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BENZO[*a*]PYRENE METABOLISM AND DNA ADDUCT FORMATION MEDIATED BY ENGLISH SOLE LIVER ENZYMES

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Abstract—Tritiated benzo[*a*]pyrene (BaP) and (±)-7,8-dihydroxy-7,8-dihydroBaP (BaP 7,8-dihydrodiol) were incubated with English sole liver microsomes in the presence of salmon testes DNA. The modified deoxynucleosides were isolated by Sephadex LH-20 column chromatography and analyzed by reverse-phase high-pressure liquid chromatography (HPLC). A single, major adduct (60–68% of the total modified deoxynucleosides) was formed when either BaP or BaP 7,8-dihydrodiol was incubated with sole liver microsomes and DNA. Although other minor BaP–DNA adducts were formed, none represented greater than 3% of the total adducts. The major adduct had a retention time on HPLC identical to that of the N^2-[10β(7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene)yl]-deoxyguanosine (7R-*anti*-BPDE/*trans*-dG) adduct formed when *anti*-BPDE, the ultimate carcinogen of BaP in mammals, was incubated with DNA. Analysis of the Bay region tetrols showed that only the 7α,8β,9β,10α-tetrahydroxy-7,8,9,10-tetrahydroBaP, a hydrolysis product of the *anti*-BPDE, was formed when BaP was incubated with sole liver microsomes. When BaP 7,8-dihydrodiol was used as the substrate, the 7α,8β,9β,10α-, 7α,8β,9α,10β-, and 7α,8β,9α,10α-tetrahydroxy-7,8,9,10-tetrahydroBaP's were formed, indicating the formation of both *anti*- and *syn*-BPDE. The ratio of tetrols of *anti*-BPDE/*syn*-BPDE was 2; however, the ratio of adducts of *anti*-BPDE/*syn*-BPDE was 20. Thus, the findings show that hepatic microsomes of English sole, a fish species having a high incidence of liver neoplasia in chemically contaminated estuaries, metabolized BaP and BaP 7,8-dihydrodiol stereoselectively to form predominantly the 7R-*anti*-BPDE/*trans*-dG adduct.

Toxic effects of certain PAHs† are exerted via metabolic activation to derivatives which interact with cellular macromolecules [1–3]. For example, BaP is metabolized to the reactive *anti*-BPDE which has been shown in mammalian test systems to possess the highest mutagenic and carcinogenic potential of the identified BaP metabolites [4–6]. The formation of this metabolite is dependent on the presence of the proximate carcinogen BaP 7,8-dihydrodiol and an enzyme system which can metabolize this precursor to the *anti*-BPDE [7–9]. Because isolation of this labile diol epoxide from biological systems is difficult, its presence is usually inferred from its hydrolysis products (e.g. tetrols; [7–9]) and DNA adducts [10–13].

Studies [14, 15] from this laboratory have demonstrated the formation of BaP 7,8-dihydrodiol in BaP-exposed English sole, a fish species with a high incidence of idiopathic liver lesions, including neoplasms, when sampled from PAH-contaminated areas in Puget Sound [16]. Moreover, significant amounts of BaP-derived intermediates were covalently bound to DNA and protein in liver of sole exposed to BaP [17, 18]. These findings prompted us to investigate whether the *anti*-BPDE is formed from both BaP 7,8-dihydrodiol and BaP in the presence of sole liver microsomes by analyzing for both the hydrolysis products and DNA adducts of *anti*-BPDE.

MATERIALS AND METHODS

Chemicals. Benzo[*a*]pyrene, deproteinized salmon testes DNA (type III), DNase I and II (from bovine pancreas), phosphodiesterase (from *Crotalus atrox*) and alkaline phosphatase (from calf intestine) were purchased from the Sigma Chemical Co., St. Louis, MO. Generally labeled tritiated BaP (70 Ci/mmol) was obtained from Amersham, Arlington, IL, and purified (>98% pure) as previously described [19]. The [^{3}H]BaP was then diluted with unlabeled BaP to a specific activity of 300 mCi/mmol. Tritiated BaP 7,8-dihydrodiol (397 mCi/mmole) and *anti*-BPDE (400 mCi/mmole), along with unlabeled BaP metabolite standards, were obtained from Midwest Research Institute, Kansas City, MO. Bay region

