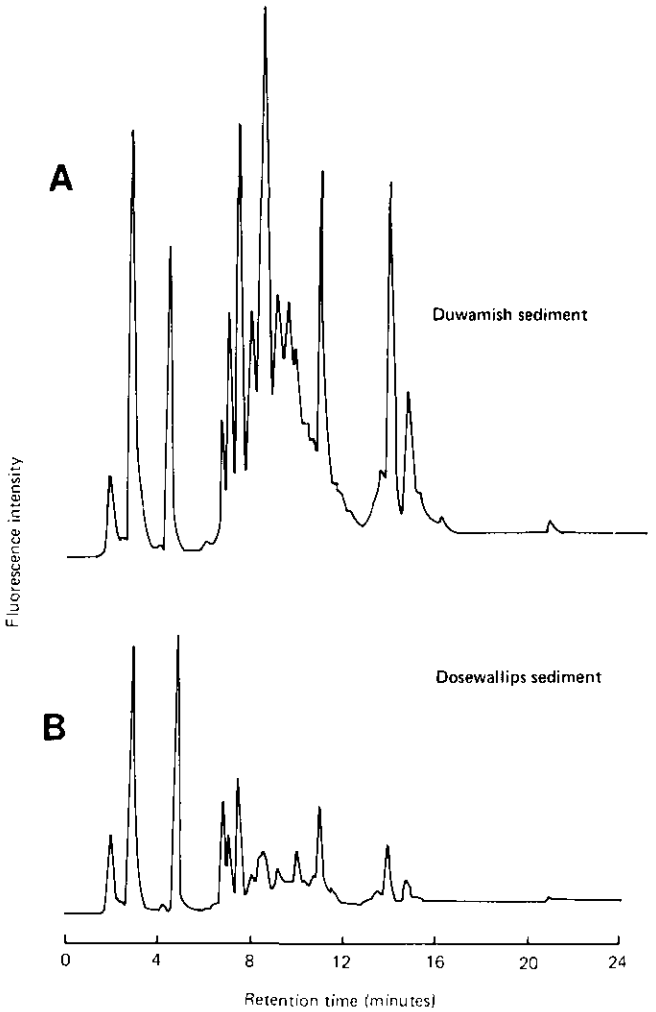


FIGURE 1. HPLC/fluorescence analyses of English sole bile for aromatic compounds fluorescing at BaP wavelengths (380/430 nm). BaP metabolites elute between 6 and 16 min. (*A*) Bile from fish exposed to Duwamish River sediment (contaminated) for 28 days. (*B*) Bile from fish exposed to Dosewallips sediment (reference) for 28 days. Contaminated sediment contained selected AHs at a total concentration of 16 μg/g wet weight and PCBs at 1.2 μg/g wet wt, plus added trace levels of $^{3}$H-BaP and $^{14}$C-PCBs; the respective concentrations in the reference sediment were 0.015 μg/g ug/g wet weight and < 0.001 μg/g wet weight. Trace levels of $^{3}$H-BaP were also added to the reference sediment. Adapted from Varanasi et al. (*16*).

prevented from direct contact. Hence, direct desorption of the xenobiotics from the particles to gut, gill and skin mucosa may constitute a significant route of uptake. Moreover, we have noted that the stomach contents of English sole examined immediately after capture comprised substantial amounts of sediment, along with various invertebrates which also contained sediment in their digestive tracts. In addition, substantial amounts of organic xenobiotics have been reported to be present in the gut contents of English sole from contaminated areas (*4,5*). Thus, both the direct uptake of particle-bound xenobiotics or indirect uptake via food organisms which ingest particles containing xenobiotics implicate sediment as a major source of contaminants for English sole and presumably other benthic fishes.

The results of study (a) show that from 24 to 168 hr, the liver concentration of NPH-derived radioactivity decreased, whereas BaP-derived radioactivity increased in fish exposed simultaneously to these compounds via sediment (Fig. 2) (*13*). Moreover, the proportion of unmetabolized NPH in the liver dropped substantially during this time. The $K_m$ value for NPH (300 μM) is considerably higher than that for BaP (2.1 μM) for fish liver microsomes (*18*), indicating that NPH is a relatively poor substrate for hepatic monooxygenases. Thus, the decrease in tissue concentrations of NPH-derived radioactivity was most probably due to facile excretion of the parent compound via skin and gills (*19,20*). PCBs are also metabolized to a lesser extent than BaP by most organisms; however, in contrast to NPH, PCBs continued to accumulate in tissues of sole exposed to these xenobiotics in studies (b) and (c) (*14,15*). These results indicate that, unlike NPH, PCB

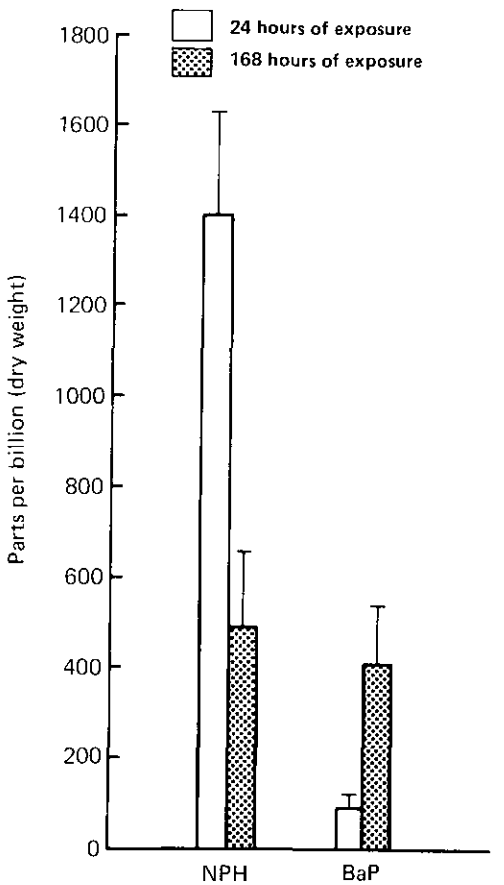


FIGURE 2. Uptake of sediment-associated NPH and BaP in liver of English sole; values include parent AHs and metabolites. $^{3}$H-BaP, $^{14}$C-NPH, and Prudhoe Bay crude oil were added to a reference sediment such that the final concentrations were 370 and 450 ng/g dry weight and 0.02 g/g dry weight, respectively. Adapted from Varanasi et al. (*13*).

congeners may not be easily excreted from fish prior to metabolism and thus will accumulate in all tissues. This conjecture is supported by the results depicted in Figure 3 showing that while BaP-derived radioactivity was primarily (ca. 65%) in the hepatobiliary system—a major site for xenobiotic metabolism and excretion—PCB-derived radioactivity was more evenly distributed within the tissues of fish (only 21% in the hepatobiliary system) exposed to both $^{3}$H-BaP and $^{14}$C-PCB in sediment (*14*). Thus, through the use of various analytical techniques (GC, HPLC-fluorimetry) we were able to show clearly that AHs and PCBs, either added to sediments or deposited in urban sediments by natural processes, were bioavailable to English sole. Further, through the use of radiolabeled compounds, we were able to show differences in both the accumulation and disposition of AHs and PCBs (*13–16*).

The results of studies (b) and (c) also reveal differences in the accumulation of BaP by English sole in the presence and absence of other xenobiotics (*14,15*). For example, concentrations of BaP-derived radioactivity in fish exposed to Duwamish River sediment with added $^{3}$H-BaP [study (c)] or reference sediment containing both $^{3}$H-BaP and $^{14}$C-PCBs [study (b)] were significantly higher than the corresponding values for fish exposed to reference sediment containing $^{3}$H-BaP alone (Fig. 4). These increased body burdens of BaP-derived radioactivity were due primarily to the increased concentrations in liver and bile. Further analysis (Table 2) by solvent extractions and enzymatic hydrolysis showed that the BaP-derived radioactivity in bile was present as glucuronide and sulfate conjugates, based on hydrolysis of these conjugates by appropriate enzymes (*21,22*), and as glutathione (GSH) conjugates, based on our earlier studies showing that metabolites remaining in the aqueous phase after enzymatic hydrolysis cochromatographed with BaP-GSH conjugates on aluminum oxide columns and thin-layer chromatography plates and were ninhydrin positive (*23*).

