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High-Pressure Liquid Chromatographic Analysis of Benzo(*a*)pyrene Metabolism and Covalent Binding and the Mechanism of Action of 7,8-Benzoflavone and 1,2-Epoxy-3,3,3-trichloropropane

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SUMMARY

High-pressure liquid chromatography is a rapid and efficient method for the separation of benzo(*a*)pyrene metabolites. This new technique readily separates eight benzo(*a*)pyrene metabolites from the parent hydrocarbon. These are three dihydrodiols (9,10-, 7,8-, and 4,5-dihydrodihydroxybenzo(*a*) pyrene), three quinones (benzo(*a*)pyrene-1,6-dione, -3,6-dione, and -6,12-dione) and two phenols (9- and 3-hydroxybenzo(*a*)-pyrene).

This method was applied to the study of the mechanism of inhibitor action on the microsomal benzo(*a*)pyrene metabolism. 7,8-Benzoflavone, an inhibitor of aryl hydrocarbon hydroxylase, inhibits the covalent binding of benzo(*a*)pyrene to DNA and inhibits the formation of each of the metabolites. The lack of selective inhibition suggests that the 7,8-benzoflavone acts on the oxidase or a prior component of the microsomal electron chain. 1,2-Epoxy-3,3,3-trichloropropane inhibits benzo(*a*)pyrene disappearance, which also suggests an inhibitory effect on oxidase activity. This compound, however, also stimulates formation of benzo(*a*)pyrene binding to DNA, reduces the ratio of 3-hydroxybenzo(*a*)pyrene to 9-hydroxybenzo(*a*)pyrene formation, and completely eliminates the formation of the three dihydrodiols. This selective effect suggests a specific inhibition of hydrase activity with a lesser effect on oxidase function.

INTRODUCTION

PAH's[1] are ubiquitous pollutants of the atmosphere, waterways, oceans, and soil and are present in marine life and in the food chain. Sources of these hydrocarbons include emissions from heat and power generation, refuse burning, transportation sources, industrial processes, and oil spills. Many componds in this class of chemicals cause cancer in experimental animals and are believed to cause cancer in humans and, in particular, lung cancer in individuals who smoke cigarettes (1). Mammals and many lower organisms metabolize polycyclic hydrocarbons primarily by enzymatic oxygenation to epoxides (13, 14, 19, 20, 36), phenols, dihydrodiols, quinones, and water-soluble conjugates (6, 8, 9, 18, 22, 37, 39). The polycyclic hydrocarbons are also covalently bound to cellular macromolecules *in vivo* (3, 16, 26), and the microsomal mixed-function oxygenases have been responsible for the activation of BP to intermediate(s) that reacts covalently with DNA (2, 11, 23, 24). In addition to the detoxification function of the microsomal mixed-function oxygenases (10, 42), this system converts polycyclic hydrocarbons to toxic or active carcinogenic forms (12).

In order to understand the mechanism of polycyclic hydrocarbon carcinogenicity it is necessary to understand the relationship between specific metabolite formation and the induction of cancer. It is thus necessary to describe the profile of metabolites formed from each PAH. The previous methods for the analysis of PAH metabolites have been laborious, destructive to some compounds, and largely inadequate for complete separation and quantitation of metabolites. We therefore felt that new methods were required in this area of research. We chose BP as the prototype hydrocarbon because BP is a major PAH pollutant and is present in smoke in relatively high concentrations; it is carcinogenic and undergoes complex enzymatic conversion to a large number of metabolites. Furthermore, the phenolic metabolites exhibit strong fluorescence, which makes BP an excellent substrate for the measurement of microsomal aryl hydrocarbon hydroxylase with respect to phenol formation (30). In this paper we describe a HPLC technique that we believe will be a major advance in the methodology of polycyclic hydrocarbon metabolite separation and isolation. A preliminary report of these studies has appeared (35).