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† Abbreviations: PAH, polycyclic aromatic hydrocarbon; BaP, benzo[*a*]pyrene; *anti*-BPDE, (±)-7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene; *syn*-BPDE, (±)-7α,8β-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pryrene; *trans*-2-tetrol, (±)-7α,8β,9β,10α-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene; *trans*-1-tetrol, (±)-7α,8β,9α,10β-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; *cis*-2-tetrol, (±)-7α,8β,9β,10β-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene; *cis*-1-tetrol, (±)-7α,8β,9α,10α-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; 7R-*anti*-BPDE/*trans*-dG, N^2-[10β-(7β,8α,9α-trihydroxy-7,8,9,10-tetra-hydrobenzo[*a*]pyrene)yl]-deoxyguanosine; BaP 7,8-dihydrodiol, (±)-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; BaP 9,10-dihydrodiol, (±)-9,10-dihydroxy-9,10-dihydrobenzo[*a*]-pyrene; BaP 4,5-dihydrodiol, (±)-4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrene; quinones, benzo[*a*]pyrene 1,6-, 3,6-, and 6,12-quinone; HPLC, high-pressure liquid chromatography; and 3-MC, 3-methylcholanthrene.

tetrols of BaP were obtained from the hydrolysis of unlabeled *syn*- and *anti*-BPDE [20].

Preparation of microsomes. English sole (120 ± 20 g; 25 ± 1.0 cm) were captured by otter trawl off Bainbridge Island in Puget Sound, WA. The fish were killed with a blow to the head, and the livers were excised and washed with ice-cold 0.25 M sucrose prior to microsome preparation. Hepatic microsomes were prepared as previously described [21]. Protein content was determined by the method of Lowry *et al.* [22] using bovine serum albumin as a standard.

In vitro *metabolism of BaP and BaP 7,8-dihydrodiol.* Tritiated BaP or BaP 7,8-dihydrodiol was incubated at 25° with sole liver microsomes (3–5 mg protein) and NADPH (0.8 to 1.0 mg) in 2 ml of 0.05 M Tris buffer as previously described [19], except that the incubation time was extended to 1 hr. The unconjugated metabolites were extracted from the aqueous phase with nitrogen-saturated ethyl acetate and prepared for HPLC analysis as described previously [15].

Preparation of DNA adducts. Benzo[*a*]pyrene- and BaP 7,8-dihydrodiol-modified DNA were prepared using the above reaction conditions with 2 mg of DNA added to the incubation mixture [19]. The metabolites were extracted as described above and the DNA was isolated from the aqueous phase by a chloroform-isoamyl alcohol extraction (2 vol.) and ethanol precipitation [17].

The DNA adduct standards of *anti*-BPDE were prepared by the addition of 20 μl (66 nmoles) of a solution of tritiated *anti*-BPDE in tetrahydrofuran : triethylamine (19 : 1, v/v) to a solution of 2 mg of salmon testes DNA in 1 ml of deionized, distilled water. The solution was stirred, incubated at 25° for 1 hr, and then extracted with nitrogen-saturated ethyl acetate (3 × 1 ml) to remove the hydrolysis products of *anti*-BPDE. The modified DNA was isolated after ethanol precipitation as described earlier [17].

Isolation of DNA adducts. The DNA samples were dissolved in deionized, distilled water (2 ml), and the deoxynucleoside adducts were isolated as previously described [23] with a few modifications. Briefly, the DNA samples were hydrolyzed using DNase I (720 units), DNase II (500 units), snake venom phosphodiesterase (0.1 units) and alkaline phosphatase (7.5 units) overnight at 37°. The extent of hydrolysis and the BaP–DNA adduct profile did not change when the incubation time was extended to 72 hr. The hydrolyzed BaP–DNA adduct mixture was added to a Sephadex LH-20* column (40 × 9 mm), and the column was washed with 20 ml of deionized, distilled water. The BaP–deoxynucleoside adducts were then eluted with 20 ml of degassed methanol, the solvent was removed under vacuum, and the adducts were analyzed by reverse-phase HPLC after dissolution in methanol.