The increased accumulation of BaP metabolites in bile and liver of sole exposed to BaP in the presence of other xenobiotics may result from induction of hepatic xenobiotic metabolizing enzymes (HXMEs) such as the mixed-function oxidases (MFOs) and conjugation enzymes [e.g., UDP–glucuronosyl transferase (UDPGT) and glutathione-S-transferase (GST)]. In rodents, exposure to PCBs results in induction of both MFO and conjugation enzymes (*24*). In fish, hepatic MFO activities are rapidly induced by xenobiotics, such as AHs and PCBs (*25–28*), known to be present in urban sediments, whereas activities of conjugating enzymes are induced much more slowly and to a lesser extent. For example, Andersson et al. (*29*) showed that exposure of rainbow trout to β-naphthoflavone and Clophen A50 markedly induced (170- and 50-fold, respectively) hepatic MFO activity in 4 to 7 days, but that both GST and UDPGT were much less induced (1- to 3-fold), and maximal induction was not reached until 2 to 3 weeks after exposure. Further, it has been shown that exposure of salmonids to known inducers of hepatic MFO activity followed by exposure to 2-methylnaphthalene or 2,6-dimethylnaphthalene resulted in higher concentrations of parent AHs and metabolites in bile than the corresponding levels found in control fish (*30,31*). We have also shown that exposure of both juvenile and adult English sole to injections of an organic-solvent extract

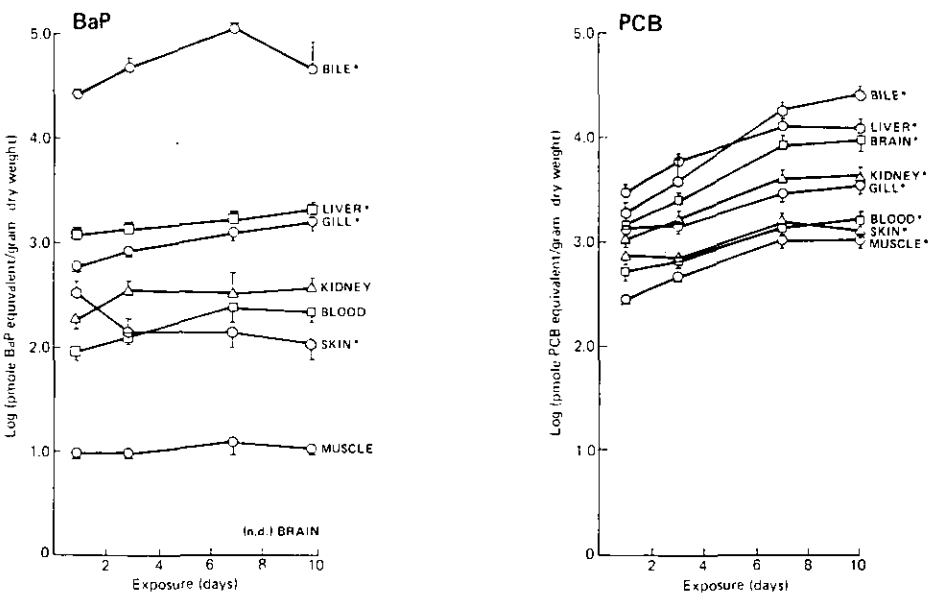


FIGURE 3. Concentrations (log-transformed) of BaP- and PCB-derived radioactivities in tissues and fluids of English sole exposed to sediment-associated BaP and PCBs. $^{3}$H-BaP and $^{14}$C-PCBs were added to a reference sediment at 3 μg/g dry wt and 1 μg/g dry weight, respectively. Adapted from Stein et al. (*14*).

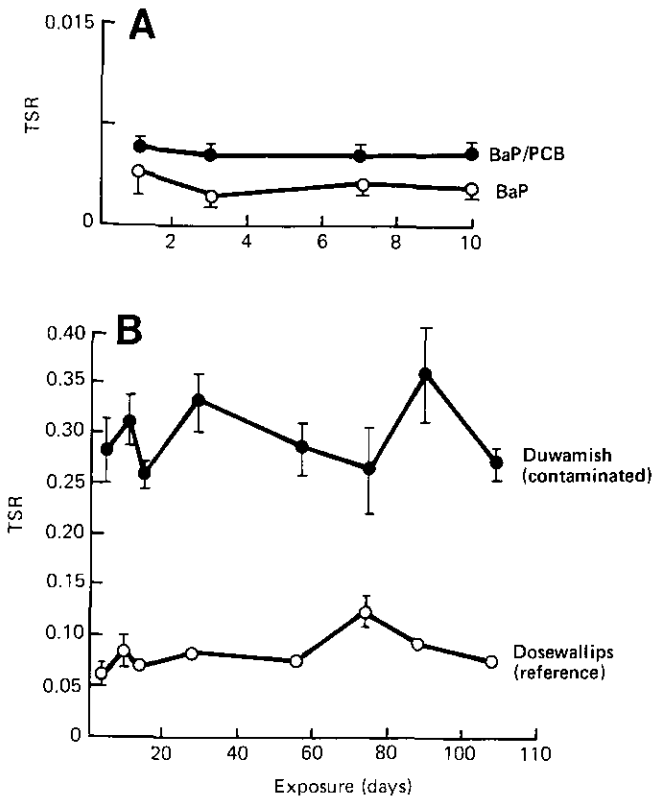


FIGURE 4. Tissue to sediment concentration ratios (TSRs) of BaP-derived radioactivity in English sole exposed to (*A*) reference sediment with added $^{3}$H-BaP or $^{3}$H-BaP and $^{14}$C-PCBs (TSRs are for whole body); (*B*) Duwamish River (contaminated) and Dosewallips (reference) sediments containing trace levels of $^{3}$H-BaP (TSRs are for the liver). See Figs. 1 and 3 and (*14,15*) for further details.

of Duwamish River sediment can substantially induce hepatic MFO activity, measured as aryl hydrocarbon (BaP) hydroxylase (AHH), within several days (*32,33*). Thus, if induction of HXMEs occurred in English sole exposed to Duwamish River sediment [study (c)], then the higher concentrations of BaP-derived radioactivity in liver and especially bile may be due to increased metabolism and turnover (uptake and secretion) of BaP. However, factors such as increased hepatic uptake of BaP as well as decreased excretion of BaP and its metabolites may also contribute to the greater accumulation of BaP-derived radioactivity seen in the liver and bile of sole exposed to sediment-associated BaP in the presence of other xenobiotics [studies (b) and (c)]. Moreover, in study (c), even though test and reference sediments had similar physiochemical properties, the possibility of higher bioavailability of BaP from the test sediment cannot be excluded.

The data from study (c) (Table 2) reveal that there was a selective retention of metabolites bound to GSH and macromolecules in liver, whereas glucuronide/sulfate conjugates appeared to be rapidly released into bile. These results are in agreement with those of Plummer et al. (*34*) showing the GSH conjugates of BaP 4,5-oxide were retained in rodent liver, whereas the BaP 4,5-diol glucuronide was more readily released into bile. Table 2 also shows that the ratios of GSH conjugates to glucuronide conjugates of $^{3}$H-BaP in liver and bile of fish chronically exposed to $^{3}$H-BaP in Duwamish River sediment averaged 42 and 3.1, respectively, whereas the corresponding values for fish exposed to $^{3}$H-BaP in the reference sediment were 15 and 1.9, respectively. These results showing that a higher proportion of BaP was converted to GSH conjugates in sole exposed simultaneously to other xenobiotics in Duwamish River sediment are suggestive of alterations in BaP metabolism by fish chronically exposed to contaminated sediments. Further, this shift to greater formation of GSH conjugates in fish exposed to Duwamish River sediment is consistent with the higher liver tissue to sediment concentration ratios (TSRs) for BaP-derived radioactivity in these fish compared to fish exposed to a reference sediment. These alterations in the ability of English sole