We have used this technique to study the mechanism of action of 7,8-BF, an inhibitor of AHH (7,43), and TCPO, an inhibitor of epoxide hydrase (33). The 7,8-BF inhibits the cytotoxicity (7) and carcinogenicity of 7,12-dimethylbenz(*a*)-anthracene in mice (21) and the conversion of BP to phenolic

[1] The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; BP, benzo(*a*)pyrene; HPLC, high-pressure liquid chromatography; 7,8-BF, 7,8-benzoflavone; AHH, aryl hydrocarbon hydroxylase; TCPO, 1,2-epoxy-3,3,3-trichloropropane; 9-OH-BP, 9-hydroxybenzo(*a*)pyrene; 9,10-diol, 9,10-dihydrodihydroxybenzo(*a*)pyrene; 7,8-diol, 7,8-dihydrodihydroxybenzo(*a*)pyrene; MCA, 3-methylcholanthrene; TLC, thin-layer chromatography; 1,6-quinone, benzo(*a*)pyrene-1,6-dione; 3,6-quinone, benzo(*a*)pyrene-3,6-dione; 6,12-quinone, benzo(*a*)pyrene-6,12-dione; 4,5-diol, 4,5-dihydrodihydroxybenzo(*a*)pyrene; 3-OH-BP, 3-hydroxybenzo(*a*)pyrene.

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metabolites (43). We have found that 7,8-BF inhibits the formation of all BP metabolites, including those that bind to DNA. TCPO completely inhibits the formation of all 3 dihydrodiols of BP, increases the ratio of 9-OH-PB to 3-OH-BP, and increases the covalent binding of BP to DNA.

MATERIALS AND METHODS

Hydrocarbon standards: 9,10-diol and 7,8-diol were obtained by incubation of BP with rat liver microsomal preparations from MCA-treated rats, followed by TLC of ethyl acetate-extractable material. Both dihydrodiols were purified by HPLC and their UV spectra were identical to those reported by Waterfall and Sims (41). 1,6-, 3,6-, and 6,12-quinones (34) were kindly supplied by Dr. C. R. Raha from the Eppley Institute for Cancer Research, Omaha, Neb. 3-OH- and 9-OH-BP and *trans*-4,5-diol were first isolated by TLC and column chromatography (22) and later obtained from Midwest Research Institute, Kansas City, Mo., under National Cancer Institute Contract N01-CP-33387 (38). 7,8-BF and TCPO were purchased from Aldrich Chemical Co., Milwaukee, Wis. BP-^{3}H and BP-^{14}C were purchased from Amersham/Searle, Arlington Heights, Ill.

Male Sprague-Dawley rats weighing 125 to 150 g were given i.p. injections of 5 mg MCA in 0.5 ml corn oil 40 hr prior to sacrifice. Liver microsomes were prepared as previously described (22).

Metabolites were formed by incubating rat liver microsomes with BP-^{3}H (specific activity, 70 mCi/mmole) of BP-^{14}C (specific activity 21 mCi/mmole) in the following manner. Each flask contained, in a total volume of 1.0 ml: 0.25 mg microsomal protein; 0.36 μmole NADPH; 3 μmoles $MgCl_2$; 50 μmoles Tris-chloride buffer, pH 7.5; and 100 nmoles of BP dissolved in 0.04 ml methanol. For inhibition studies 7,8-BF was used at 1×10^{-4} M final concentration, and TCPO was used at 2×10^{-3} M final concentration. The flasks were incubated for 10 min at 37° under red illumination and the reaction was stopped by the addition of 1.0 ml acetone. The mixture was then extracted with 2.0 ml ethyl acetate. Five ethyl acetate extracts were pooled, dried over 1.0 g anhydrous magnesium sulfate, and evaporated under vacuum to dryness, and the metabolites were dissolved in 0.1 ml methanol. The formation of BP:DNA was performed with the incubation conditions described above in the presence of 1.36 mg microsomal protein with 2 mg calf thymus DNA present in the incubation medium. The BP-DNA was isolated and purified as previously described (11).

TLC. Ethyl acetate extracts of metabolites were spotted under red illumination on silica gel plates (Camag D-B) and developed in benzene:methanol (19:1). Compounds were visualized under UV light, scraped from the plate, and eluted with 95% ethanol. UV spectra (Cary 15) of dihydrodiols and phenols were identical to authentic samples and literature references (37, 39, 41).

