Biochemical Pharmacology, Vol. 37, No. 11, pp. 2173–2182, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

RADICAL CATIONS AS PRECURSORS IN THE METABOLIC FORMATION OF QUINONES FROM BENZO[*a*]PYRENE AND 6-FLUOROBENZO[*a*]PYRENE

FLUORO SUBSTITUTION AS A PROBE FOR ONE-ELECTRON OXIDATION IN AROMATIC SUBSTRATES

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(*Received* 14 *April* 1987; *accepted* 23 *October* 1987)

Abstract—Three classes of products are formed when benzo[*a*]pyrene (BP) is metabolized by cytochrome P-450: dihydrodiols, phenols and the quinones, BP 1,6-, 3,6- and 6,12-dione. These products have been thought to arise from attack of a catalytically-activated electrophilic oxygen atom. In this paper we report chemical and biochemical experiments which demonstrate that BP quinones arise from an initial one-electron oxidation of BP to form its radical cation. BP, 6-fluorobenzo[*a*]pyrene (6-FBP), 6-chlorobenzo[*a*]pyrene (6-ClBP), and 6-bromobenzo[*a*]pyrene (6-BrBP) were metabolized by uninduced and 3-methylcholanthrene-induced rat liver microsomes in the presence of NADPH or cumene hydroperoxide (CHP) as cofactor. BP and 6-FBP produced similar metabolic profiles with induced microsomes in the presence of NADPH or 2 mM CHP. With NADPH both compounds produced dihydrodiols, phenols and quinones, whereas with CHP, they yielded only quinones. Metabolism of BP and 6-FBP was also similar with uninduced microsomes and 2 mM CHP, yielding the same BP quinones. With uninduced microsomes in the presence of NADPH, BP produced all three classes of metabolites, whereas 6-FBP afforded only quinones. At a low concentration of CHP (0.10 mM), BP was metabolized to phenols and quinones, whereas 6-FBP gave only quinones. 6-ClBP and 6-BrBP were poor substrates, forming metabolites only with induced microsomes and NADPH. One-electron oxidation of BP by $Mn(OAc)_3$ occurred exclusively at C-6 with predominant formation of 6-acetoxyBP and small amounts of BP quinones. In the one-electron oxidation of 6-FBP by $Mn(OAc)_3$, the major products obtained were 6-acetoxyBP, a mixture of 1,6- and 3,6-diacetoxyBP, and BP quinones. Reaction of BP and 6-FBP radical cation perchlorates with water produced the same BP quinones. Conversely, electrophilic substitution of 6-FBP with bromine or deuterium ion afforded C-1 and/or C-3 derivatives with retention of the fluoro substituent at C-6. These results indicate that metabolic formation of BP quinones from BP and 6-FBP can only derive from their intermediate radical cation.

A one-electron transfer process has been implicated as the initial step in the cytochrome P-450-catalyzed metabolism of cyclopropylamine [1, 2], dihydropyridine [3], tertiary amines [4], sulfides [5] and sulfoxides [6]. Aminium and sulfinium radicals are intermediates in the metabolism of the aforementioned compounds. Similarly quadricyclane is metabolized via an intermediate radical cation [7].

Three classes of products are formed when benzo[*a*]pyrene (BP§) is metabolized by cytochrome P-450 monooxygenase: phenols, dihydrodiols and quinones [8–10]. The major phenols formed are the 3- and 9-hydroxyBP. The dihydrodiols are the BP 4,5-, 7,8- and 9,10-dihydrodiol, and the quinones are the BP 1,6-, 3,6- and 6,12-dione. Formation of dihydrodiols occurs in two enzymatic steps catalyzed by cytochrome P-450 monooxygenase and epoxide hydratase. The mechanism of formation of 3- and 9-hydroxyBP has not been demonstrated, although these metabolites have been reported to arise from nonenzymatic isomerization of the intermediate epoxides [10, 11].

Quinones have been postulated to derive by attack of an electrophilic oxygen atom at C-6 of BP with formation of the putative 6-hydroxyBP precursor, which has never been isolated [12–16]. Auto-oxidation of 6-hydroxyBP [15] would produce BP quinones. The same quinones are also formed by metabolism of 6-FBP [17] with displacement of fluorine from position 6.

