Metabolism and Activation of 7,8-Dihydrobenzo[a]pyrene during Prostaglandin Biosynthesis

INTERMEDIACY OF A BAY-REGION EPOXIDE*

(Received for publication, May 7, 1982)

Gregory A. Reed‡ and Lawrence J. Marnett§
From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202

A Tween 20-solubilized preparation of prostaglandin endoperoxide synthase has been shown to metabolize 7,8-dihydrobenzo[a]pyrene (H₂BP) to a form highly mutagenic to Salmonella typhimurium strain TA98. The arachidonic acid-dependent metabolism of H₂BP by microsomal and purified prostaglandin endoperoxide synthase has been studied and the products identified. A spectral investigation of the metabolism indicated the bay-region double bond as the primary site of metabolism. Radiolabeled H₂BP was synthesized and incubated with the enzyme preparations and the metabolites were separated by reverse phase high performance liquid chromatography and quantitated by liquid scintillation counting. Radioactive products were characterized by co-chromatography with chemically synthesized standards, UV-visible spectra, and mass spectrometry of acetate derivatives. The major polar products were determined to be trans- and cis-9,10-dihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene and 7,8,9,10-tetrahydrobenzo[a]pyrene-9-one in a ratio of 1:120.4. The inclusion of 5 mm 3,3,3-trichloropropene-1,2-oxide, an epoxide hydrolase inhibitor, produced the same products but in a ratio of 1:2.3:1.2. Incubations with purified prostaglandin endoperoxide synthase yielded the three products in a ratio of 1:28:0.7. The major nonpolar product was identified as benzo[a]pyrene. The polar products of metabolism, the effects of 3,3,3-trichloropropene-1,2-oxide on their distribution, and the detection of a mutagenic intermediate support the conclusion that H₂BP is co-oxygenated during prostaglandin biosynthesis to 9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene.

PAH§ are a class of ubiquitous environmental contaminants.

---

*This project was supported by Research Grant BC 244 from the American Cancer Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Present address, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709.
§Recipient of American Cancer Society Faculty Research Award FRA 243. To whom correspondence should be addressed.

1The abbreviations and trivial names used are: PAH, polycyclic aromatic hydrocarbon; BP, benzo[a]pyrene; H₂BP, 7,8-dihydrobenzo[a]pyrene; H₂BP, 7,8,9,10-tetrahydrobenzo[a]pyrene; H₂BP, 7,8,9,10-tetrahydrobenzo[a]pyrene-9-one; 7,8,9,10-tetrahydrobenzo[a]pyrene-9-one; trans-H₂BP-diol, trans-9,10-dihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; cis-H₂BP-diol, cis-9,10-di-hydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; H₂BP-epoxide, 9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; BP, 7,8-diol, trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene; 304, 5,8,11,14-eicosatetraenoic acid; 15-HPETE, 15-hydroxy-5,8,11,14-eicosatetraenoic acid; TCPo, 3,3,3-trichloropropene-1,2-oxide; RLM, rat liver microsomes; RSV, ram seminal vesicles; RP-HPLC, reverse phase high performance liquid chromatography; THF, tetrahydrofuran.
Dihydrobenzo[a]pyrene Metabolism

20:4

PGG₂

Oxidizing Equivalent

PGH₂

Xenobiotic

Oxidized

Xenobiotic

Metabolism (24, 25) and the nucleic acid adducts formed (26) strongly suggested that the initial product of BP-7,8-diol cooxygenation was 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. Since no one has ever isolated a bay-region diolepoxide from an in vitro incubation mixture, all of the evidence for their formation is indirect and is based upon the identification of stable derivatives formed by the attack of nucleophiles at the benzylic epoxide carbon. An alternative approach to detecting unstable epoxide intermediates would be to determine the effect of epoxide-specific reagents on the profile of stable epoxide-derived products. An example of an epoxide-specific reagent is epoxide hydrolase which catalyzes the hydrolysis of arene oxides and other epoxides to trans-dihydriodiol (50, 51). Unfortunately, microsomal epoxide hydrolase exhibits little or no catalytic activity towards dihydriodiolepoxides derived from PAH (30, 52). As a result, the relative distribution of diolepoxide solvolysis products is unchanged by the presence of epoxide hydrolase. Bay-region epoxides which lack hydroxyl groups in the tetrahydrobenzene ring do appear to be substrates for epoxide hydrolase so that the level of epoxide hydrolase activity should modulate the relative distribution of solvolysis products (29, 46). H₂BP has been shown by others to be metabolized by mixed-function oxidases to derivatives mutagenic to S. typhimurium TA98 (46) and H₂BP-epoxide is mutagenic (30). In addition, H₂BP-epoxide undergoes spontaneous solvolysis to a mixture of products in which the cis-H₂BP-diol is the major product and microsomal-mediated hydrolysis to the trans-H₂BP-diol (Equation 3). We have, therefore, investigated the metabolism of H₂BP by RSV enzyme preparations in an attempt to test for the hydroperoxide-dependent epoxidation of PAH derivatives by PES. Our results are described herein.