HPLC. Metabolite separation was accomplished with a Dupont Model 830 high-pressure liquid chromatograph fitted with a 1-M ODS Permaphase column. Peak resolution was best using gradient elution with reversed phase where polar compounds elute first. Solvents were spectro-grade and glass distilled from Burdick and Jackson Laboratories, Inc., Muskegon, Mich. The initial solvent composition was 30% methanol: 70% water, and the final composition was 70% methanol:30% water with the gradient rate set at 3%/min.

Column pressure was 450 psi and oven temperature was 50°. Effluent was monitored at 254 nm through a 8-μl flow cell.

Fractions (0.2 ml) were collected at 20-sec intervals and measured for radioactivity in a Beckman scintillation counter using Aquasol (New England Nuclear, Boston, Mass.) as counting solution.

RESULTS

Chart 1 shows the separation by HPLC of 8 BP metabolites formed by the incubation of BP-^{3}H with liver microsomes from MCA-treated rats. A small unidentified peak is eluted first. This is followed by 3 clearly separated vicinal glycols: 9,10-diol, 4,5-diol and, 7,8-diol. These are followed by 3 quinones, 2 of which are not completely separated: 1,6-quinone, 3,6-quinone, and 6,12-quinone. These are followed by 2 phenols, 9-OH-BP and 3-OH-BP, which have not been separable by ordinary TLC. BP elutes considerably behind the metabolites, so that tailing effects common to TLC are minimized. There is a corresponding peak of absorbance at 254 nm with each of the radioactive peaks. In addition there are several peaks of absorbance at 254 nm that do not contain radioactivity; these represent other materials extracted from the microsomal incubation mixture. The nonradioactive UV-absorbing peak between the diols and quinones appears only in extracts of microsomes from MCA-treated rats and is probably a metabolite of MCA. The pattern shown represents the material removed from the incubation mixture by a single ethyl acetate extraction. Further extractions of the incubation mix (not shown) removes considerably more material, which appears on the HPLC as new peaks of absorbance at 254 nm with no radioactivity.

Chart 2 *B* to *G* shows HPLC patterns of individual BP-^{14}C metabolites previously isolated from microsomal incubation mixtures by TLC and then injected into the liquid chromatograph. The entire BP-^{3}H metabolite pattern was cochromatographed on HPLC with each BP-^{14}C metabolite in Chart 2, *B* to *G*, and is identical to that shown in Chart 2*A*. The BP-^{3}H metabolite patterns are not shown in Chart 2, *B* to *G*, in order to simplify reading of the graph.

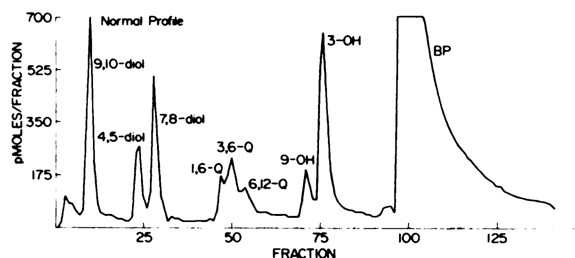

Chart 1. Pattern of metabolites after incubation of BP-^{3}H with liver microsomes prepared from MCA-treated rats. Fractions (0.2 ml) were collected at 20-sec intervals. ——, pmoles of hydrocarbon formed. Aliquot contained 35×10^{3} pmoles. *Q*, quinone; *OH*, hydroxy.