Combined studies of the radical cation chemistry and metabolism of BP and 6-FBP have enabled us to demonstrate that metabolic quinone formation

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§ Abbreviations: BP, benzo[*a*]pyrene; $BP^{\cdot+}$, benzo[*a*]pyrene radical cation; $BP^{\cdot+}ClO_4^-$, benzo[*a*]pyrene radical cation perchlorate; 6-OAcBP, 6-acetoxybenzo[*a*]pyrene; 6-BrBP, 6-bromobenzo[*a*]pyrene; 6-ClBP, 6-chlorobenzo[*a*]pyrene; 6-FBP, 6-fluorobenzo[*a*]pyrene; CHP, cumene hydroperoxide; MC, 3-methylcholanthrene; and PAH, polycyclic aromatic hydrocarbons.

occurs via an initial one-electron oxidation of the substrate. These results suggest that displacement of a fluoro substituent from an aromatic substrate serves as a probe for metabolism by one-electron transfer. Furthermore, the comparative metabolic study of BP, 6-FBP, 6-ClBP and 6-BrBP with uninduced and 3-methylcholanthrene (MC)-induced rat liver microsomes in the presence of NADPH or cumene hydroperoxide (CHP) as the cofactor indicates that BP and 6-FBP exhibit a similar metabolic profile, whereas 6-ClBP and 6-BrBP resemble one another.

MATERIALS AND METHODS

Chemicals. BP (Aldrich Chemical Co., Milwaukee, WI) was purified by column chromatography on activated alumina eluted with hexane–benzene (1:1) and recrystallized from benzene–methanol, m.p. 176–178°. 6-BrBP was synthesized by reaction of BP and pyridinium bromide perbromide in glacial acetic acid (unpublished method). Recrystallization of the compound from acetone–methanol yielded a product having m.p. 216–218° (m.p. 223–224° [18]). 6-FBP was prepared by a two-step synthesis from 6-BrBP with *n*-butyl lithium and then with perchloryl fluoride (unpublished method). The product was chromatographed on silica gel eluted with hexane and recrystallized from acetone–methanol. The final product had m.p. 167–169° (m.p. 165° [19]). 6-ClBP was obtained by reaction of BP and $CuCl_2$ in CCl_4 according to the method of Nonhebel [20] for 9-chloroanthracene. The compound was recrystallized from acetone–methanol to give 6-ClBP, m.p. 210–212° (m.p. 210° [21]).

BP, 6-FBP, 6-ClBP and 6-BrBP were purified by high pressure liquid chromatography (HPLC) prior to metabolism studies, using a Spectra Physics 8100 or 8700 system with an analytical Altex Ultrasphere ODS 5 μm column. The column was eluted with 50% methanol in water for 10 min, followed by a linear gradient to 100% methanol in 60 min at a flow rate of 1 ml/min. The eluant was monitored for absorbance at 254 nm using a Kratos Spectroflow monitor.

MC was purchased from the Aldrich Chemical Co., while CHP, NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Co. (St. Louis, MO). Authentic samples of BP metabolites were obtained from the Chemical Carcinogen Repository, National Cancer Institute, Bethesda, MD.

Preparation of microsomes. Male Wistar rats (8 weeks old, Eppley Colony) were induced by intraperitoneal injection of 20 mM MC in olive oil on 2 consecutive days at 100 μmol/kg and killed 24 hr after the last injection. Both uninduced and MC-induced rats were fasted overnight before being killed by cervical dislocation.

Rat liver microsomes were prepared at 0–4°. The liver was minced and homogenized in 40 ml of 0.25 M sucrose–0.1 mM EDTA, pH 7.5, and the homogenate was centrifuged at 9000 *g* for 10 min. The supernatant fraction was centrifuged at 100,000 *g* for 60 min, and the resultant microsomal pellets were resuspended in the sucrose solution at 1 ml/g liver and stored in small aliquots at −80°. The protein concentration of the microsomal fraction was determined by the method of Lowry *et al.* [22], and the cytochrome P-450 content was determined by the technique of Omura and Sato [23].

Metabolism by rat liver microsomes. Metabolic reactions of PAH were performed in 2-ml incubation mixtures. The CHP-supported microsomal metabolism was begun by preincubation of 2 mg of microsomal protein, 150 mM KCl and 5 mM $MgCl_2$ in 50 mM Tris–HCl, pH 7.5, for 3 min at 37°, after which 160 nmol of substrate dissolved in 25 μl acetone was added, followed immediately by the appropriate amount of CHP. After further incubation for 10 min, the reactions were terminated by addition of 2 ml of acetone. In the NADPH-supported metabolism, 2 mg of microsomal protein, NADPH-generating system (0.43 mM NADP, 1.29 mM glucose-6-phosphate, 0.43 units/ml glucose-6-phosphate dehydrogenase), 150 mM KCl and 5 mM $MgCl_2$ in 50 mM Tris–HCl, pH 7.5, were preincubated for 5 min at 37° and the incubation was continued for 30 min after addition of the substrate. The reactions were terminated by addition of 2 ml acetone, and the metabolites were extracted three times with 3 ml ethyl acetate.