HPLC analyses. The BaP–DNA adducts were separated using a Varian 5020 HPLC fitted with an Altex Ultrasphere ODS column (0.46 × 25 cm; Beckman Instruments, Berkeley, CA) and equipped with a Varian Fluorichrome fluorescence detector (ex 340–380 nm; em >423 nm). A water (solvent A) : methanol (solvent B) gradient was used and changed as follows: 45–50% B in 20 min, held isocratic for 30 min, 50–70% B in 15 min, 70–100% B in 15 min, then held isocratic for 10 min. The flow rate was changed linearly from 0.6 to 1.0 ml in 20 min, then held at 1 ml/min. The column temperature was 25° and both solvents were continuously purged with helium. An internal standard of *trans*-2-tetrol was added to each adduct sample and was detected by its fluorescence emission. One-minute fractions were collected for the first 20 min, after which 0.5-min fractions were collected. After addition of Insta-gel (Packard Instrument Co., Downers Grove, IL) to each fraction, radioactivity of each fraction was determined using a Packard 300 C liquid scintillation spectrometer. Tetrols formed from BaP and BaP 7,8-dihydrodiol by microsomes were also separated using reverse-phase HPLC as described above. Primary metabolites of BaP were separated by reverse-phase HPLC as previously described [15].

* Mention of trade names is for information only and does not constitute endorsement by the U.S. Department of Commerce.

RESULTS

Metabolism of BaP by sole liver microsomes. With [^{3}H]BaP as the substrate and in the absence of DNA, the major primary metabolites formed were BaP 9,10- and 7,8-dihydrodiols, and the 1- and 3-hydroxyBaPs (Fig. 1A). Quinones were also present in appreciable amounts; the identity of their monohydroxy precursor(s) has not yet been established. Furthermore, the proportions of BaP 4,5-dihydrodiol and 9-hydroxyBaP were <1%. Significant radioactivity eluted with fractions having retention times of 10.5 (peak A) and 15.8 min (peak B; Fig. 1A). Although the identity of peak B is unknown, peak A was shown to cochromatograph with the *trans*-2-tetrol standard (Fig. 2A).

When DNA was added to the incubation mixture, the metabolite profile remained essentially the same (Fig. 1B). However, there was an increase in the proportion of 9-hydroxyBaP from <1% to 5 ± 4% of the radioactivity in the organic phase, and a concomitant decrease in the proportion of BaP 9,10-dihydrodiol. The total amount of products derived from BaP 9,10-oxide remained apparently unchanged. Other minor changes in the proportions of metabolites included an apparent increase in the amount of *trans*-2-tetrol (Table 1). BaP 4,5-dihydrodiol was not detected in the organic extract when DNA was present in the incubation mixture.

Metabolism of BaP 7,8-dihydrodiol by sole liver microsomes. When [^{3}H]BaP 7,8-dihydrodiol was metabolized by liver microsomes in the absence of DNA, HPLC analysis of the ethyl acetate-soluble fraction showed that the major metabolite eluted with *trans*-2-tetrol standard (Fig. 3A; Table 1), which is a hydrolysis product of the *anti*-BPDE [7, 9]. Equal but lesser amounts of radioactivity eluted with the *cis*- and *trans*-1-tetrols (Table 1) which are formed from the hydrolysis of *syn*-BPDE [7, 9]. The *cis*-2-tetrol, a hydrolysis product of *anti*-BPDE, was not detected. When DNA was present in the incubation