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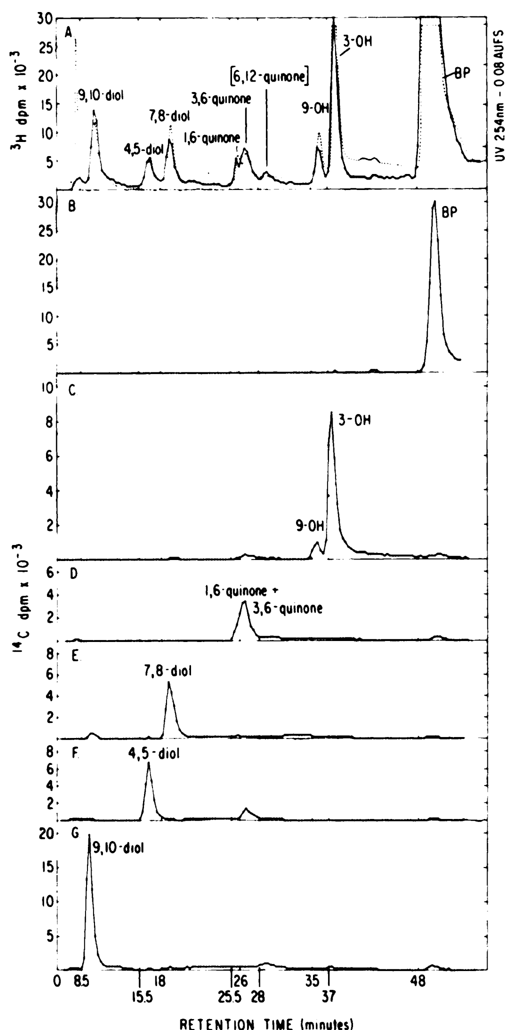

Chart 2. HPLC identification of total and individual BP metabolites. *A*, metabolite pattern after incubation of BP-^{3}H with liver microsomes from MCA-treated rats. ----, absorbance at 254 nm; —— pmoles of hydrocarbon formed. *B* to *G*, pattern of BP-^{14}C metabolites first isolated by TLC (22) and then analyzed by HPLC. Each spot was eluted from the TLC plate and reanalyzed by HPLC. Retention times and UV spectra were the same for corresponding ^{14}C and ^{3}H peaks. *C*, 9-OH-BP resolved from the single TLC spot corresponding to 3-OH-BP.

We previously reported the isolation by TLC and the identification of the metabolites shown in Chart 2, *B* to *G* (22). In the present study we used these characterizations but did additional mass and UV spectroscopy. Identification of the structure of each of the compounds other than the 9,10-diol and the 7,8-diol was further confirmed by comparison of retention time with synthetic standards. There is an exact correspondence of retention time on the HPLC for the ^{14}C peaks individually isolated from TLC and the ^{3}H peaks from the metabolite mixture (Chart 2, *C* to *G*).

Chart 2*C* shows the separation of 9-OH-BP and 3-OH-BP. With TLC, these 2 phenols have not been separated and move together as a single spot. We have recently demonstrated 9-OH-BP as a metabolite (22), after isolating it by laborious and extensive chromatography using conventional columns. The new HPLC technique accomplished this difficult separation easily and rapidly. Mass spectral analysis confirmed the molecular weight of each of the metabolites (Table 1). In addition, each of the isomeric dihydrodihydroxy compounds produced a characteristic fracture pattern (Chart 3), which will be useful for identifying unknowns in future metabolism studies.

All 3 dihydrodihydroxy compounds have the same molecular ion *m/e* 286 and lose water (M – HOH) *m/e* 268 followed by a CHO fragment (M – HOH – CHO) yielding *m/e* 239. The doubly charged ion is seen at *m/e* 119.5 for all 3 compounds. Some of the fragments below *m/e* 190 are probably due to lipid extracted from the incubation mixture, since the majority of these lines are absent in mass spectra of synthetic samples of 4,5-diol. The *m/e* 242 peak for the 9,10-diol

Table 1

The retention time and molecular weight of BP metabolites separated by HPLC

Metabolite	Retention time (min)	*m/e*[a]
9,10-diol	8.5	286
4,5-diol	15.5	286
7,8-diol	18.0	286
1,6-quinone	25.5	282
3,6-quinone	26.0	282
6,12-quinone[b] (tentative)	28.0	
9-OH-BP	35.0	268
3-OH-BP	37.0	268
BP	48.0	252

[a] Molecular weight determinations performed on a Jeol JMS-01SG-2 at 70 eV with a solid probe. Temperature range, 90° –150°.