Analysis of metabolites by HPLC. The ethyl acetate extracts were evaporated to dryness under argon, redissolved in 0.2 ml methanol–dimethyl sulfoxide (1:1), and analyzed by HPLC as described above. The same-sized aliquots were injected for all reactions to obtain proportional metabolic profiles. In the absence of cofactor, no significant metabolites were observed from BP or the 6-halogenated derivatives.

BP metabolites were identified by elution with authentic standards. 6-FBP dihydrodiols were identified by comparing their spectra to published ones [17]. UV spectra of the 6-FBP phenols indicated that the major elution peak (see Fig. 1B) was predominantly 3-OH-6-FBP, which had maxima at 300, 310, 353, 369, 387, 406 and 430 nm, each red-shifted 5–8 nm with respect to 3-hydroxyBP. The BP 1,6-, 3,6- and 6,12-dione obtained from 6-FBP, as well as 6-ClBP and 6-BrBP below, were identified by comparison of elution time, UV absorbance spectra, and mass spectra with authentic samples.

The metabolism of 6-ClBP is reported here for the first time. The dihydrodiols were identified by their UV spectra, which resembled those of the analogous BP dihydrodiols. However, the maxima were red-shifted 2–8 nm. The peak eluting at 26 min (see Fig. 1C) was identified as 6-ClBP, 4,5-dihydrodiol, with maxima at 265, 274, 315 and 330 nm. 6-ClBP 7,8-dihydrodiol eluted at 33 min, and its spectrum had maxima at 258, 285, 297, 337, 353 and 371 nm. The spectrum of 6-ClBP 9,10-dihydrodiol, at 39 min, exhibited maxima at 273, 280, 303, 334 and 351 nm. The peaks eluting at 45 and 47 min were identified as BP 1,6- and 3,6-dione by comparison of their elution time and UV absorbance with authentic samples. The 67-min peak in the phenol region contained mostly 3-OH-6-ClBP, as evidenced by the resemblance of the UV spectrum, with maxima at 303, 313, 357, 374, 392, 412 and 435 nm, to 3-hydroxyBP, but other phenols appeared to be present.

In the metabolism of 6-BrBP (see Fig. 1D), 6-BrBP 4,5-dihydrodiol eluted at 28 min and 6-BrBP 7,8-dihydrodiol eluted at 35 min and had UV spectra identical to published ones [24]. 6-BrBP 9,10-dihydrodiol eluted at 40 min and its spectrum had maxima at 280, 302, 336 and 352 nm, similar to that of BP 9,10-dihydrodiol but red-shifted 4–9 nm. The two peaks at 47 and 49 min were BP 1,6- and 3,6-dione, respectively, whereas the peak at 68 min contained predominantly 3-OH-6-BrBP, as indicated by its UV spectrum.

Manganic oxidation of BP and 6-FBP. BP or 6-FBP (1 mmol) was dissolved in 20 ml of glacial acetic acid at 40° under argon. $Mn(OAc)_3 2H_2O$ (2 mmol) was added as a solid, and the brown suspension was stirred for 10 min (BP) or 30 min (6-FBP) at 40°. By the end of that time, the mixture had become a clear brown–orange solution. The reaction mixture was cooled to room temperature and poured into an equal volume of 1 M sodium thiosulfate, resulting in a yellow–orange solid. The solid-containing mixture was extracted repeatedly with $CHCl_3$, and the organic layer was washed with sodium bicarbonate until neutrality, then with water, and dried over sodium sulfate. Evaporation of the solvent gave a crude material. The products were purified by column chromatography on silica gel with hexane–benzene (1:1). In the BP reaction, 6-OAcBP was isolated in 90% yield, m.p. 209–210° (m.p. 209.5–210° [25]), and BP 1,6-, 3,6- and 6,12-dione in 5% yield. The structures of these compounds were elucidated by comparison with authentic synthesized samples. In the oxidation of 6-FBP, using similar purification procedures, the major products were 6-OAcBP (31%), a mixture of 1,6- and 3,6-$(OAc)_2$BP (48%), and BP 1,6-, 3,6- and 6,12-dione (19%). Details of the structure elucidations of the diacetoxy derivatives will be described elsewhere.*

Synthesis of BP and 6-FBP radical cation perchlorates. The radical cation perchlorates of BP and 6-FBP were synthesized by a modification of the

* P. Cremonesi, E. L. Cavalieri and E. G. Rogan, manuscript in preparation.