EXPERIMENTAL PROCEDURES²

Materials—7,8,9,10-tetrahydro-[7,10-¹³C]benzo[a]pyrene-7-one and [7-¹³C]styrene oxide were purchased from Amersham. 20:4 was from NuChek Preps, Elysian, MN. Indomethacin and soybean lipoxigenase were from Sigma, and hematin was from Calbiochem. HPLC solvents and scintillation chemicals were from Fisher; NMR solvents were purchased from Wilmad. S. typhimurium strain TA98 was generously provided by Professor Bruce Ames, University of California, Berkeley. 15-HPETE was prepared by the method of Funk et al. (33). All other chemicals and solvents were reagent grade.

Analytical Procedures—UV-visible spectra and spectrophotometric assays were performed on a Cary 210 spectrophotometer. NMR spectra were obtained using a Nicolet NTCD-1180 spectrometer at 300 MHz. Mass spectra were recorded on a Hewlett-Packard 5985 mass spectrometer at the Regional Mass Spectrometry Facility, located at Michigan State University, E. Lansing, MI. Liquid scintillation counting was carried out in a dioxane-based mixture on an Isocap 300. Quench correction was made using the sample channels ratio technique. Protein was measured by the method of Lowry et al. (34).

Synthetic Procedures—All synthetic procedures were carried out under subdued light, and reactions were run under argon. H₂BP was prepared by a modification of published procedures (35, 36). Trans-H₂BP-diol was prepared as previously described (38), while cis-H₂BP-diol was synthesized using a modified Prevost reaction (39). H₂BP-9-one was prepared from the H₂BP-diols by an acid catalyzed dehydration (35). All compounds exhibited appropriate UV-visible spectra, and the acetate derivatives of the diols and the ketone were analyzed by proton NMR and direct probe mass spectrometry. The diols were additionally characterized by formation of an acetamide derivative (42) and by differential reactivity with potassium triacetylosmate (41, 42). Details of the syntheses and characterizations may be found in the miniprint.

Enzyme Preparations—RSV were collected at a local slaughterhouse and stored at ~80 °C. Microsomal and Tween-20-solubilized enzymes were prepared as previously described (43). Purified PES from RSV, exhibiting a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis slab gels, was prepared by a procedure developed in this laboratory. Rat liver microsomes were prepared by the method of Oesch et al. (44).

Epoxide Hydrolase Assays—The epoxide hydrolase activities of various preparations were determined using the radiometric assay of Oesch et al. (44). [7-¹³C]Styrene oxide was added to a concentration of 2 mM and incubated for 15 min at 37 °C. All values are corrected for control incubations consisting of styrene oxide and buffer without enzyme.

Spectrophotometric Assay of H₂BP Metabolism—Determinations were carried out in a stirred cuvette thermostatted at 37 °C. Mixtures containing 0.1 M KPO₄ (pH 7.8), enzyme, and H₂BP were monitored at 370 nm for 3 min to establish the initial absorbance at 370 nm. 20:4 or 15-HPETE was added by syringe and the change in absorbance with time was recorded. The initial rate of the oxidation and the total consumption of H₂BP were calculated using ε = 3.79 × 10⁴ M⁻¹ cm⁻¹.

Incubations with solubilized enzyme or with purified PES also contained 0.1% Tween 20 (w/v).

Mutagenicity Experiments—Assays with S. typhimurium strain TA98 were carried out essentially as described previously (23, 45). Overnight cultures in nutrient broth were used, however, without further concentration, and hemoglobin was not added to the incubations.