**Table 2. Distribution of BaP- and PCB-derived radioactivities in liver and bile of English sole exposed to Duwamish River (test) and Dosewallips (reference) sediments having added $^{3}$H-BaP and $^{14}$C-PCBs, and $^{3}$H-BaP, respectively.[a]**

| | | % of total radioactivity[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | $^{3}$H-BaP | | | | $^{14}$C-PCBs, test sole | |
| | | Test sole | | Reference sole | | | |
| Metabolic compartment | Exposure, days | Liver | Bile | Liver | Bile | Liver | Bile |
| Parent compounds | 56 | 1.0 ± 0.1 | < 1 | 2.9 ± 0.3 | < 1 | 92 ± 3 | 60 ± 4 |
| | 108 | 1.1 ± 0.3 | < 1 | 1.3 ± 0.3 | < 1 | 95 ± 1 | 64 ± 2 |
| Unconjugated metabolites | 56 | 3.9 ± 0.8 | 3 ± 1 | 7.1 ± 0.6 | 1 ± 1 | 3.5 ± 0.5 | < 4 |
| | 108 | 3.2 ± 0.5 | 4 ± 1 | 7.0 ± 1.2 | 2 ± 2 | 2.4 ± 0.3 | < 4 |
| Glucuronides and sulfates | 56 | 1.7 ± 0.2 | 25 ± 2 | 5.6 ± 0.3 | 33 ± 2 | 0.6 ± 0.3 | 30 ± 4 |
| | 108 | 1.9 ± 0.4 | 22 ± 1 | 4.1 ± 0.4 | 34 ± 2 | 0.3 ± 0.1 | 27 ± 1 |
| Glutathione-derived conjugates | 56 | 70 ± 2 | 72 ± 2 | 70 ± 2 | 66 ± 1 | 3 ± 2 | 10 ± 2 |
| | 108 | 80 ± 2 | 74 ± 1 | 70 ± 2 | 64 ± 3 | 1.4 ± 0.2 | 9 ± 1 |
| Metabolites bound to macromolecules | 56 | 20 ± 2 | — | 16 ± 1 | — | 0.7 ± 0.2 | — |
| | 108 | 20 ± 2 | — | 16 ± 1 | — | 0.5 ± 0.2 | — |

[a] Adapted from Stein et al. (*15*).
[b] $\bar{X}$ ± SEM ($n$ = 3 for liver values and 4–8 for bile values).

to metabolize carcinogenic AHs are intriguing in view of the findings from the field studies showing that the interaction between levels of PCBs and AHs in sediments may negatively correlate with the prevalences of hepatic neoplasms in English sole from Puget Sound (D. C. Malins, personal communication). It should also be noted that simultaneous exposure to PCBs and AHs in rodents inhibits AH-induced carcinogenesis (*35*) and that simultaneous exposure of rainbow trout to PCBs and aflatoxin $B_1$ ($AFB_1$) results in a reduced incidence of liver cancer in these fish (*36*). However, it is also reported that the time of the exposure to PCBs (i.e., pre- or post-initiation phase) as well as numerous endogeneous (e.g., age, sex, nutritional status) and exogeneous (e.g., promoting agents, water temperature, etc.) factors may modulate the effect of xenobiotics, such as PCBs, on chemically induced carcinogenesis (*2*).

In addition to showing that the ability of fish to process carcinogens may be altered due to concomitant exposure to other xenobiotics, the studies described above also provided valuable information on differences in metabolic activation between BaP and PCBs (*14,15*). The data in Figure 5 show that in English sole exposed for 108 days to $^{3}$H-BaP and $^{14}$C-PCBs added to Duwamish River sediment, the level of BaP metabolites bound covalently to hepatic macromolecules was 5 times greater than the value for PCB metabolites, although the total concentration of PCB-derived radioactivity in the liver was 5-fold higher than BaP-derived radioactivity. The results (Table 2) also show that a substantially greater proportion of BaP than PCBs was converted by sole liver to reactive electrophilic metabolites which can serve as substrates for GSH conjugation. This finding suggests different mechanisms of metabolism between BaP and PCBs by English sole. A recent study (*37*) with phenobarbital-induced rat liver microsomes suggested that 2,2′,5,5′-tetrachlorobiphenyl, a major component of Aroclor 1254, was predominantly (> 90%) metabolized by a nonarene oxide pathway. In contrast, BaP is primarily metabolized via arene oxide pathways (*38*). Metabolism by a non-arene oxide pathway may result in less conjugation with GSH and less binding to hepatic macromolecules because a non-arene oxide mechanism may lead to an intermediate that is less reactive towards either GSH or cellular macromolecules. As discussed above, GSH-derived BaP conjugates were apparently retained in liver relative to BaP glucuronides and sulfates; thus, the low proportion of PCB me-

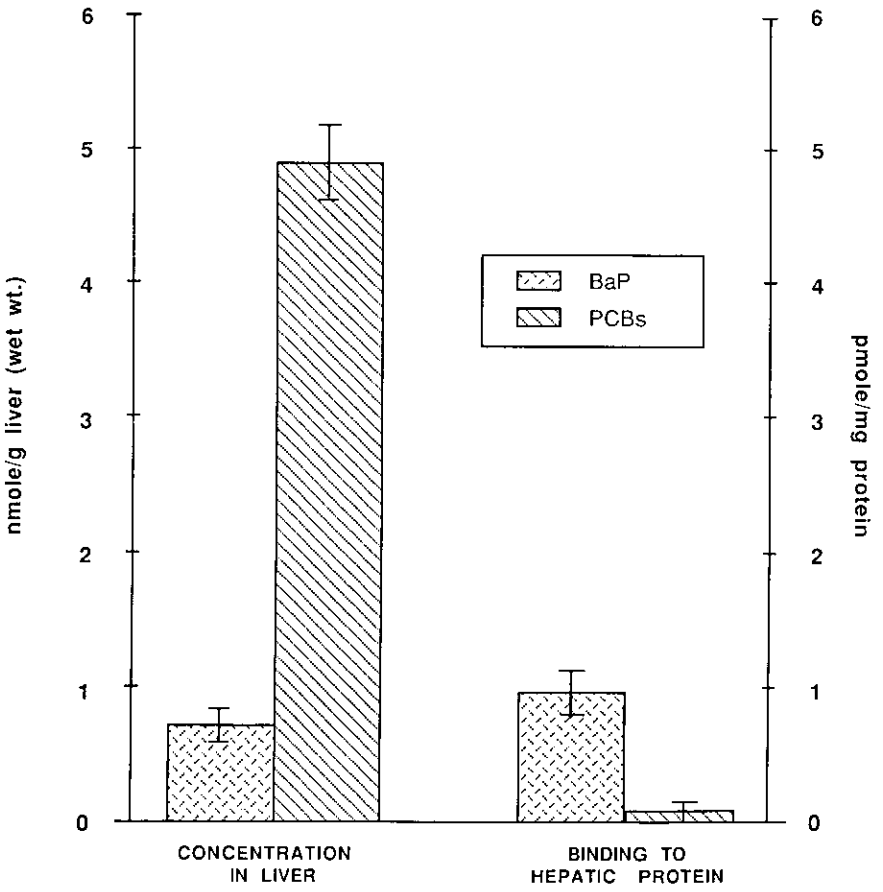


FIGURE 5. Concentrations in liver and levels of covalent binding to hepatic proteins of BaP- and PCB-derived radioactivities in English sole exposed to Duwamish River sediment for 108 days (see Fig. 1 for further details). Adapted from Stein et al. (*15*).

tabolites in liver (< 8% of total PCB-derived radioactivity) of sole exposed to sediment-associated PCBs may be due to both a low rate of phase I metabolism and rapid conjugation of hydroxylated PCB metabolites to form glucuronides or sulfates and subsequent secretion of these compounds into bile (*14,15*). While considering this information, however, it must be remembered that we have used a mixture of PCB congeners (Aroclor 1254) in these studies, and thus the possibility that specific PCB congeners may be efficiently activated to reactive electrophilic metabolites cannot be excluded.

The results described above serve as initial studies to draw attention to differences in the mechanisms of metabolism of different classes of organic pollutants. Concomitantly, we have studied the metabolic activation of carbazole (CBZ), a nitrogen-containing aromatic compound that has been detected in sediment from a highly polluted estuary in Puget Sound (*39*). CBZ is implicated in one study as a hepatocarcinogen in mice (*40*), and electron spin resonance spectroscopy of the hepatic microsomal fractions isolated from English sole with liver lesions indicated the presence of CBZ intermediates associated with these fractions (*41*). Our preliminary results show that 24 hr after juvenile English sole was given intraperitoneal (IP) injections containing equimolar concentrations of either $^{3}$H-BaP or $^{14}$C-CBZ dissolved in acetone, no detectable level of covalent binding of $^{14}$C-CBZ intermediates to hepatic DNA was observed, whereas the binding of BaP intermediates to hepatic DNA was an order of magnitude higher than that observed for rat liver DNA (see discussion below for more details). Moreover, covalent binding of $^{14}$C-CBZ to hepatic proteins was about 20-fold lower than the value for $^{3}$H-BaP. Thus, these results again emphasize differences in the metabolic activation of different classes of organic xenobiotics by English sole.