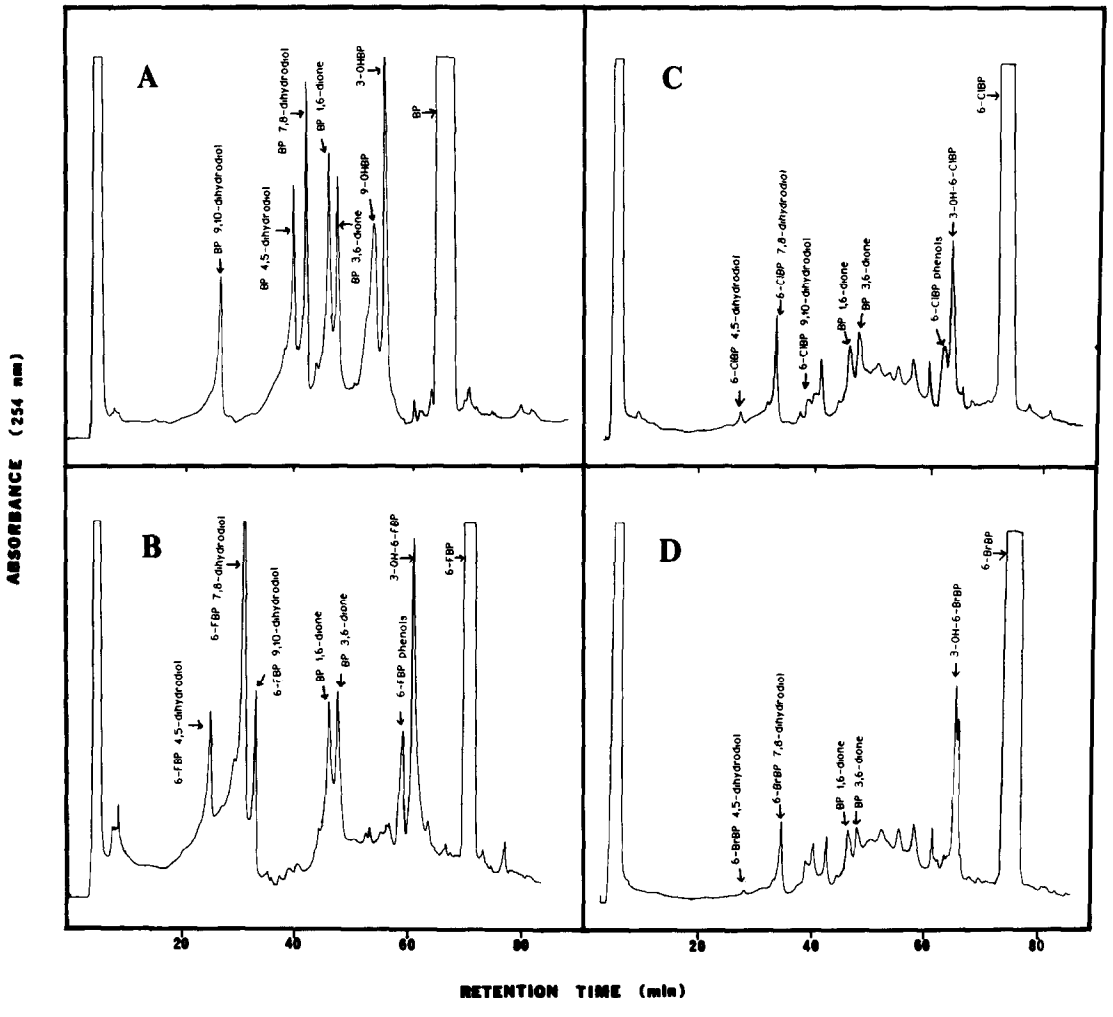

Fig. 1. NADPH-supported metabolism by MC-induced rat liver microsomes. Key: (A) BP; (B) 6-FBP; (C) 6-ClBP; and (D) 6-BrBP.

method of Sato *et al.* [26] and Ristagno and Shine [27]. A solution of BP or 6-FBP (1 mmol) and $AgClO_4$ (1 mmol) in 13.5 ml benzene was oxidized with iodine (0.45 mmol) in 2 ml benzene at room temperature under argon atmosphere. A black precipitate was formed instantaneously. The suspension was poured immediately into two test tubes, flushed with argon, sealed and centrifuged. The supernatant fraction was decanted and replaced with an equal volume of degassed benzene. The tubes were flushed with argon and sealed. The washing process was repeated three to four times until the iodine color disappeared. A final wash was done with hexane. The black solid containing the radical cation adsorbed on AgI was determined iodometrically using platinum and calomel electrodes. The yields of $BP^{+\cdot}ClO_4^-$ and $6\text{-}FBP^{+\cdot}ClO_4^-$ were 35 and 39% respectively. The BP radical cation was characterized further by electron spin resonance [28].