H₂BP Metabolism Studies—Product studies were carried out in 2.0 ml incubations in 0.1 M K₂HPO₄, pH 7.8. Microsomal incubations contained 0.5–1.0 mg/ml of microsomal protein, 10 μM [¹³C]H₂BP.

² Portions of this paper (including "Experimental Procedures" and one figure) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 82M-1210, cite the authors and include a check for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

³ W. R. Pagels, R. Sachs, M. T. Leithauer, and L. J. Marnett, submitted for publication.
and 5 mM TCP0 where indicated. These mixtures were preincubated at 37 °C for 3 min, whereupon 20:4 was added to a concentration of 100 nM. After a 10-min incubation at 37 °C with vigorous shaking, each mixture was extracted thrice with 2 ml of ethyl acetate/acetone (4:1) each time. Extracts were taken to dryness using a rotary evaporator, and the residues were taken up in methanol for HPLC analysis.

Preparative incubations for product characterization were identical with the above, except that H2BP was 20 nM. Extracts from 6-8 tubes were pooled and stripped at reduced pressure and the residue dissolved in methanol for HPLC separation.

Incubations with purified PES were carried out in 0.1 M KPO4, pH 7.8, containing 0.1% Tween 20 (w/v) and 1 µM hematin. The incubation procedure was identical with the above, except that the metabolism was initiated by 50 µM 15-HPETO rather than by 20:4.

**HPLC Conditions**—All HPLC was performed using methanol/water mixtures on C-18 columns. Analyses were performed using a Waters HCM-100 module containing a Radial Pak B cartridge. Metabolites and H2BP were separated by a linear gradient from 75:25 methanol/water to 95:5 at 2% min⁻¹, then from 95:5 to straight methanol at 1% min⁻¹. The flow rate was 1.5 ml min⁻¹. Elution was monitored at 344 nm using a Varian Varichrom detector. Metabolites isolated by this gradient were purified further by isocratic elution using 75:25 methanol/water at 1.0 ml min⁻¹. Acetate derivatives were purified by elution with 90:10 methanol/water at 1.0 ml min⁻¹.

**RESULTS**

**Metabolic Activation of H2BP by Co-oxygenation**—H2BP has no intrinsic mutagenic activity but it is activated to a highly mutagenic species by Tween 20-solubilized RSV preparations. The activation is dependent on RSV protein (Table I), and this dependence is linear up to 400 µg of protein ml⁻¹. Maximal activation requires the addition of either 20:4 or 15-HPETE, but a clear concentration dependence is not routinely obtained. This may be due to the extreme sensitivity of the system to lipid hydroperoxides. In the presence of 10 µM H2BP, as little as 0.5 µM 15-HPETE produces a 2-fold increase in the reversion rate (data not shown). Even lipids endogenous to the enzyme preparation itself may become oxidized and thus serve to activate H2BP by co-oxygenation. The activation may be linked to prostaglandin biosynthesis in that the PES inhibitor indomethacin inhibits H2BP activation by up to 95% when 20:4 is added (Table I). The dependence of reversion rate on H2BP concentration is shown in Fig. 1. H2BP is activated to a potent mutagenic species, while the isomeric 9,10-dihydrobenzo[a]pyrene is not. This is in agreement with the results of Wood et al. (46), using a mixed-function oxidase-dependent activating system for the two BP derivatives (30).

The concentration dependence of H2BP activation is shifted to lower concentrations relative to the analogous curve for BP-7,8-diol activation (23). This is probably due both to the greater extent of metabolism of H2BP than of BP-7,8-diol (see below), and to the higher inherent mutagenic activity of the H2BP epoxide than of the diol epoxides (30).