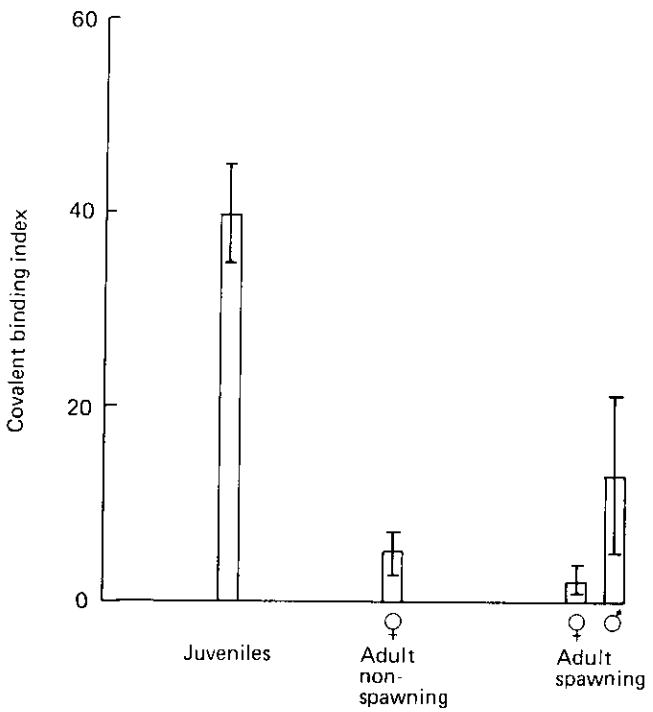


FIGURE 6. Covalent binding indices of benzo[a]pyrene metabolites to hepatic DNA of juvenile and adult English sole exposed PO to 0.1 mg $^{3}$H-BaP/kg body weight. Fish were sacrificed 24 hr later and hepatic DNA was isolated by previously described methods (*46*). The covalent binding index (CBI) = (μmole BaP-equivalents/mole nucleotide)/(mmole BaP administered/kg body weight). Adapted from Varanasi et al. (*23,46,47*).

## Metabolic Activation of BaP

The findings described above definitively show that BaP, a carcinogenic AH present in sediment from industrialized waterways, is taken up by English sole and converted to metabolites that bind to hepatic macromolecules. As many xenobiotics exert their carcinogenic effects only after metabolic activation, we are conducting detailed studies on the activation and detoxication of BaP, used as a model carcinogenic AH, in various fish species. Debate continues, however, about the validity of using biochemical parameters, such as covalent binding of a carcinogen to DNA or metabolite profiles of a procarcinogen, to measure quantitatively the carcinogenic potency of a compound or susceptibility of a tissue or species. Nevertheless, it is generally agreed that information from such studies, if used prudently, can be useful in our attempts to understand why certain fish species are susceptible to chemically induced carcinogenesis.

Field studies have shown that the prevalence of hepatic neoplasms increases with age for English sole captured from the same area (*42,43*). However, our experiments conducted in the summer show that the level of covalent binding of BaP intermediates to hepatic DNA in juvenile sole is an order of magnitude higher than that obtained for nonspawning adult female sole exposed to equivalent doses of BaP (Fig. 6). It should also be noted that rainbow trout embryos exposed only once to very low levels of BaP develop hepatic tumors within 10 months (*44*), whereas adults must be exposed to chronic high levels of BaP to achieve a similar effect (*45*). Thus, it seems possible that the field data described above reflect the length of time for initiation to be manifested as discernible tumors, rather than any enhanced senstivity of older fish to chemically induced carcinogenesis.

The data in Figure 6 also show that the binding values for nonspawning adult female sole and spawning adult male sole were higher than the values for the spawning adult females (*23,46,47*). Concomitant with this, our data (*33*) have shown that AHH activity towards BaP is very low in hepatic microsomes from spawning female English sole (10 pmole BaP metabolized/mg protein/min). Lowered levels of AHH activity in hepatic microsomes of gonadally mature female fish are generally shown to occur, and to be due to decreases in the levels of microsomal cytochrome P-450 (*48*). It should also be

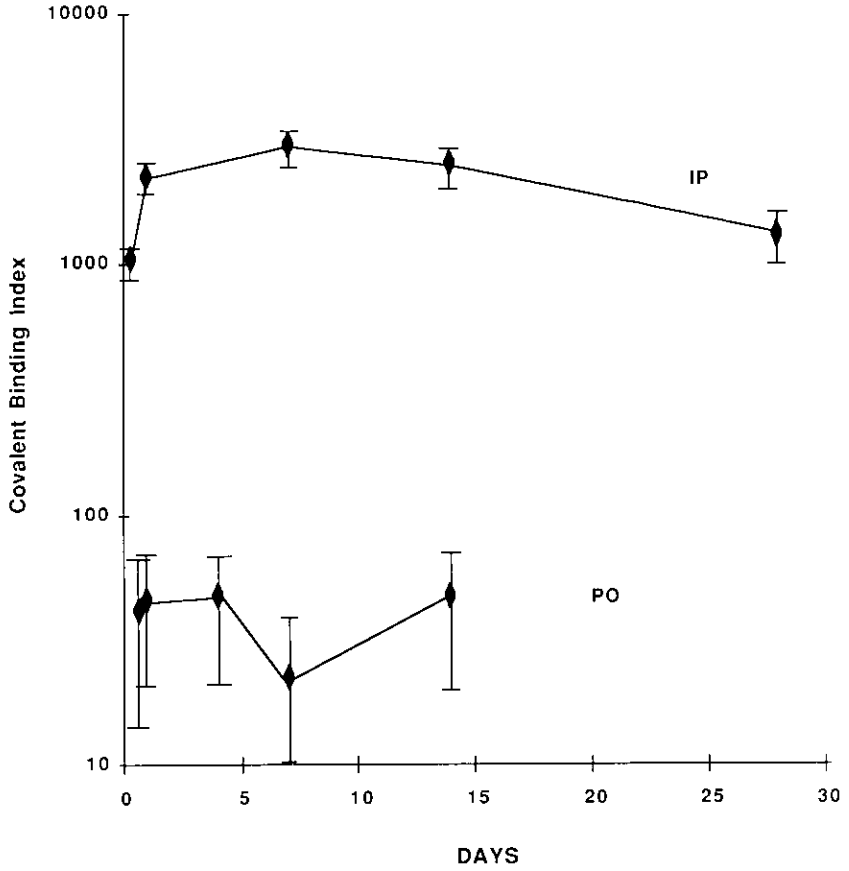


FIGURE 7. Persistence of BaP-DNA adducts in liver of juvenile English sole exposed to $^{3}$H-BaP via IP injection [(2 mg BaP, dissolved in acetone)/kg body weight] or PO [(0.1 mg BaP, dissolved in corn oil)/kg body weight]. Hepatic DNA was isolated as previously described (*46,51*), and radioactivity was determined by using liquid scintillation spectrometry. Data for the PO study were adapted from Varanasi et al. (*46*).

noted that English sole spawn in winter and that it appears that both gonadal steroids (e.g., estradiol-17β) and water temperature play important roles in the modulation of the levels of cytochrome P-450 in fish (*49*). Thus, more detailed studies are needed to evaluate sex-related differences in metabolic activation of BaP by adult sole. Accordingly, because our results with juvenile sole show no significant sex-related differences in the level of covalent binding to hepatic DNA, and also because of the greater activation of BaP by juvenile sole into intermediates that bind to hepatic DNA, we have used juvenile sole as our model to conduct more detailed studies on the *in vitro* and *in vivo* metabolism of BaP by English sole and to compare these results with other juvenile fish and rodent species.