RESULTS

Metabolism by uninduced and MC-induced microsomes. Metabolic studies of 6-halogenated BP derivatives with CHP as cofactor were dictated by the finding that CHP selects one or a few constitutive isozymes of cytochrome P-450 to metabolize BP [29, 30]. Both MC-induced and uninduced rat liver microsomes were used to study metabolism under these conditions. These metabolic profiles were compared to those from NADPH-supported reactions, which are catalyzed by both constitutive and inducible isozymes.

With MC-induced microsomes and NADPH as cofactor, BP (Fig. 1A) and 6-FBP (Fig. 1B) were metabolized in a similar manner, as already described by Buhler *et al.* [17]. The 4,5- and 7,8-dihydrodiol produced in the 6-FBP metabolism eluted before the 9,10-dihydrodiol because these compounds become diaxial with substituents at the 6 position [17]. In both metabolic profiles, BP 1,6- and 3,6-dione were the major quinones, and the phenolic region contained in both cases predominantly 3-hydroxy derivatives. 6-ClBP (Fig. 1C) and 6-BrBP (Fig. 1D) produced relatively small amounts of metabolites, although the same patterns dihydrodiols, quinones and phenols as with BP and 6-FBP.

When uninduced microsomes were used with

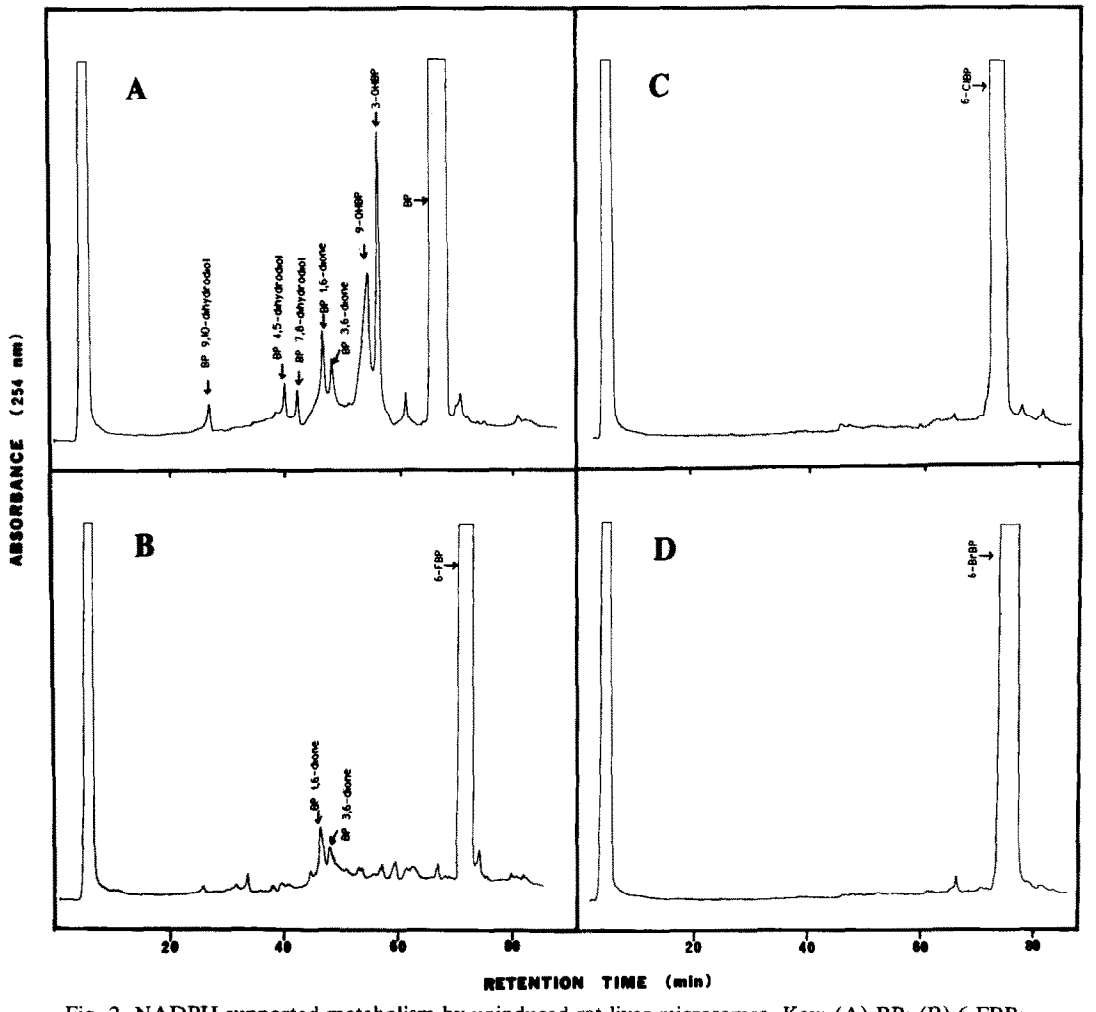

Fig. 2. NADPH-supported metabolism by uninduced rat liver microsomes. Key: (A) BP; (B) 6-FBP; (C) 6-ClBP; and (D) 6-BrBP.

NADPH (Fig. 2), BP yielded a significant amount of metabolites. In contrast 6-FBP produced only a small amount of quinones, and 6-ClBP and 6-BrBP did not afford any metabolites.

In the presence of 2 mM CHP, MC-induced (Fig. 3) and uninduced (Fig. 4) microsomes yielded similar metabolic profiles. With BP and 6-FBP, quinones were the predominant metabolites (Figs. 3A, 3B, 4A and 4B). Much larger amounts of BP 6,12-dione were observed compared to NADPH-supported metabolism, which produced only traces of this metabolite. The relative amount of the 6,12-dione is even greater than it appears in the HPLC profiles because the extinction coefficient of BP 6,12-dione at 254 nm is only half that of the other two diones. 