**Spectrophotometric Assay of H2BP Metabolism**—Based on the established precedent for PAH activation, and on the mutagenicity results with the isomeric dihydrobenzo[a]pyrenes, the bay-region double bond of H2BP appeared to be the most likely site for metabolism and activation. Spectrophotometric determinations demonstrated that this was indeed the major site of metabolism. H2BP contains an extended pyrene chromophore with a major absorbance peak at 370 nm in the mixed aqueous-detergent media used. Any saturation of the 9,10 double bond yields a pyrene chromophore, shifting the long wavelength maximum from 370 to 349 nm. Any metabolism altering the H2BP chromophore will cause a decrease in the absorbance at 370 nm, and if the pyrene chromophore remains, there will be a corresponding increase in the absorbance at 349 nm (data not shown). The time course of H2BP metabolism was virtually identical with that of prostaglandin biosynthesis as measured by oxygen uptake. Similar

**TABLE I**

| Metabolic activation of H2BP | TA98 revertants/plate* |
|-------------------------------|-------------------------|
| None                          | 20 ± 3                  |
| Complete system†              | 471 ± 61                |
| + Indomethacin†               | 45 ± 3                  |
| - 20:4                        | 29 ± 13                 |
| - Enzyme                      | 35 ± 10                 |
| - H2BP                        | 24 ± 6                  |

*Values are the average of quadruplicate incubations ± S.D.
†The complete system consisted of a Tween 20-solubilized preparation from RSV (100 µg of protein), 5 µM H2BP, and bacteria in 0.5 ml of 0.1 M K2HPO4, pH 7.8, containing 0.1% Tween 20. Metabolism was triggered by the addition of 200 µM 20:4. Details are described under "Experimental Procedures."
‡Enzyme was preincubated with 100 µM indomethacin.

![FIG. 1. Concentration dependence of mutations in S. typhimurium strain TA98. Incubations contained 10° bacteria, RSV-solubilized enzyme (200 µg of protein), and the indicated concentration of H2BP in 0.1 M KPO4, pH 7.8. Total volume was 0.5 ml. Metabolism was initiated by the addition of 100 µM 20:4. Incubations, plating, and counting were carried out as described under "Experimental Procedures."](image)

![FIG. 2. Spectrophotometric assay of H2BP metabolism. A mixture containing 40 µM H2BP and RSV-solubilized enzyme (200 µg of protein ml⁻¹) in 0.1 M KPO4, pH 7.8, was stirred in a cuvette at 37 °C. Total volume was 2.50 ml. Co-oxidation of H2BP was initiated by 100 µM 20:4, and the reaction monitored at 370 nm.](image)
results have been obtained for the co-oxygenation of BP-7,8-diol (27). HBP appears to be a better substrate for co-oxygenation than is BP-7,8-diol. Both the initial rate and the total metabolism of HBP are approximately 2-fold higher than the values for BP-7,8-diol (data not shown). It has also been shown using this assay that the addition of 100 μM butylated hydroxytoluene to the cuvette at any point before or after the addition of 20:4 or 15-HPETE would immediately and totally abolish the consumption of HBP. This further establishes the sensitivity of co-oxygenation to antioxidant inhibition.

**Epoxide Hydrolase Assays**—RSV microsomes and solubilized preparations contain an active epoxide hydrolase. Using the radiometric assay of Oesch et al. (44) with labeled styrene oxide, as substrate. This enzyme appears similar to the microsomal epoxide hydrolase of liver (Table II). The RSV activity is destroyed by boiling the preparation, and marked inhibition by TCPO is also seen. These results are also obtained using

| Enzyme | Conditions | Activity (nmol diol min⁻¹ mg protein⁻¹) |
|--------|------------|--------------------------------------|
| RLM    | Native     | 4.0 ± 0.5                             |
|        | Boiled     | 0.1 ± 0.2                             |
|        | + TCPO     | 0.6 ± 0.1                             |
| RSV    | Native     | 2.4 ± 0.1                             |
|        | Boiled     | 0.1 ± 0.1                             |
|        | + TCPO     | 0.6 ± 0.2                             |

**Table II**

*Comparison of epoxide hydrolase activity in RLM and RSV microsomes*

Incubations were performed by the method of Oesch et al. (44). RLM (1.4 mg of protein) or RSV microsomes (0.6 mg of protein) were added to 100 μl of 0.5 M Tris (pH 9) containing 0.1% (w/v) Tween 20 and 5 mM TCPO where indicated. Water was added to a final volume of 400 μl. Following a 3-min preincubation, 2 mM [7-¹H]styrene oxide was added and incubation continued for 15 min at 37 °C. Boiled preparations were placed in a 100 °C water bath for 15 min.