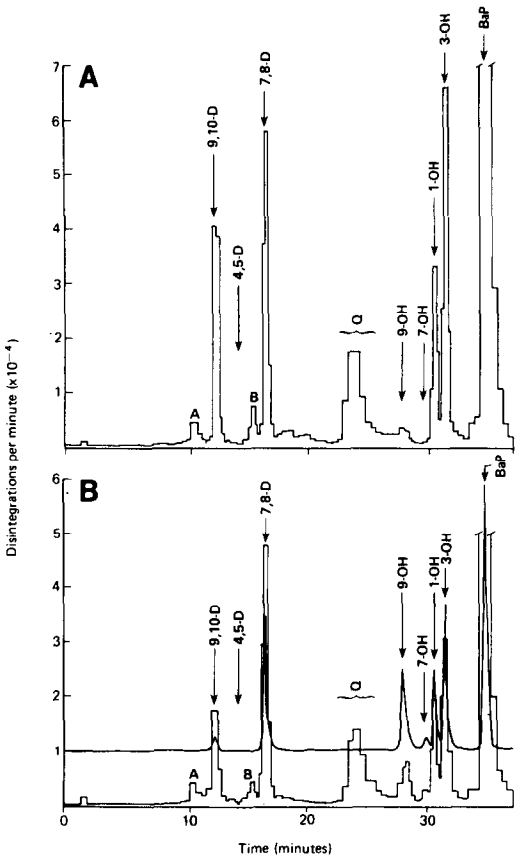

Fig. 1. Reverse-phase HPLC analyses of metabolites formed from [^{3}H]BaP by English sole liver enzymes in the (A) absence or (B) presence of DNA (see Materials and Methods). Fractions were collected at 0.4-min intervals except in the dihydrodiol (11–17 min) and phenol (28–31 min) regions where 0.2-min fractions were collected. BaP metabolite standards were cochromatographed with each sample and monitored by fluorescence emission (solid line trace) and also by u.v. absorbance.

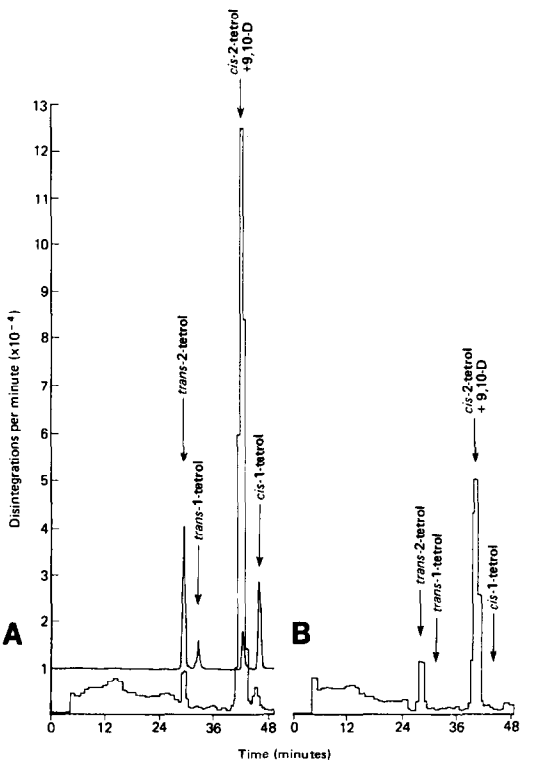

Fig. 2. Analyses of tetrols formed from BaP by English sole liver enzymes in the (A) absence or (B) presence of DNA. One-minute fractions were collected for 24 min, after which 0.5-min fractions were collected. Non-radiolabeled tetrols formed by the hydrolysis of *syn*- and *anti*-BPDE were cochromatographed with each sample and monitored by fluorescence emission (solid line trace).

mixture, the hydrolysis products of both *syn*- and *anti*-BPDE increased; for example, the amount of *trans*-2-tetrol increased 1.4-fold while the amount of tetrols formed from the *syn*-BPDE increased 1.3 times (Table 1). No *cis*-2-tetrol was formed, even though DNA was present to catalyze the hydrolysis of the *anti*-BPDE [24]. Interestingly, when DNA was added to the incubation mixture, the ratio of *cis*- to *trans*-1-tetrol increased to *ca.* 3 from a value of *ca.* 1.