We conducted a number of studies (*13–15,21,46,50–54*) in which juvenile English sole were used to study the biochemical fate of radiolabeled BaP, both *in vivo* and *in vitro*. Experimental details are given with the figures and tables. In these studies, we found that the levels of chemical modification of hepatic DNA in sole (Fig. 7) reaches a maximum value between 8 and 48 hr after administration of BaP IP or PO and persists for a period of 2 to 4 weeks (*46*). Higher levels of BaP intermediates are bound covalently to hepatic DNA in sole given IP injections of BaP dissolved in acetone as compared to corn oil at 24 hr (Table 3) (*51*). The level of binding of BaP intermediates to hepatic protein (data not shown) and hepatic DNA (Table 3) was 20- to 40-fold higher in fish exposed to 2 mg BaP/kg body weight than in those exposed to 0.1 mg BaP/kg body weight (*46,51*), indicating a dose-dependent increase in the level of modification of hepatic macromolecules by BaP. Regardless of dose or route of exposure, reverse- and normal-phase HPLC of phase I metabolites released after treatment of bile from BaP-exposed fish with β-glucuronidase and arylsulfatase show that BaP 7,8-diol and 1- and 3-hydroxy BaP were the major identifiable metabolites (Fig. 8) (Table 4). Sole liver microsomes in the presence of exogenous DNA metabolized BaP into essentially a single adduct, identified as (+)*anti*-7,8-diol-9,10-epoxy-7,8,9,10-tetrahydroBaP (*anti*-BPDE)-dG by reverse-phase HPLC and boronate column chromatography (Fig. 9) (*52,53*). For hepatic microsomes of sole from two sites in Puget Sound, there was a direct relationship between AHH activity and the amount of

**Table 3. Covalent modification of hepatic DNA by metabolites of benzo(a)pyrene (BaP) in several fish and rodent species.**

| Species[a] | Dose, mg BaP/ kg body weight | Vehicle | Route of exposure | BaP, fmole equivalents/ mg DNA | % admin. dose in liver | CBI[b] | Reference |
|---|---|---|---|---|---|---|---|
| California killifish | 0.6 | Corn oil | IP | 8.9 | NA | 1.2 | (*62*) |
| Speckled sanddab | 0.6 | Corn oil | IP | 14 | NA | 1.8 | (*62*) |
| English sole | 0.1 | Corn oil | PO | 51 | 0.8 | 45 | (*46*) |
| | 2.0 | Corn oil | PO | 2100 | 2.1 | 85 | (*51*) |
| | 2.0 | Corn oil | IP | 250 (880)[c] | 0.78 (1.59)[c] | 9.8 (31)[c] | (*50*) |
| | 2.0 | Acetone | IP | 27,000 | 6.0 | 1050 | (*51*) |
| Starry flounder | 2.0 | Corn oil | PO | 540 | 0.5 | 21 | |
| | 2.0 | Acetone | IP | 14,000 | 3.5 | 540 | |
| Rat (Sprague-Dawley) | 2.0 | Acetone | IP | 300 | 1 | 13 | (*51*) |
| Mouse (C57B1/6J) | 0.6 | Corn oil | IP | 72 | NA | 9.3 | (*62*) |

[a] All values are for both sexes combined, except for rat and mouse, where only females and only males were used, respectively; animals were killed 24 hr after exposure.

[b] Covalent binding index (CBI) = $\frac{\text{μmole BaP equivalents/mole nucleotides}}{\text{mmole BaP administered/kg body weight}}$

[c] Values in parentheses are for 48 hr after exposure—note that all show increases over 24-hr values.

protein that cross-reacted with the rabbit antibody to a trout cytochrome P-448 type isozyme, $LM_{4b}$ (Fig. 10) (*54*).

## Comparative Metabolism of Benzo[a]pyrene and Covalent Binding to Hepatic DNA in Fish and Rodent Species

Feral fish species exhibit a range of susceptibility to hepatocarcinogenesis in chemically polluted estuaries. For example, of the pleuronectid fish studied, English sole and rock sole exhibit high prevalences of liver neoplasms when sampled from chemically polluted estuaries in Puget Sound, WA, whereas starry flounder exhibits a very low prevalence of liver neoplasms (*3,55*). In other field studies, winter flounder in Boston Harbor, MA, (*56*) and brown bullhead in Niagara River, NY (*57*), show a high prevalence of liver neoplasms. Moreover, laboratory studies with salmonid fish (*2*) have demonstrated that the Mount Shasta strain of rainbow trout is susceptible to both BaP- and $AFB_1$-induced carcinogenesis, whereas coho salmon is relatively resistant to these carcinogens. Another interesting species-specific difference is noted when comparing target tissues for AH-induced carcinogenesis in fish species and rodents. For example, AHs such as DMBA and BaP cause liver cancer in *Poeciliopsis* and rainbow trout, respectively (*44,45,58*), whereas these AHs tend to cause cancer in extrahepatic tissues (e.g., skin, mammary gland, and lung) in adult rodents (*59–61*). Numerous studies (*51–53,62–65*) on hepatic microsomal metabolism of AHs, such as BaP, by fish and rodent species show several quantitative differences (Table 5). Generally, rodent liver microsomes metabolize BaP at a substantially higher rate than fish liver microsomes. However, only a small proportion (< 10%) of BaP is metabolized to BaP 7,8-diol by rat and mouse liver microsomes, compared to a value of 20 to 30% for several fish species. The *in vitro* metabolite profiles obtained with AH-induced fish are similar to those for untreated fish (Table 5). When hepatic microsomes from rat are incubated with BaP in the presence of DNA, a major adduct formed is the BaP-9-hydroxy-4,5-oxide-dG (*65*), whereas for English sole (*52,53*) the major adduct is *anti*-BPDE-dG (Fig. 11). These differences in the metabolism of BaP by rat and fish liver enzymes are consonant with the findings *in vivo*, showing that BaP 4,5-diol is the major diol formed in rat liver (*66*), whereas BaP 7,8-diol was the major diol released after enzymatic hydrolysis of aqueous soluble metabolites in bile of English sole (*21*). Another consistent difference in microsomal metabolism of BaP between fish and rodent species is that lower proportions of quinones are formed by fish liver microsomes (Table 5). In one study (*51*) when English sole, starry flounder, and Sprague-Dawley rat were given IP injections containing equimolar concentrations of $^3$H-BaP dissolved in acetone, the level of BaP intermediates bound to hepatic DNA in both fish species at 24 hr after BaP exposure was 50 to 90 times greater than that for rat (Table 3). Thus, the result that in rat liver relatively small proportions of BaP were converted into BaP 7,8-diol, the precursor of reactive intermediates such as *anti*-BPDE, helps explain the very low level of binding of BaP intermediates to rat liver DNA compared to sole or flounder liver DNA. In addition, differences in rates of excision-repair of BaP-modified DNA may also contribute significantly to the observed differences in binding levels between rat and fish species. Our results showed that high levels of modification of hepatic DNA in juvenile English sole persisted for up to 4 weeks after administration of BaP (Fig. 7), which supports findings that fish cells have a very low rate of excision-repair compared to rodent cells (*67*).

Interestingly, recent work by von Hofe and Puffer (*62*) shows that when California killifish (*Fundulus parvipinnis*), speckled sanddab (*Citharicthys stigmaeous*),