---

**Fig. 3.** HPLC of H₂BP metabolites and standards. *a*, absorbance profile of authentic standards of *trans*- and *cis*-H₂BP-diol, H₂BP-9-one, and H₂BP monitored at 344 nm. *b*, ethyl-acetate-extracted metabolites and H₂BP from an incubation containing 10 μM H₂BP, RSV microsomes (1 mg of protein/ml), and 100 μM 20:4, incubated for 10 min at 37 °C and worked up as described under “Experimental Procedures.” *c*, identical with *b*, except the incubation contained 5 mM TCPO.

**Fig. 4.** UV-visible spectra of biosynthetic and chemically synthesized metabolites. All spectra were recorded in methanol. ——*, biosynthetic; ---, chemically synthesized. Both compounds were purified by isocratic HPLC as described under Experimental Procedures. *a*, *trans*-H₂BP-diol. *b*, 9-acetoxy-H₂BP.

rat liver microsomes. The specific and total activities are much higher in RLM than in RSV microsomes.

**HPLC Profiles of H₂BP Metabolism**—Incubations were performed using [¹⁴C]H₂BP with RSV microsomes in the presence and absence of the epoxide hydrolase inhibitor TCPO. Ethyl acetate-extractable material was subjected to reverse phase HPLC analysis. The column effluent was mon-
**Fig. 5.** Mass spectra of acetylated metabolites of H$_2$BP. Metabolites were purified by HPLC, acetylated, and repurified as described under "Experimental Procedures." Direct probe mass spectra were then recorded. **a,** mass spectrum of biosynthetic cis-H$_2$BP diol diacetate. **b,** mass spectrum of biosynthetic 9-acetoxy-H$_2$BP.
Acetates, and mass spectra of the acetates, the metabolites of ring (53).

Successive loss of acetate and then contraction of the benzo-

HlBP formed by co-oxygenation are the trans-H4BP-diol, cis-

H4BP-diol, and H4BP-9-one. The ratios in Table III are the mean ± S.D. of the 12 ratios. The purified PES ratios were calculated in the same manner from quadruplicate incubations in a single experiment.

A striking change in the ratio of trans- to cis-H4BP-diol may be seen when epoxide hydrolysis is inhibited or is removed by purification. This increase in the cis-H4BP-diol and the accompanying increase in the amount of H4BP-9-one are consistent with the metabolic scheme shown in Fig. 6. In this scheme, the initial product of H4BP co-oxygenation is the H4BP-epoxide. This unstable intermediate may be enzymatically hydrated by epoxide hydrolyase to form the trans-H4BP-diol, or it may spontaneously hydrolyze and rearrange to form a mixture of three products. The changes in product ratios shown in Table III support the generation of the H4BP-epoxide which is further transformed by both the enzymatic and the spontaneous pathways. When the enzymatic pathway is inhibited, the stable product profile reflects the increased contribution of the spontaneous pathway. The changing stable product profile resulting from epoxide hydrolyase inhibition or removal, and the functional group specificity and stereospecificity of epoxide hydrolyase (31, 32) indicate that the unstable initial product of H4BP co-oxygenation is the H4BP-epoxide.

Identification of Polar Products—Samples of each of the three metabolites were collected by HPLC and their UV-visible spectra were taken in methanol. All three exhibited UV-visible spectra indistinguishable from those of the metabolites themselves. The third metabolite when acetylated gave the UV-visible spectrum shown in Fig. 4b. This spectrum, shifted to longer wavelengths relative to the metabolite spectrum, is identical with the spectrum of the authentic 9-acetoxy-H2BP synthesized by the acetylation of H4BP-9-one.

The three acetylated metabolites were analyzed by direct probe mass spectrometry. Attempts at gas chromatography-mass spectrometry were unsuccessful due to extensive decomposition of the compounds during gas chromatography. Identical molecular ions and fragmentation patterns were seen for the acetate derivatives from the diol peaks. The spectrum of the cis-H4BP diol diacetate is shown in Fig. 5a. The molecular ion is seen at m/e = 372.2. Major fragments are seen at m/e = 312.2 (loss of CH3COOH), 270.1 (base peak, loss of CH3COOH + CH2CO), 252.2 (loss of 2CH3COOH), and 239.1 (loss of 2CH3COOH + CH). The spectrum of the ketone derivative, seen in Fig. 5b, exhibits a molecular ion at m/e = 312.2 and shows a similar fragmentation pattern featuring peaks at m/e = 270.2 (base peak), 239.1, and 226.2. These mass spectra are consistent with H4BP-diol diacetates for the larger mass derivatives, and 9-acetoxy-7,8-H2BP for the smaller mass derivative. As expected, most fragmentation occurs leaving the pyrene nucleus intact, progressing through successive loss of acetate and then contraction of the benzo-