Analyses of BaP–DNA adducts. After the DNA was modified by [^{3}H]BaP intermediates and hydrolyzed with enzymes, the BaP–deoxynucleoside adducts were isolated by Sephadex LH-20 chromatography. The BaP–DNA adducts eluting in the methanol fraction represented 73 ± 6.1% (N = 3) of the total radioactivity bound to DNA. This value was similar to the value [70 ± 7.5% (N = 3)] obtained when DNA was modified by [^{3}H]*anti*-BPDE. The methanol fraction was analyzed by HPLC and a typical BaP–DNA adduct profile is presented in Fig. 4A. The major adduct (peak A) comprised 60 ± 4.9% (N = 3) of the radioactivity applied to the HPLC column (Fig. 4A). This adduct had a retention time identical to that of the major adduct formed between [^{3}H]*anti*-BPDE and DNA (Fig. 4B; peak 6) which has been identified as the 7R-*anti*-BPDE/*trans*-dG adduct by others [25]. In addition to peak A, several smaller peaks, each consisting of no more than 3% of total adducts, were observed in the chromatogram given in Fig. 4A. Radioactivity eluting with a retention time of *ca.* 72 min may represent deoxyadenosine adducts which have been shown to elute later than deoxyguanosine adducts [25].

Analyses of BaP 7,8-dihydrodiol–DNA adducts. The DNA adduct profile obtained from the interaction of metabolized [^{3}H]BaP 7,8-dihydrodiol with DNA (Fig. 5) was similar to that for [^{3}H]BaP-modified DNA in that the major adduct (*ca.* 62%) had similar chromatographic properties to the 7R-*anti*-BPDE/*trans*-dG. The difference in retention times between peak A for BaP- and BaP 7,8-dihydrodiol-modified DNA appears to be due to column use. However, the standard *anti*-BPDE–DNA adducts were chromatographed immediately prior to the analysis of BaP 7,8-dihydrodiol–DNA adducts, and peak A had a retention time identical to the *anti*-BPDE/*trans*-dG. Unlike the BaP-deoxynucleoside adduct profiles of BaP (Fig. 4A) and *anti*-BPDE (Fig. 4B), adducts derived from BaP 7,8-dihydrodiol contained a peak B (Fig. 5) which was a minor prod-

Table 1. Formation of Bay region tetrols from BaP and BaP 7,8-dihydrodiol by English sole liver microsomes in the absence or presence of DNA

Substrate	% Radioactivity of metabolites in the organic phase			
	trans-2-Tetrol*	*cis*-2-Tetrol	*trans*-1-Tetrol	*cis*-1-Tetrol
BaP†	1.3 ± 0.11‡	—	ND§	ND
BaP + DNA	1.8 ± 0.23	—	ND	ND
(±)-BaP 7,8-dihydrodiol	27 ± 3.6	ND	6.4 ± 0.50	6.2 ± 2.9
(±)-BaP 7,8-dihydrodiol + DNA	39 ± 13	ND	4.4 ± 0.01	12 ± 3.1

* The *trans*- and *cis*-2-tetrols are products of the hydrolysis of the *anti*-BPDE, whereas the *trans*- and *cis*-1-tetrols are from the *syn*-BPDE [7, 9].
† See Fig. 1 for analysis of other metabolites of BaP.
‡ Mean ± S.D. (N = 2).
§ Not detected.

uct (*ca.* 3%) and was tentatively identified as an adduct formed from *syn*-BPDE. The ratio of *anti*-BPDE deoxynucleoside adducts to *syn*-BPDE deoxynucleoside adducts was *ca.* 30.

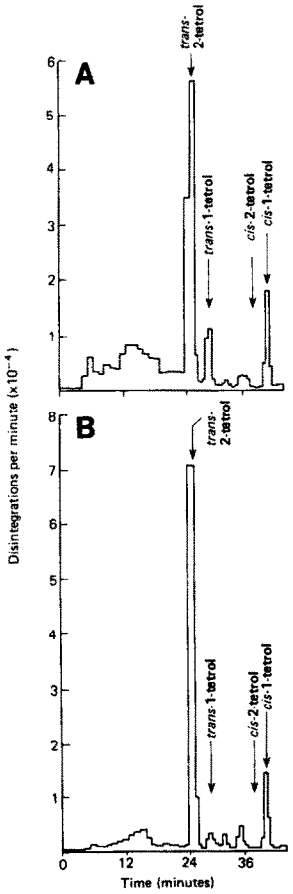

Fig. 3. Reverse-phase HPLC analyses of [^{3}H]BaP 7,8-dihydrodiol metabolites formed by English sole liver enzymes in the (A) absence or (B) presence of DNA. One-minute fractions were collected for 24 min, after which 0.5-min fractions were collected.