6-ClBP and 6-BrBP again were demonstrated to be poor substrates, because they produce virtually no metabolites (Figs. 3C, 3D, 4C and 4D).

When 0.1 mM CHP, instead of 2 mM CHP, was used to support metabolism by MC-induced microsomes, BP yielded quinones and phenols (Fig. 5A), as previously observed [29, 30], whereas 6-FBP gave almost exclusively quinones (Fig. 5B). As anticipated 6-ClBP and 6-BrBP yielded no metabolites (data not shown).

Generation and nucleophilic trapping of BP and 6-FBP radical cations by manganic acetate in acetic acid. Manganic oxidation of BP yielded predominantly 6-OAcBP and a small amount of BP diones (see Materials and Methods). Completion of this reaction required two equivalents of Mn^{3+}. One-electron oxidation of BP produced a radical cation (Fig. 6) with positive charge appreciably localized at C-6, whereas C-1 and C-3 are the positions of second-highest charge density [31]. Nucleophilic attack of acetate ion occurred selectively at C-6. The resulting radical cation underwent a rapid second one-electron oxidation to an arenonium ion, which by loss of a proton yielded 6-OAcBP. No formation of 1- or 3-OAcBP was ever observed.

The initially-formed $BP^{+\cdot}$ can also be attacked by water, which is present in trace amounts in glacial acetic acid and as water of crystallization in manganic acetate. Reaction of water with $BP^{+\cdot}$ at C-6 represents the first step in the formation of the three BP diones.

Manganic oxidation of 6-FBP yielded 6-OAcBP, 1,6- and 3,6-$(OAc)_2$BP and BP diones (see Materials and Methods). In this case the reaction was complete when 6-FBP consumed one equivalent of $Mn(OAc)_3$.