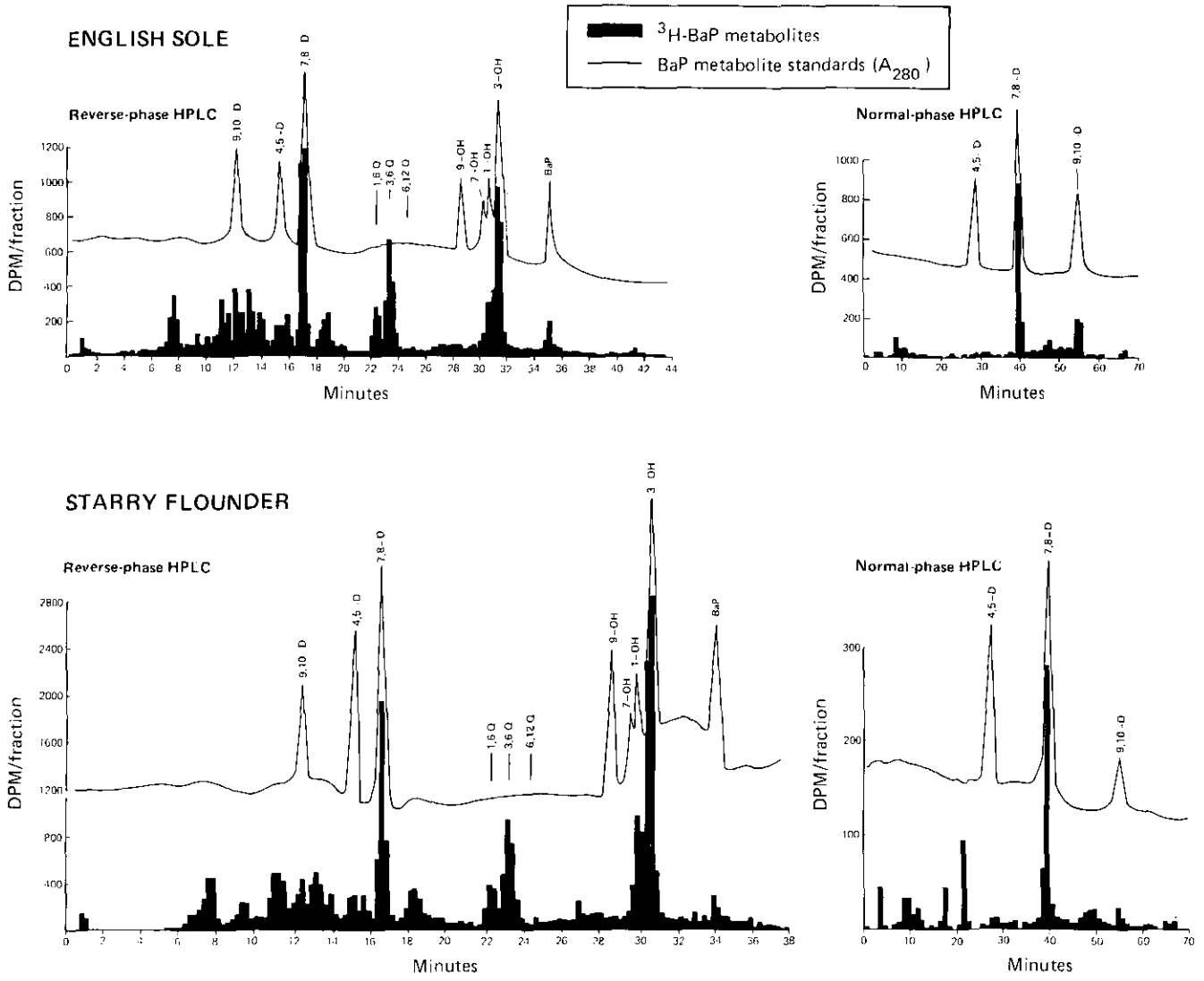


FIGURE 8. HPLC profiles of BaP metabolites released after β-glucuronidase and arylsulfatase treatment of bile of English sole and starry flounder exposed to 2 mg $^3$H-BaP/kg body weight for 24 hr. The fractions from 12–23 min were isolated from a second reverse-phase HPLC and an aliquot was analyzed further by normal-phase HPLC (*51*). DPM = disintegrations per minute.

**Table 4. Reverse-phase HPLC analyses of metabolites of benzo(a)pyrene released after enzymatic hydrolysis of bile of English sole and starry flounder.[a]**

| | | % total radioactivity in the organic phase[b] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Species | Route | 9,10-Diol | 4,5-Diol | 7,8-Diol | Quinones | 9-OH | 1-OH | 3-OH |
| Experiment 1 | | | | | | | | |
| English sole, ($n$ = 9) | PO | 3.6 ± 0.4 | tr[c] | 12 ± 1* | 7.8 ± 0.8 | 1.5 ± 0.2* | 7.2 ± 0.6 | 29 ± 1 |
| Starry flounder, ($n$ = 7) | PO | 3.7 ± 0.5 | tr | 8.1 ± 0.6 | 11 ± 2 | 2.2 ± 0.2 | 5.7 ± 0.8 | 24 ± 2 |
| Experiment 2 | | | | | | | | |
| English sole, ($n$ = 7) | IP | 4.2 ± 0.9 | tr | 14 ± 1* | 14 ± 1 | 1.8 ± 0.2 | 7 ± 1 | 14 ± 1* |
| Starry flounder, ($n$ = 9) | IP | 2.7 ± 0.4 | tr | 11 ± 1 | 11 ± 1 | 1.9 ± 0.2 | 10 ± 1 | 23 ± 2 |

[a] Adapted from Varanasi et al. (*51*); fish were exposed to 2 mg $^3$H-BaP/kg body weight and sampled after 24 hr.
[b] Values are expressed as $\bar{X}$ ± SEM.
[c] tr = trace (< 1%).
*Significantly different ($p < 0.05$) from corresponding values for starry flounder as analyzed by Student's $t$-test.