[b] Insufficient material for complete analysis.

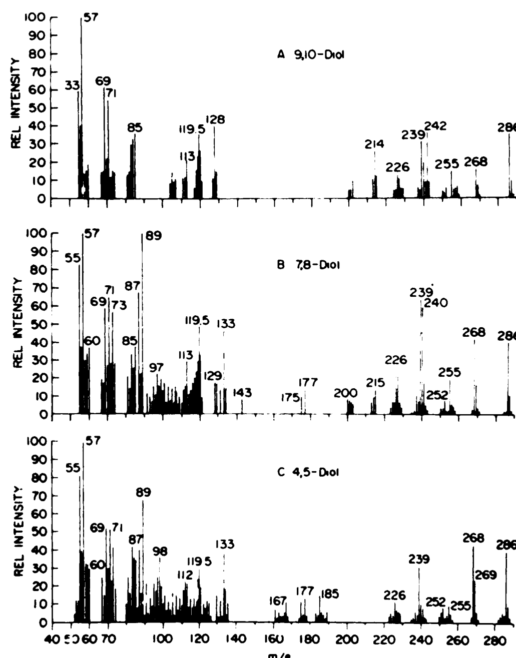

Chart 3. Mass spectral analysis of 3 dihydrodihydroxy metabolites of BP. Molecular weight determinations of the 3 dihydrodiols were made from peaks collected from the HPLC. Three peaks were pooled for each analysis. Analyses were performed on a Jeol JMS-01SG-2 at 70 eV with a solid probe; temperature was at 120°. *Rel.*, relative.

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probably corresponds to M − HOH − C_2H_2 while the *m/e* 240 peak for the 7,8-diol represents M − HOH − CO. Neither peak is observed in the K-region 4,5-diol spectrum, thus allowing identification of the 3 isomers.

Chart 1 and Table 2 (control) show the amount of each metabolite and the unmetabolized BP extracted after a 10-min incubation of microsomes with BP-^{3}H. The total radioactivity recovered after extraction and chromatography was greater than 95%.

The most abundant metabolite formed by these microsomes from MCA-treated rats was the 3-OH-BP. This agrees with our previous report showing that the combined 3-OH-BP and 9-OH-BP fraction was the single largest metabolite fraction (22).

The 2nd most abundant metabolite was the 9,10-diol. Of the 3 diols formed, the K-region 4,5-diol was the least abundant. As a class, the total amount of the 3 diols formed was of the same order but somewhat greater than total phenol formation. The quinones were formed to a lesser extent than either the diols or phenols.

These results are in general agreement with our previous studies utilizing TLC (22), except that the ratio of diols to phenols was somewhat greater in the present study. This may reflect the more efficient method of metabolite separation where drying in air is kept to a minimum and thus oxidation to quinones is diminished. The data presented in Table 2 are typical of results obtained in more than 6 experiments done under similar conditions. We have routinely monitored these experiments by comparison of the percentage of each metabolite, based on the total amount of BP metabolized. While the overall metabolism of BP is dependent upon the amount of inducible enzyme present, the relative yield of each metabolite is reproducible to within 10 to 15%.

Effect of 7,8-BF on BP Metabolism. 7,8-BF is a strong inhibitor of extrahepatic AHH and AHH in hepatic microsomes from MCA-treated rats (43). These studies were based on AHH activity as measured by the fluorescence of phenolic metabolites. Table 3 confirms these findings and shows a large inhibitory effect of 7,8-BF on phenol formation as measured by fluorescence. Table 2 and Chart 4, however, show that 7,8-BF inhibits not only phenol formation but also the formation of diols and quinones. Furthermore, there is a large increase in the amount of unmetabolized BP. Thus, 7,8-BF apparently interacts with the enzyme complex to inhibit the oxygenation at all sites of the benzopyrene molecule. However, diol formation is inhibited by 7,8-BF to a relatively greater extent (76 to 79%) than are phenol and quinone formations, which are inhibited from 55 to 68%. If the diols represent the products of epoxide hydrase action on epoxide intermediates, the 7,8-BF would seem to have somewhat greater inhibitory effect on epoxide formation than on phenol formation. This suggests that the enzymatic mechanism for oxygenation at position 3 may be different than the mechanism at positions 4, 5, 7, 8, 9, and 10.