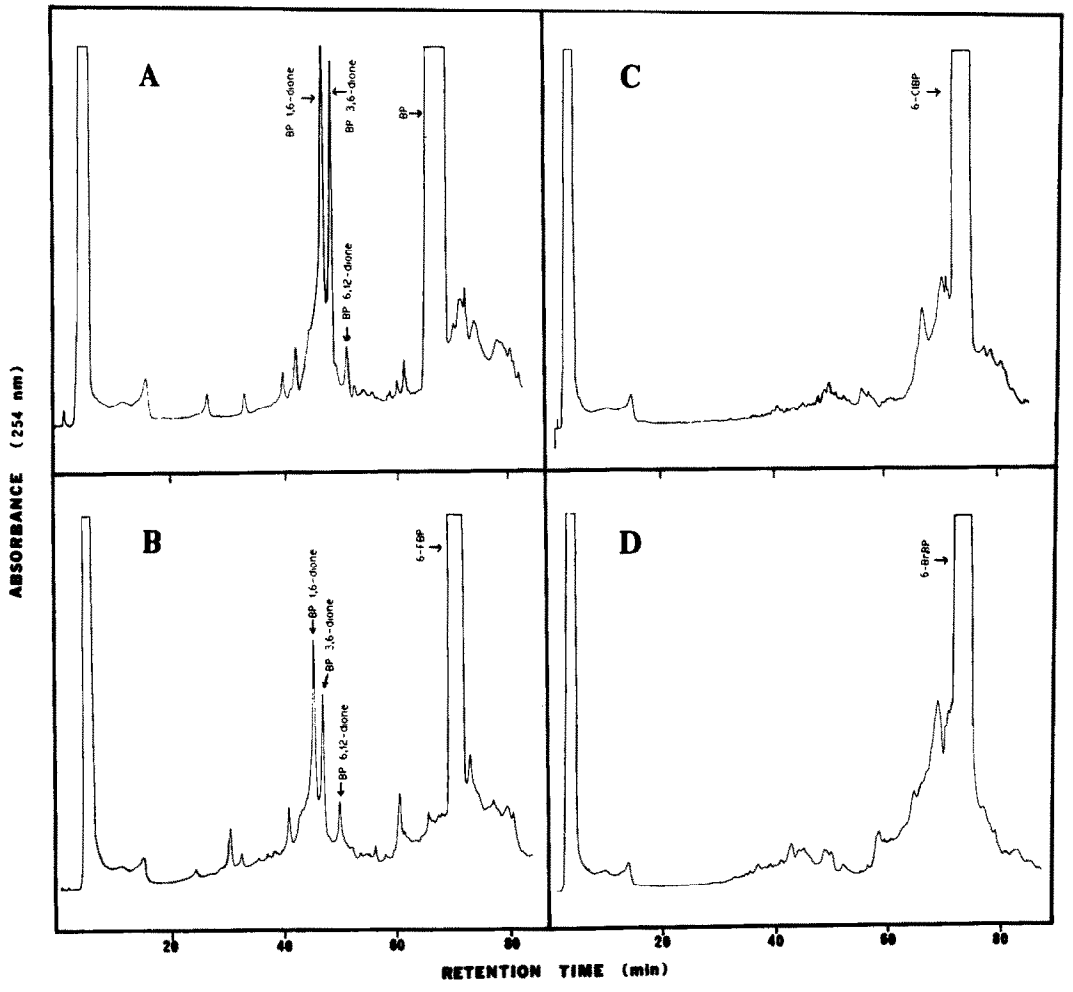

Fig. 3. CHP (2 mM) supported metabolism by MC-induced rat liver microsomes. Key: (A) BP; (B) 6-FBP; (C) 6-ClBP; and (D) 6-BrBP.