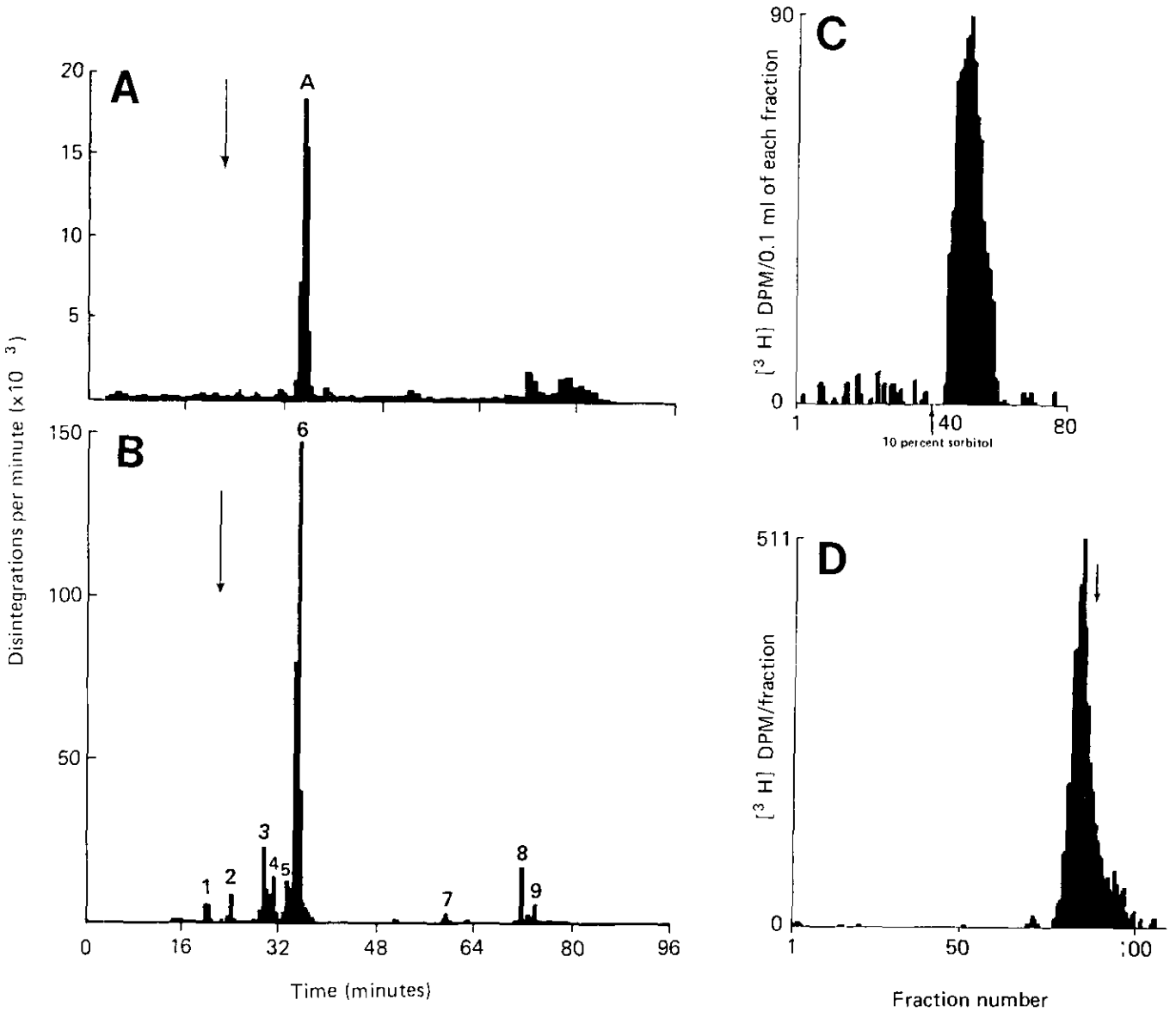


FIGURE 9. Tritiated benzo[a]pyrene was incubated with hepatic microsomes of English sole in the presence of salmon testes DNA, NADPH, and Tris buffer (25°C) and DNA was isolated. The modified DNA was hydrolyzed to deoxyribonucleosides using DNase I, snake venom phosphodiesterase and alkaline phosphatase, and the BaP-DNA adducts were isolated by Sephadex LH-20 column chromatography. The adducts were characterized by (*A*) HPLC analysis of the BaP-DNA adducts using a Beckman Ultrasphere ODS column, (*C*) boronate column chromatography using a morpholine (fractions 1-40) morpholine/10% sorbitol (fractions 41–80) mobile phase to separate *cis*- and *trans*-diol moieties, and (*D*) HPLC analysis of the major adduct, isolated by boronate column chromatography, using a Beckman Ultrasphere $C_8$ column. Figure 9*B* represents HPLC analysis of DNA adduct standards formed by the reaction of *anti*-BPDE with DNA. The arrows in Figures 9*A* and 9*B* represent where *trans*-2-tetrol chromatographs, whereas the arrow in Fig. 9*D* represents where the $^{14}$C-(+) *anti*-BPDE-dG adduct chromatographs. The $^{14}$C-labeled adduct has a slightly greater retention time than the $^{3}$H-labeled adduct. Adapted from Nishimoto and Varanasi (*53*).

and mice were given IP injections containing equimolar concentrations of BaP dissolved in corn oil, the level of binding of BaP intermediates to hepatic DNA in both fish species, at 24 hr after BaP exposure, was five to eight times lower than the binding for mouse (Table 3). Evaluation of HPLC profiles of phase I metabolites produced by the fish liver microsomes (Table 5) showed that in both fish species BaP 7,8-diol was a major metabolite, whereas mice are known to produce high proportions of BaP 4,5-diol (*64*). Thus, it is obvious from the two studies (*51,62*) described here that differences in metabolic activation as discerned from microsomal metabolism of BaP by fish and rodent species alone cannot adequately explain the differences in level of covalent binding of BaP to hepatic DNA *in vivo*. This is not too surprising, because *in vivo* both activation and detoxication systems will be operating in concert. For example, comparison of phase I metabolites produced

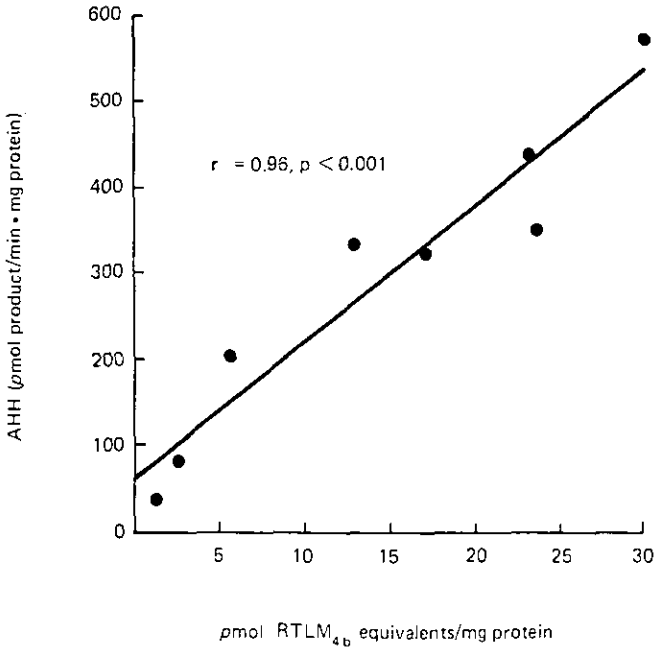


FIGURE 10. Correlation between levels of AHH activity in hepatic microsomes of English sole and levels of a cytochrome P-450 isozyme that reacts with the rabbit IgG to rainbow trout $LM_{4b}$, the major cytochrome P-450 isozyme induced by β-naphthoflavone in rainbow trout. Adapted from Varanasi et al. (*54*).

by liver microsomes of five fish species shown in Table 5 [sole, flounder, sanddab, killifish, and southern flounder (*Paralichthys lethostigma*)] show striking similarities, with BaP 7,8-diol constituting 20 to 30% of total metabolites (*51,62,63*). However, the range of the covalent binding indices (CBIs) for BaP-modified hepatic DNA in four of these fish species was quite large (Table 3), indicating that other factors, such as different solvent vehicles used (corn oil versus acetone), differential detoxication of reactive intermediates, and excision-repair of BaP-DNA adducts may play important roles in ultimately determining the level of DNA modification. Because of the different solvent vehicles used and different water temperatures (20°C with sanddab and killifish versus 12°C for sole and flounder), detailed comparisons of the data in Tables 3 and 5 are not possible. General observations are that the CBI for hepatic DNA in mouse and rat in these studies (Table 3) were comparable (9 versus 13) with each other and with values reported in literature (*61*). However, use of corn oil as a vehicle in the experiment (*62*) with fish at 20°C and mouse at 37°C may have resulted in much slower absorption of the administered dose by fish at 24 hr. In support of this possibility, we find that when BaP was dissolved in corn oil and administered IP to English sole, the CBI for hepatic DNA at 24 hr was 100 times lower than the value obtained for fish given IP injections containing equimolar concentrations of BaP dissolved in acetone (Table 3). Moreover, the CBI for fish given BaP in corn oil had increased 3-fold by 48 hr whereas that for fish given BaP in acetone remained unchanged, suggesting that absorption from the peritoneal cavity of BaP dissolved in corn oil was considerably slower. Nevertheless, the data given in Table 3 show that English sole was able to activate BaP (dissolved in corn oil and administered IP) to a greater extent, as indicated by higher CBI for hepatic DNA, than did sanddab or killifish, even though hepatic microsomes from all three

**Table 5. Metabolism of benzo(a)pyrene by hepatic microsomes of fish and rodents.**

| | | | % of total identifiable metabolites[d,e] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Species | Treatment[a] | AHH[b] | 9,10-Diol | 4,5-Diol | 7,8-Diol | Quinones[c] | 9-OH | 1-OH | 3-OH | Reference |
| English sole | U | 190 | 17 | 1.1 | 26 | 7 | 2.3 | 21 | 26 | (*51*) |
| | I | 550 | 20 | 1.3 | 26 | 6.7 | 1.0 | 18 | 26 | |
| Starry flounder | U | 180 | 24 | 3.3 | 22 | 7 | 5 | 19 | 20 | (*51*) |
| | I | 630 | 26 | 4.1 | 27 | 9.6 | 0.8 | 9.8 | 22 | |
| California killifish | U | 120 | 19 | ND | 20 | 8 | ND | — | 52 | (*62*) |
| | I | 400 | 24 | ND | 28 | 9 | 4 | — | 35 | |
| Speckled sanddab | U | 43 | 21 | ND | 26 | ND | ND | — | 53 | (*62*) |
| | I | 100 | 18 | ND | 28 | 6 | ND | — | 48 | |
| Southern flounder | U | 16 | 22 | 3.6 | 28 | 10 | 10 | — | 28 | (*63*) |
| | I | 840 | 24 | 2.6 | 29 | 11 | 7 | — | 13 | |
| C57BL/6J mouse | U | 875 | 0.2 | 3 | 4 | 46 | 6 | — | 41 | (*77*) |
| | I | 4560 | 1 | 2 | 9 | 31 | 14 | — | 37 | |
| Sprague-Dawley rat | U | 600 | 16 | 15 | 6.7 | 20 | 12 | 12 | 19 | (*51*) |

[a] U = uninduced, I = induced. Animals were exposed IP to BaP or 3-methylcholanthrene (3-MC) for induction of AHH as follows: English sole, starry flounder, and rat (2 mg BaP/kg body weight, 24 hr before sacrifice); killifish and sanddab (20 mg 3-MC/kg body weight, 48 hr before sacrifice); southern flounder (15–33 mg 3-MC/kg body weight 4 days before sacrifice); mouse (25 mg 3-MC/kg body weight, three daily doses before sacrifice).

[b] AHH [aryl hydrocarbon (BaP) hydroxylase] values are expressed as pmole BaP metabolized/mg protein/min.

[c] Contains BaP 1,6-, 3,6-, and 6,12-quinones.