Product Distributions—Based on co-chromatography of the three stable metabolites with chemically synthesized standards, UV-visible spectra of the metabolites and their acetates, and mass spectra of the acetates, the metabolites of H2BP formed by co-oxygenation are the trans-H4BP-diol, cis-

H4BP-diol, and H4BP-9-one. As varying amounts of H2BP were metabolized by different enzyme preparations, the absolute amounts of the three products cannot be compared directly. Rather, the ratios of the products have been compared from a number of incubations. The results using RSV microsomes (Table III) are the ratios obtained from the product distributions in five experiments, a total of 12 separate incubations. The relative amounts of the three products were calculated for each separate incubation, based on the trans-

H4BP-diol as 1.0. The ratios in Table III are the mean ± S.D. of the 12 ratios. The purified PES ratios were calculated in the same manner from quadruplicate incubations in a single experiment.

| Conditions | trans | cis | ketone |
|------------|-------|-----|--------|
| RSV microsomes* | 1 | 1.2 ± 0.2 | 0.4 ± 0.2 |
| RSV microsomes + TCPO* | 1 | 2.3 ± 0.4 | 1.2 ± 0.4 |
| Purified PES* | 1 | 2.8 ± 0.6 | 0.7 ± 0.2 |

* Incubations contained 1.0-2.0 mg of microsomal protein and H2BP in 2 mL of 0.1 M K2HPO4, pH 7.8. [TCPO] was 5 mM. Metabolism was initiated by the addition of 100 μM 20:4.

* Purified PES (6 μg of protein) and H2BP were incubated in 2 mL of 0.1 M K2HPO4, pH 7.8, containing 0.1% Tween 20 and 1 μM hematin. Metabolism was initiated by 50 μM 15-HPETE.
DISCUSSION

Co-oxygenation of BP-7,8-diol catalyzed by PES preparations has been shown to yield stable metabolites (24, 25) and nucleoside adducts (26) consistent with the intermediacy of the 7,8,9,10-tetrahydrobenzo[a]pyrene. The production of a highly mutagenic product and the observed saturation of the 9,10-double bond of H$_2$BP both suggest that a related epoxide intermediate is formed by the co-oxygenation of H$_2$BP. This intermediacy has been established in the present study.

We have shown that RSV microsomal preparations contain an active epoxide hydrolase for which the H$_4$BP-epoxide is a substrate. This enzyme will specifically hydrolyze cyclic 1,2-epoxides to their respective trans-diols (31, 32). In the absence of this enzyme, the H$_4$BP-epoxide will spontaneously hydrolyze and rearrange, forming primarily the cis-H$_4$BP-diol and the H$_4$BP-9-one and a small amount of trans-H$_4$BP-diol (28). If the H$_4$BP-epoxide is formed in these incubations, then any inhibition of epoxide hydrolase should shift the product distribution toward the cis-H$_4$BP-diol and the H$_4$BP-9-one at the expense of the trans-H$_4$BP-diol. This is indeed the case.

The ratios of cis- to trans-H$_4$BP-diol formed in the presence and absence of TCPO are particularly important in reaching this conclusion. Equal amounts of the two diols are formed by the microsomal system, whereas 2.3 times more cis- than trans-H$_4$BP-diol results when epoxide hydrolase is inhibited. Incubations with purified PES, which is devoid of epoxide hydrolase activity, produced the two diols in a ratio of 1.28, favoring the cis-H$_4$BP-diol even more strongly than in the TCPO-inhibited microsomal incubations. These results compare favorably with the results of Waterfall and Sims (29), who identified the products of metabolism of H$_2$BP and H$_4$BP-epoxide by the mixed-function oxidases in rat liver preparations. H$_2$BP metabolism produced both diols, with the trans predominating. However, when the H$_4$BP-epoxide itself was added, the ratio of the diols was 1:1. The results of their work and those of the current study underscore the key role of epoxide hydrolase in the interpretation of these stable product profiles. The common intermediate, the H$_4$BP-epoxide, is converted to the trans-H$_4$BP-diol by epoxide hydrolase. If, however, the catalytic capacity of the hydrolase is exceeded, either by inactivation of the enzyme or by excess substrate challenge, then the stable product profile will reflect the growing contribution of the spontaneous hydrolysis and rearrangement to the total product profile. The higher specific activity of epoxide hydrolase in the rat liver allows very little H$_4$BP epoxide to spontaneously hydrolyze, while the spontaneous pathway is far more pronounced in the RSV incubations of H$_2$BP.