DISCUSSION

We have shown that English sole liver microsomes metabolized both [^{3}H]BaP and [^{3}H]BaP 7,8-dihydrodiol to the *anti*-BPDE and that reaction between BaP intermediates and DNA produced essentially a single adduct. Analyses by HPLC showed that this adduct had a retention time identical to that of the major adduct formed between [^{3}H]*anti*-BPDE and DNA which others have identified as 7R-*anti*-BPDE/*trans*-dG [25]. In tissues which are susceptible to

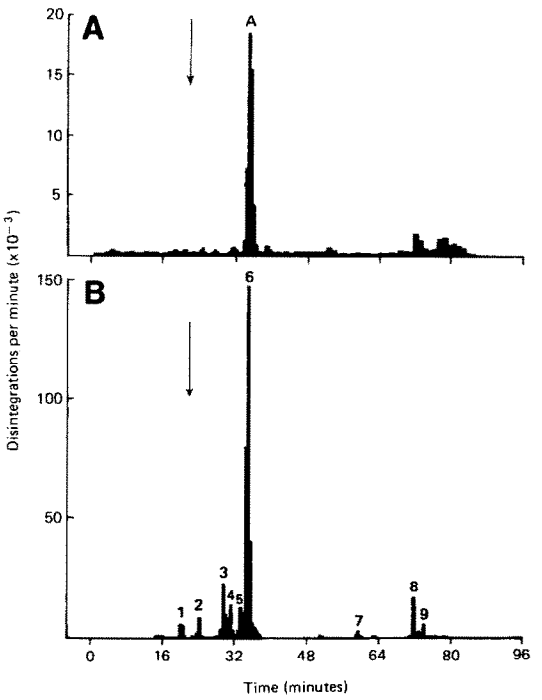

Fig. 4. Typical HPLC profile of BaP–DNA adducts formed by English sole hepatic microsomes (see Materials and Methods). (A) BaP–DNA adducts formed in the presence of sole liver microsomes; (B) DNA adduct standards formed by the reaction of *anti*-BPDE with DNA. Peak 6 represents the 7R-*anti*-BPDE/*trans*-dG adduct, whereas the identities of peaks 1–5 and 7–9 are currently being confirmed. The arrows indicate where the internal standard of BaP *trans*-2-tetrol chromatographs.

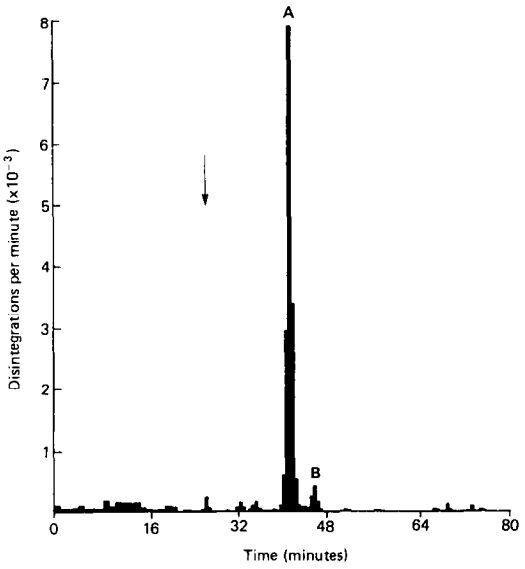

Fig. 5. HPLC profile of BaP 7,8-dihydrodiol–DNA adducts formed in the presence of English sole liver microsomes. The arrow represents where the internal standard of BaP *trans*-2-tetrol chromatographs.