Effect of TCPO on BP Metabolism. TCPO is a potent inhibitor of the epoxide hydrase (33). Table 2 shows that

Table 3

The effect of 7,8-BF and TCPO on AHH activity and BP-DNA formation

Additions	AHH (units)[a]	%	BP:DNA (cpm/mg)[b]	%
None	11,617		1,724	
7,8-BF	5,643	49	938	54
TCPO	9,128	79	2,478	143

[a] pmoles of hydroxylated BP with the fluorescence equivalent to 3-OH-BP formed in 30 min.

[b] The pmoles equivalent to BP-^{3}H are 10.7, 4.4, and 12.7 for no addition, 7,8-BF, and TCPO, respectively.

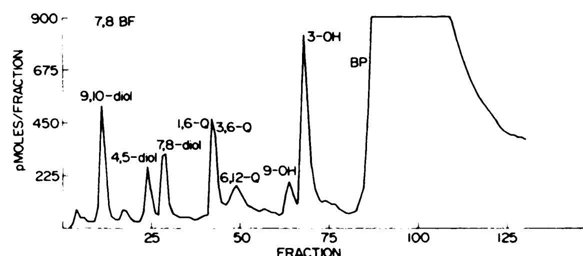

Chart 4. Effect of 7,8-BF on the metabolism of BP by liver microsomes from MCA-treated rats. BP-^{3}H was incubated with microsomes in presence of 10^{-4}M 7,8-BF, and the metabolites were analyzed by HPLC. Aliquot contained 94 × 10^3 pmoles. *Q*, quinone; *OH*, hydroxy.

Table 2

The effect of 7,8-BF and TCPO on BP metabolite formation

Each experiment consisted of 5 pooled incubations containing a total of 5000 × 10^{-2} pmole BP as substrate. Similar results were obtained in 5 other experiments performed under similar conditions. The percentage of each metabolite varied between 10 and 15% in these experiments.

	pmole × 10^{-2}								
	9,10-diol	4,5-diol	7,8-diol	Quinones	9-OH-BP	3-OH-BP	Total metabolites	BP	% metabolism[a]
Control	215.0	81.4	146.4	172.2	51.2	248.8	915.0	4035.0	18.3
7,8-BF	48.5 (22.5)[b]	19.6 (24.1)	31.5 (21.5)	77.7 (45.1)	15.7 (30.7)	95.8 (38.5)	288.8	4650.8	5.9
TCPO	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	95.8 (55.6)	123.1 (240)	250.8 (100.8)	469.4	4360.4	9.4

[a] Ethyl acetate-extractable material. The water-soluble radioactivity was <1% in all 3 incubations.

[b] Numbers in parentheses, percentage of control value.

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TCPO inhibits total BP metabolism by almost 50%, suggesting that TCPO may interfere with BP oxygenation as well as with epoxide hydration. In contrast to 7,8-BF, TCPO has a more selective effect on the metabolism of BP. Chart 5 and Table 2 show that TCPO completely prevents the formation of all 3 diols. The formation of 3-OH-BP, however, is not affected by TCPO. It is possible, although unlikely, that TCPO has a selective effect on the oxidase. A more reasonable explanation for the absence of dihydrodiols is the inhibitory action of TCPO on the hydrase activity. The 3-OH-BP apparently is not a secondary reaction product formed from either the 9,10-BP epoxide, 7,8-BP epoxide, or 4,5-BP epoxide, since interference with their hydration would be expected to alter 3-OH-BP levels. It may be possible that 3-OH-BP is not derived from an epoxide intermediate, since no 2,3-diol has been detected and the hydration of a 2,3-epoxide would be expected to be inhibited by the TCPO in the same manner as the other epoxides.