The relative amounts of H$_2$BP-9-one formed under the different incubation conditions are less easily interpreted. Though some increase in the relative amount of H$_2$BP-9-one is seen in the TCPO-inhibited microsomal incubations and in the purified PES incubations, the magnitude of the increase is less than might be expected, based on the reported extent of spontaneous rearrangement (28). The ketone has been noted to be unstable at basic pH, making quantification difficult (28). Thus, the ketone may be decomposing during the course of the incubations. An alternative explanation may come from the results of Waterfall and Sims (29). In their studies with both H$_2$BP and H$_4$BP-epoxide in rat liver preparations they reported only the two diols as products. These results may indicate that the spontaneous hydrolysis and rearrangement of the H$_4$BP epoxide in purely aqueous media and in the mixed aqueous-protein-lipid media may not produce identical product distributions.

In addition to H$_2$BP-epoxide, the co-oxygenation product of H$_2$BP, a nonoxygenated product is also formed in this system. Approximately 10% of the total metabolism of H$_2$BP by RSV preparations results in the formation of H$_2$BP. This co-oxidation product results from the dehydrogenation of H$_2$BP. The dehydrogenation of dihydroarenes has been shown in various chemical systems to be a facile process (48) leading to the establishment of a fully aromatic system. The production of BP (this system is dependent on the intact RSV preparations and 20,4, and is inhibited by antioxidants. These similarities to the co-oxygenation reaction indicate that both BP and the H$_4$BP epoxide are formed from H$_2$BP by interaction with an oxidizing species generated during prostaglandin biosynthesis.

As has been noted, co-oxygenation of BP does not involve epoxidation (21) nor does it activate BP as a mutagen (23). Co-oxygenation, however, is capable of epoxidizing the isolated double bonds of BP-7,8-diol and H$_2$BP to form their bay-region epoxides, the ultimate mutagenic and carcinogenic forms of these molecules. Additionally, work by Eling has shown that 3,4-dihydroxy-3,4-dihydrobenzo[a]anthracene and 1,2-dihydroxy-1,2-dihydrochrysene are both co-oxygenated to bay-region diol epoxides by RSV preparations, indicating that co-oxygenation can be a general pathway for PAH activation.

The cytochrome P-450-dependent mixed-function oxygenases appear absolutely required for the initial oxygenation step in PAH activation, but the ultimate activation step may be catalyzed by either mixed-function oxidases or PES. Microsomal preparations from skin and lung, target tissues for BP carcinogenesis, have both been found to catalyze epoxidation of BP-7,8-diol by both pathways in vitro (47). Further studies are underway to establish if both pathways function in vivo as well.

Acknowledgments—NMR spectra were skillfully obtained by Paul E. Weller. Mass spectra were run by Betty Baltzer. Purified PES was generously provided by Robert Sachs.