BaP-induced carcinogenesis, such as mouse skin, the major DNA adduct formed is the 7R-*anti*-BPDE/*trans*-dG [26, 27]. The initiating event of PAH-induced carcinogenesis is believed to be the covalent interaction of electrophilic intermediates with DNA [28, 29], and dose–response curves show a possible causal relationship between the presence of the 7R-*anti*-BPDE/*trans*-dG lesion and mouse skin papillomas [26]. It has also been shown that the rate of excision of the 7R-*anti*-BPDE/*trans*-dG adduct from mouse skin is relatively slow compared to the rat skin [30], which is relatively resistant to BaP-induced carcinogenesis. Moreover, the rate of removal of 7R-*anti*-BPDE/*trans*-dG adduct in mouse skin is slower than that of the DNA adduct of BaP 4,5-oxide [31]. Therefore, the formation of the 7R-*anti*-BPDE/*trans*-dG adduct as the major adduct by sole liver microsomes, along with the persistence of high levels of BaP binding to hepatic DNA *in vivo* [17, 18], suggests that English sole liver may be a target tissue for BaP-induced carcinogenesis.

Unlike English sole liver microsomes, hepatic microsomes from rainbow trout [32] and 3-MC-treated rats [32, 33] produce at least two major adducts. The two adducts formed by trout and rat liver microsomes were identified by Sephadex LH-20 chromatography as the BPDE and 9-hydroxy-4,5-oxide BaP–deoxynucleoside adducts [32, 33]. The differences among the BaP–DNA adduct profiles obtained with sole, trout and rat liver microsomes could be attributed to the differences in proportions of 9-hydroxyBaP and BaP 4,5-dihydrodiol formed by each microsomal system. Sole liver microsomes do not biotransform BaP into 9-hydroxyBaP or BaP 4,5-dihydrodiol to an appreciable extent ([19]; Fig. 1) except when DNA is present to catalyze the rearrangement of the BaP 9,10-oxide to the 9-hydroxyBaP, as shown with rat liver microsomes [34]. In addition, sole does not produce these metabolites *in vivo* over a 20-fold dose range [14, 15]. The absence of 9-hydroxyBaP may be due to high levels of epoxide hydrase, whereas the absence of BaP 4,5-dihydrodiol may be due to insufficient levels of the necessary cytochrome P-450 isozyme(s) required for K-region metabolism. Thus, the contribution of phenol epoxides to total DNA binding in English sole represented a very small fraction due to the absence of the required metabolite precursor(s).

The present results show that, of the four possible Bay region tetrols that can be formed on secondary oxidation of BaP [7, 9], only the *trans*-2-tetrol was detected, indicating a stereoselective formation and hydrolysis of *anti*-BPDE by sole liver microsomes. When racemic BaP 7,8-dihydrodiol was used as a substrate, the hydrolysis products of both *syn*- and *anti*-BPDE were formed; however, a greater proportion of hydrolysis products of *anti*-BPDE than *syn*-BPDE was detected. Thakker *et al.* [7, 9] have reported similar distributions of tetrols derived from racemic BaP 7,8-dihydrodiol metabolized by liver microsomes of 3-MC-treated rats. Moreover, in good agreement with liver microsomes from 3-MC-treated rats [35], a substantially greater proportion of *anti*-BPDE–DNA adduct was formed compared to the *syn*-BPDE–DNA adduct when racemic [^{3}H]BaP 7,8-dihydrodiol was the substrate for sole liver microsomes. This demonstrates that *syn*-BPDE–DNA adducts are formed in substantially smaller amounts than the *anti*-BPDE–DNA adducts even when their required precursors are present in equal amounts.

In summary, the results show that, although the metabolism and DNA adduct formation of racemic BaP 7,8-dihydrodiol were similar in hepatic microsomes from both English sole and 3-MC-treated rats, English sole liver microsomes, unlike the rat liver microsomes, metabolized [^{3}H]BaP stereoselectively and regioselectively to form predominantly the 7R-*anti*-BPDE/*trans*-dG adduct. Because of the absence of K-region metabolism by English sole [14, 15], binding of BaP-intermediates to hepatic DNA *in vivo* may result solely from Bay region diol epoxides.

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