In the presence of TCPO the amount of 9-OH-BP relative to 3-OH-BP is largely increased, suggesting that the latter is a rearrangement product of an intermediate 9,10-epoxide the hydration of which has been inhibited by TCPO. There was no evidence of similar rearrangement products for the 4,5- or 7,8-epoxides of BP based on the liquid chromatography analysis of authentic samples of 7- and 8-hydroxy-BP and the phenolic rearrangement product (4-hydroxy or 5-hydroxy) of 4,5-epoxide of BP.

The Effect of 7,8-BF and TCPO on BP-DNA Formation. Table 3 shows that 7,8-BF inhibits AHH and the formation of BP-DNA by about 50%. Microsomes convert BP to an intermediate that binds covalently to DNA (11) and this correlates well with the degree of inhibition of oxygenated metabolite formation shown in Table 2. The intermediate(s) binding to DNA may also be a precursor of one or more of the metabolites shown. Our results show that the 7,8-BF inhibits the formation of the intermediates for both diol formation and BP:DNA formation. The nature of all of the BP metabolites binding to DNA are not known and may well include some of the other epoxides, *i.e.*, 7,8-BP-epoxide or 9,10-epoxide or other precursors of phenol or quinone formation.

Table 3 shows that TCPO stimulates BP:DNA formation. In the presence of TCPO the formation of all 3 dihydrodiols is completely inhibited (Table 2). This would suggest that the binding of BP:DNA is largely through an epoxide intermediate

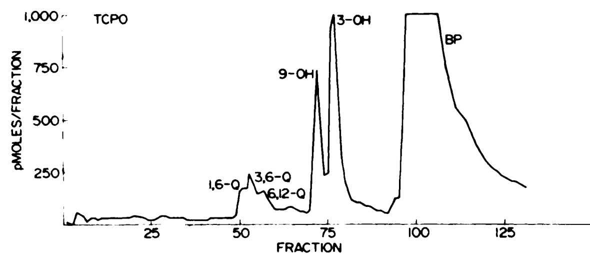

Chart 5. Effect of TCPO on the metabolism of BP by liver microsomes from MCA-treated rats. BP-^{3}H was incubated with microsomes in the presence of 2 × 10^{-3} M TCPO, and the metabolites were analyzed on HPLC. Aliquot contained 63 × 10^3 pmoles. *Q*, quinone; *OH*, hydroxy.

and that, although TCPO may inhibit overall oxygenation of BP as indicated by a reduction in BP disappearance (Table 2), it probably raises the steady-state level of reactive epoxide by inhibiting the epoxide hydrase. Thus, the amount of epoxide available for DNA binding would be increased and more BP:DNA would be formed.

DISCUSSION

The results demonstrate that HPLC is a most effective method for the separation and isolation of BP metabolites. The closed and solvent-filled system minimizes the breakdown of metabolites caused by their exposure to air and light. The metabolites can be separated and collected without the need for drying, and the amount of each product can be quantitated by spectrometry, spectrofluorometry, and scintillation counting. The procedure is much shorter than techniques involving TLC or column chromatography, and the possibility of metabolite loss or destruction is greatly reduced. The efficacy of the technique is shown by the easy separation of 3 isomeric dihydrodiols, 2 phenols, and 3 quinones. The epoxides, which are probably intermediates in dihydrodiol formation, were not detected under these conditions. This may be due to their instability in the reaction mixture or to the presence of epoxide hydrase enzymes, which rapidly convert epoxides to dihydrodiols. It is reasonable to extrapolate from these results to the promise of successful application of this method to the general class of PAH's and their metabolites.

The mechanism of inhibition of BP by 7,8-BF appears more complex than inhibition of a single phenolic product. The reduction of metabolism in the formation of each metabolite indicates that 7,8-BF affects BP metabolism at the oxidase level rather than at subsequent metabolic steps.