[d] ND = not detected.

[e] — = not separated by HPLC from other phenolic metabolites.

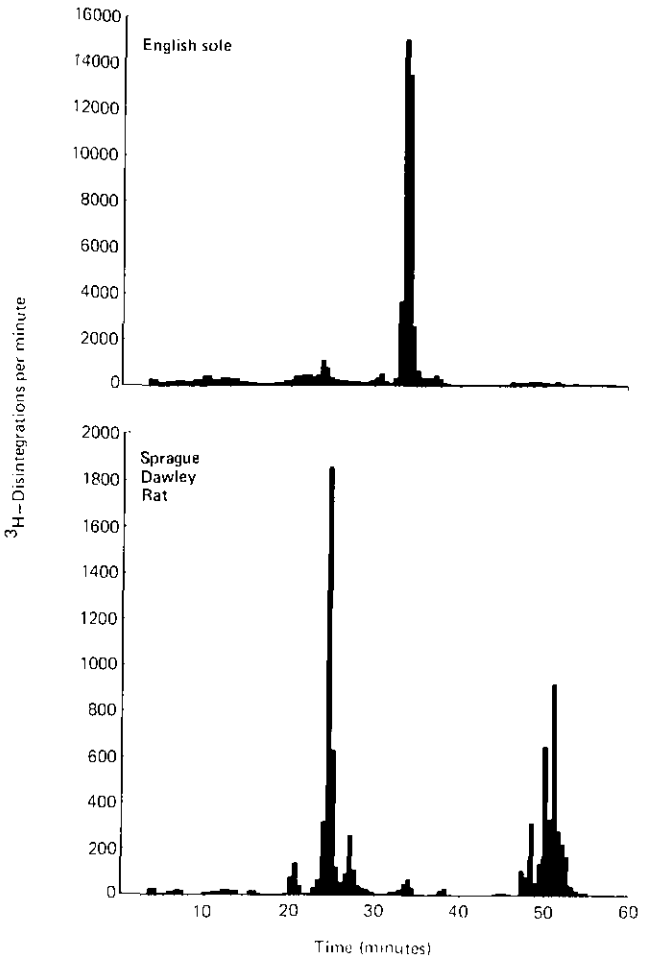


FIGURE 11. Tritiated BaP was incubated with hepatic microsomes from either English sole (25°C) or Sprague-Dawley rat (37°C) in the presence of salmon testes DNA, NADPH and Tris buffer (pH 7.5) for 30 min. DNA was isolated and hydrolyzed to deoxyribonucleosides. BaP-deoxyribonucleoside adducts were isolated by Sephadex LH-20 column chromatography and analyzed by HPLC using a Beckman Ultrasphere ODS column. The bar (⊢⊣) indicates where the $^{14}$C-(+)-*anti*-BPDE-dG adduct chromatographs. Adapted from Nishimoto and Varanasi (*52*).

species produced remarkably similar profiles of phase I metabolites (Table 5). Again, these results show that microsomal metabolism of carcinogens need not quantitatively reflect the ability of fish to form carcinogen-DNA adducts *in vivo*.

In order to evaluate further the role of detoxication processes in the metabolism of carcinogenic AHs *in vivo*, a detailed study (*51*) was conducted with English sole and starry flounder, two closely related pleuronectid fishes (*68*). As mentioned earlier, English sole appear to be more susceptible to chemically induced cancer than starry flounder, because it shows higher prevalences of hepatic neoplasms than starry flounder, even when both species are sampled from the same contaminated environment (*55*).

The results given in Tables 3 and 4 show that 24 hr after administration of BaP (2 mg/kg body weight) to fish either PO or IP, the level of binding of BaP metabolites to hepatic DNA was two to four times higher in sole than in flounder, and bile had significantly higher proportions of BaP 7,8-diol-glucuronide than did bile from flounder in both experiments. These findings along with our results showing that both English sole (*53*) and starry flounder (*50*) liver microsomes metabolize BaP essentially to a single DNA adduct, namely the (+)-*anti*-BPDE-dG, suggest that the higher binding of BaP intermediates to hepatic DNA in sole may be mostly due to more BPDE available in sole than in flounder. Although binding of other reactive intermediates (e.g., free radicals, phenol epoxides) to DNA *in vivo* cannot be excluded, no differences were observed in the proportions of phenols or quinones released after enzymatic hydrolyses of bile from the two species (Table 4).

Higher levels of BaP 7,8-diol glucuronide in the bile of sole and higher DNA binding in sole liver can be explained if a greater proportion of BaP was converted into BaP 7,8-diol in sole liver. However, our results show that both the rate of BaP metabolism and the proportion of BaP 7,8-diol formed by sole liver microsomes were essentially the same as the corresponding values for flounder (Table 5). It appears from studies with mammals that the formation of BaP 7,8-oxide is the rate-limiting step in the formation of BaP 7,8-diol (*69*), which suggests that the rate of formation of BaP 7,8-oxide was comparable for both fish species, whether they were untreated or BaP-induced (Table 5). The higher concentrations of BaP 7,8-diol glucuronide in sole bile and the higher binding of BaP to sole liver DNA can also be explained if, relative to sole, greater conjugation of BaP 7,8-oxide with GSH occurred in flounder liver. In support of this conjecture, we find that cytosolic GST activity in flounder liver (2500 ± 750 nmole/mg protein/min) is substantially higher than that in sole liver (900 ± 140 nmole/mg protein/min), measured with 1-chloro-2,4-dinitrobenzene as the substrate (*70*). The more effective conjugation of BaP 7,8-oxide with GSH in flounder liver could result in less BaP 7,8-diol available for conjugation with glucuronic acid and subsequent release into bile, as well as less formation of BPDE (Fig. 12). In addition, BPDE could be more effectively conjugated with GSH in flounder liver than in sole liver, thereby further reducing the availability of BPDE for binding to hepatic DNA in flounder. It should be noted that both BaP 7,8-oxide and BPDE are shown to be substrates for mammalian GST isozymes, with BPDE being a better substrate than BaP 7,8-oxide (*71*). Studies with mammalian systems show that both the concentration of cellular GSH and the level of GST activity are inversely related to DNA binding of BaP metabolites (*72–75*); however, there appears to be a better correlation between GST activity and DNA modification by BaP. Although the more effective detoxication of epoxides by GSH in flounder than sole can explain the present results, it should be emphasized that the efficiency of GSH conjugation apparently depends on substrate specificities of GST isozymes (*74,75*), and thus cannot be predicted only from total GST activity. More-

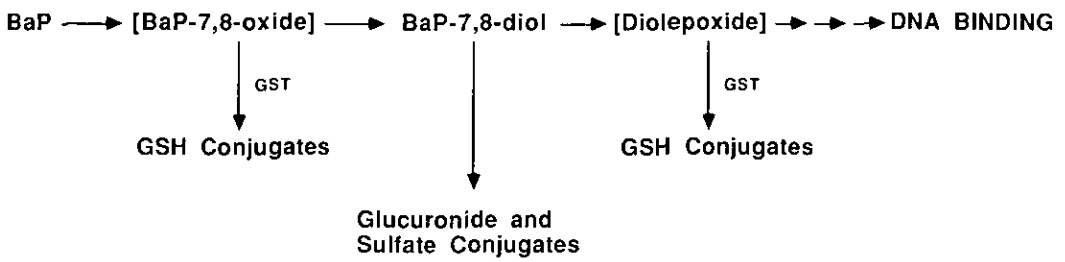


FIGURE 12. Activation and detoxication pathways for BaP.

over, no information is currently available on how exposure to contaminants affects hepatic GST activity in juvenile English sole or starry flounder. Further studies are needed to characterize isozymes of hepatic GST in uninduced and induced fish; to identify the adducts of BaP intermediates with both GSH and hepatic DNA; and to measure differences in the rates of excision-repair of modified DNA in these two fish species. In addition, the possible role of other enzymatic reactions, such as dihydrodiol dehydrogenase (*76*), in the metabolism of BaP 7,8-diol by these species needs to be investigated. Such studies should provide a clearer understanding of the present results showing a higher level of chemical modification of hepatic DNA in BaP-exposed sole than in flounder. Nevertheless, it is evident from these results that detoxication processes must be taken into account when evaluating the relative abilities of aquatic animals to activate carcinogens, and their subsequent susceptibility to chemically induced carcinogenesis.

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