REFERENCES

1. Particulate Polycyclic Organic Matter (1972) National Academy of Sciences, Washington D. C.
2. Miller, J. A. (1970) Cancer Res. 30, 559–576
3. Heidelberger, C. (1975) Annu. Rev. Biochem. 44, 79–121
4. Miller, J. A., and Miller, E. C. (1977) in Origins of Human Cancer (Hiatt, H. H., Watson, J. D., and Winters, J. A., eds) pp. 605–627, Cold Spring Harbor Laboratory
5. Cook, J. W., Hewett, C. L., and Hieger, I. (1933) J. Chem. Soc. (Lond.) 395–405
6. Boyland, E., and Sims, P. (1965) Biochem. J. 97, 7–16
7. Holder, G., Yagi, H., Dansette, P., Jerina, D. M., Levin, W., Lu, A. Y. H., and Conney, A. H. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4366–4367.
8. Selkirk, J. K, Croy, R. G., Roller, P. P., and Gelboin, H. V. (1974) Cancer Res. 34, 3474–3480
9. Borgen, A., Darvy, H., Castagnoli, N., Crocker, T. T., Rasmussen, R. E., and Wang, I. Y. (1975) J. Med. Chem. 18, 502–506
10. Sims, P., Grover, P. L., Swaisland, A., Pal, K., and Hewer, A. (1974) Nature (Lond.) 252, 226–228
11. Huberman, E., Sachs, L., Yang, S. K., and Gelboin, H. V. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 607–611
12. Wislocki, P. G., Wood, A. W., Chang, R. L., Levin, W., Yagi, H., Hernandez, O., Jerina, D. M., and Conney, A. H. (1976) Biochem. Biophys. Res. Commun. 68, 1006–1012
13. Jeffrey, A. M., Weinstein, I. B., Jentette, K. W., Grzeszkowski, K., Nakashishi, K., Harvey, R. G., Atrup, H., and Harris, C. (1977) Nature (Lond.) 269, 345–350
14. Meehan, T., Straub, K., and Calvin, M. (1977) Nature (Lond.) 269, 725–727
15. Kapitulnik, J., Wislocki, P. G., Levin, W., Yagi, H., Jerina, D. M., and Conney, A. H. (1978) Cancer Res. 38, 354–358
16. Grover, P. L., and Sims, P. (1968) Biochem. J. 119, 159–160
17. Gelboin, H. V. (1969) Cancer Res. 29, 1272–1276
18. Yang, S. K., McCourt, D. W., Roller, P. P., and Gelboin, H. V. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2584–2598

* T. E. Eling, unpublished results.
Dihydrobenzo[α]pyrene Metabolism

Supplementary Material to Metabolism and Activation of 7,12-Dimethylbenz[a]anthracene and Benzo[a]pyrene: Biochemical and Environmental Considerations

Gregory A. Reid and Lawrence J. Marnett

Synthesis of 7,12-Dimethylbenz[a]anthracene: The method used for the synthesis of 7,12-dimethylbenz[a]anthracene has been described in detail elsewhere (1,2). Briefly, the starting material was 7,12-dimethylbenz[a]anthracene, which was synthesized by the method of Houben and W. (3). The reaction was carried out at 100°C for 24 h. The crude product was purified by recrystallization from ethanol. The yield was 0.9 g, mp 187°C.

Synthesis of Dihydrobenzo[a]pyrene: The procedure used for the synthesis of dihydrobenzo[a]pyrene has been described in detail elsewhere (4). Briefly, the starting material was 7,12-dimethylbenz[a]anthracene, which was synthesized by the method of Houben and W. (3). The reaction was carried out at 100°C for 24 h. The crude product was purified by recrystallization from ethanol. The yield was 0.9 g, mp 187°C.

The spectra of the aromatic hydrocarbons were obtained using a Varian A-6000 spectrophotometer. The spectra were recorded at ambient temperature (25°C) and the spectra were corrected for the solvent contribution. The spectra were recorded in the ultraviolet (UV) and visible (VIS) regions. The spectra were recorded in triplicate and the average spectra were used for the analysis. The spectra were recorded at a scan rate of 100 nm/min.

The spectra of the aromatic hydrocarbons were obtained using a Varian A-6000 spectrophotometer. The spectra were recorded at ambient temperature (25°C) and the spectra were corrected for the solvent contribution. The spectra were recorded in the ultraviolet (UV) and visible (VIS) regions. The spectra were recorded in triplicate and the average spectra were used for the analysis. The spectra were recorded at a scan rate of 100 nm/min.

The spectra of the aromatic hydrocarbons were obtained using a Varian A-6000 spectrophotometer. The spectra were recorded at ambient temperature (25°C) and the spectra were corrected for the solvent contribution. The spectra were recorded in the ultraviolet (UV) and visible (VIS) regions. The spectra were recorded in triplicate and the average spectra were used for the analysis. The spectra were recorded at a scan rate of 100 nm/min.