Early *in vivo* studies showed arene oxides to be relatively less carcinogenic than the parent hydrocarbon (27). However, more recent studies indicate that most arene oxides exhibit greater transforming activity in cell culture than any other derivative or the parent hydrocarbon (17, 25). These results still make arene oxides suspect as the reactive carcinogenic species. The occurrence, inducibility, and specificity of epoxide hydrase have been reviewed by Oesch (31). In most of the studies with this enzyme, styrene oxide has been used as the substrate. TCPO inhibits the formation of all 3 BP-dihydrodiols, suggesting that BP-4,5-epoxide, BP-7,8-epoxide, and BP-9,10-epoxide are the precursors of the 3 diols. Grover *et al.* (14) indirectly demonstrated the formation of the K-region BP-4,5-oxide by rat liver microsomes, but the latter did not detect either the 7,8- or 9,10-arene oxides of BP. We have not detected directly the intermediate arene oxides with this HPLC system. This may be due to the instability of the epoxides, their rapid hydration by epoxide hydrase, or inappropriate conditions for their isolation. TCPO causes a marked increase in 9-OH-BP formation and is suggestive of a 9,10-oxide as the precursor of this phenol. We have not found evidence for phenol formation at positions 4,5 or 7,8. The formation of 3-OH-BP, as measured by HPLC, is not significantly affected by TCPO, which confirms similar reports by Nebert *et al.* (29) and Oesch *et al.* (32). Using fluorescence assay techniques, however, we observe a decrease in phenol formation. It is possible that other unidentified metabolites

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that fluoresce poorly are present in this region of the chromatogram.

The observation that formation 3-OH-BP continues in the presence of TCPO inhibition of epoxide hydrase and the absence of 2,3-BP-diol, the expected hydrated product of 2,3-BP-oxide, suggests that the 3-OH-BP may be formed by a different mechanism than the 9-OH-BP.

Cavalieri and Calvin (5) have proposed a model in which there is an electrophilic attack at position 6 of BP with subsequent labilization of positions 1 and 3, which can then be substituted. The formation by rat liver microsomes of the free radical 6-oxo-BP has been described by Nagata *et al.* (28) using electron spin resonance analysis. The reaction is NADPH dependent and the activity is increased by inducers of microsomal enzymes. This 6-oxo-BP radical may be a precursor of one or more quinones. Quinones may also be formed nonenzymatically from highly labile intermediates. This is suggested by the finding that quinones are produced by simple air oxidation of 3-OH-BP on silica gel (2) and is converted to 3,6-quinone when incubated with heat-inactivated microsomes (unpublished results).

The finding that TCPO also inhibits the disappearance of BP suggests that the TCPO may not only be acting as an inhibitor of the hydrase but also may interfere with oxide formation. In fact, the reduction in BP metabolism is only partly due (Table 3) to a reduction of 3-OH-BP formation and thus seems to be more related to oxygenation resulting in oxide formation.

7,8-BF reduces the binding of BP-^{3}H to DNA and the formation of BP metabolites. However, these experiments do not identify the species bound to DNA. On the other hand, TCPO stimulates the binding of BP-^{3}H to DNA. One possible explanation is that a BP epoxide is a metabolite that interacts covalently with DNA. This is consistent with the work of Bresnick *et al.* (4, 40) showing a TCPO enhancement of the microsomal catalyzed binding of MCA-^{3}H to DNA. Grover and Sims (15) have shown that several hydrocarbon epoxides can nonenzymatically bind covalently to DNA. Our results, however, do not preclude the possibility that other BP metabolites also bind to DNA. In other studies, Lesko *et al.* (24) and Nagata *et al.* (28) have shown that 6-OH-BP binds covalently to DNA, and it is possible that the latter, quinones, or diols are involved in the binding.

It is likely that PAH's are oxygenated by several mechanisms, both enzymatic and nonenzymatic, and that the amount of products formed varies between different tissues and species. The final products will also depend on stability of reaction intermediates, affinity of the substrate for the mixed-function oxidases and epoxide hydrase, and the availability of target macromolecules that become substrates for alkylation. These parameters are more easily studied with the methodology presented. A knowledge of the metabolic pathways of carcinogen activation and detoxification may be significant to understanding individual susceptibility to PAH carcinogenesis and in the development of effective prophylactic measures.

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