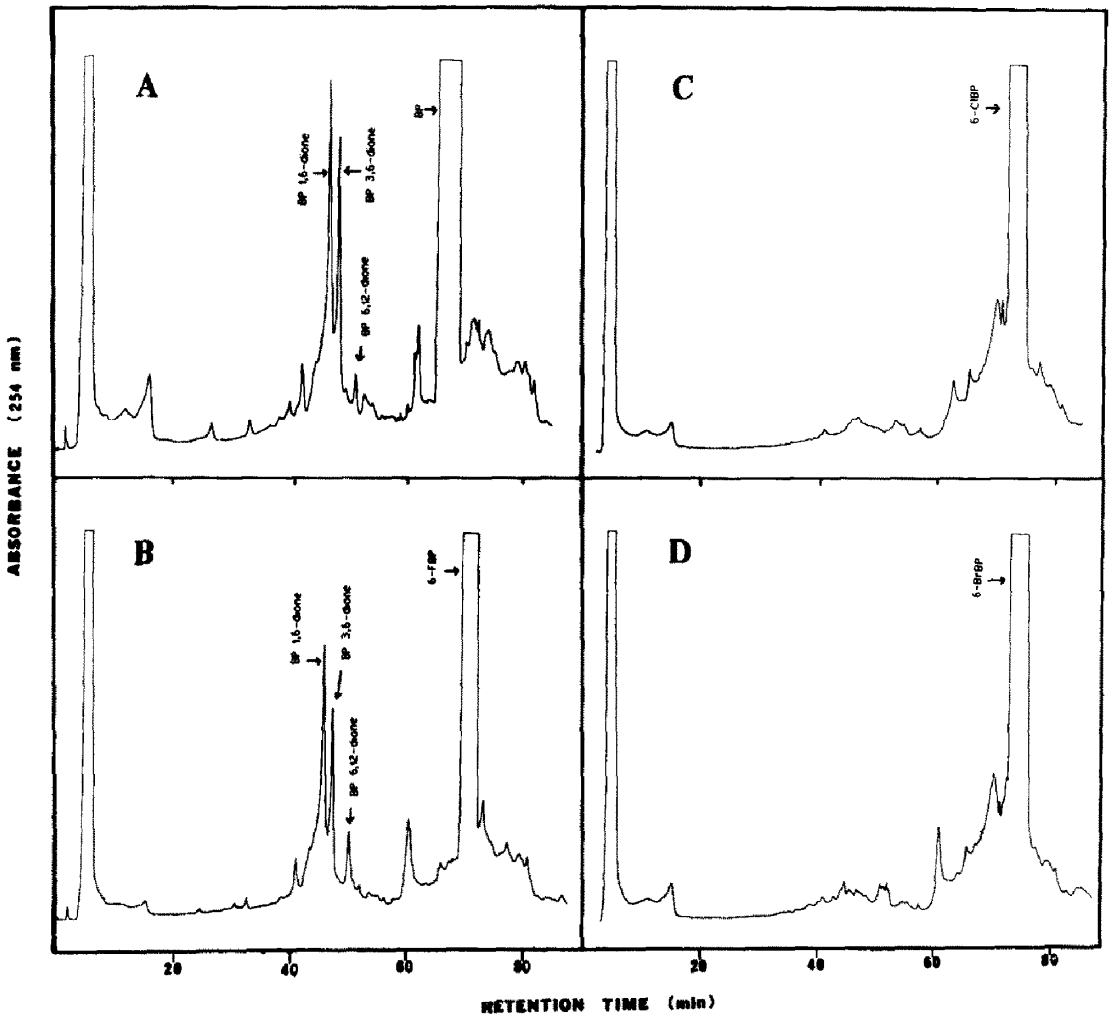

Fig. 4. CHP (2 mM) supported metabolism by uninduced rat liver microsomes. Key: (A) BP; (B) 6-FBP; (C) 6-ClBP; and (D) 6-BrBP.

This led us to suggest that the formation of acetoxy and diacetoxy derivatives occurs by an initial nucleophilic attack of acetate ion at C-6 of 6-FBP$^{+\cdot}$ (Fig. 7), followed by loss of a fluoro ion, to generate a second radical cation which yields 6-OAcBP by a final one-electron reduction by $Mn(OAc)_2$. If nucleophilic attack of a second acetate ion occurred before the final one-electron reduction, 1,6- and 3,6-$(OAc)_2$BP were obtained (not shown in Fig. 7).

The high percentage of BP diones obtained from 6-FBP when compared to the oxidation of BP can be explained in terms of two pathways. The first is analogous to BP (see above) and involves an initial nucleophilic attack of water on the 6-FBP$^{+\cdot}$ at C-6. Loss of HF affords 6-oxyBP, which by three further one-electron oxidations yields 1,6-, 3,6- and 6,12-dione. BP 1,6- and 3,6-dione are also formed very efficiently by hydrolysis of 1,6- and 3,6-$(OAc)_2$P catalyzed by $Mn(OAc)_3$. The mechanistic details of this reaction will be presented elsewhere. Thus, from one-electron oxidation of 6-FBP, it is evident that the most reactive position in 6-FBP$^{+\cdot}$ is C-6.

Reaction of BP and 6-FBP radical cation perchlorates with water. To provide further evidence that C-6 is the most reactive position in BP$^{+\cdot}$ and 6-FBP$^{+\cdot}$ toward nucleophilic substitution, synthesis of the radical cation perchlorates of these two compounds was conducted by reaction of the PAH with iodine and $AgClO_4$ in anhydrous benzene. When 1 mmol of BP$^{+\cdot}ClO_4^-$ or 6-FBP$^{+\cdot}ClO_4^-$ was added to acetonitrile–water (1:9, 7 ml) under argon, the reaction yielded a mixture of BP 1,6- 3,6- and 6,12-dione, in addition to the parent hydrocarbon. The BP diones were obtained in 29% yield from BP$^{+\cdot}ClO_4^-$ and 31% yield from 6-FBP$^{+\cdot}ClO_4^-$. The initial steps in the reaction are shown in Fig. 8 for both compounds. Therefore, displacement of fluorine at C-6 occurs when a radical cation is generated.

In fact, electrophilic substitution of 6-FBP with CF_3COOD in chloroform (volume ratio 1:1) afforded the 1,3-dideuterated derivative of 6-FBP after 24 hr at room temperature, indicating that electrophilic substitution does not occur at C-6, but at C-1 and C-3, which are the positions of highest

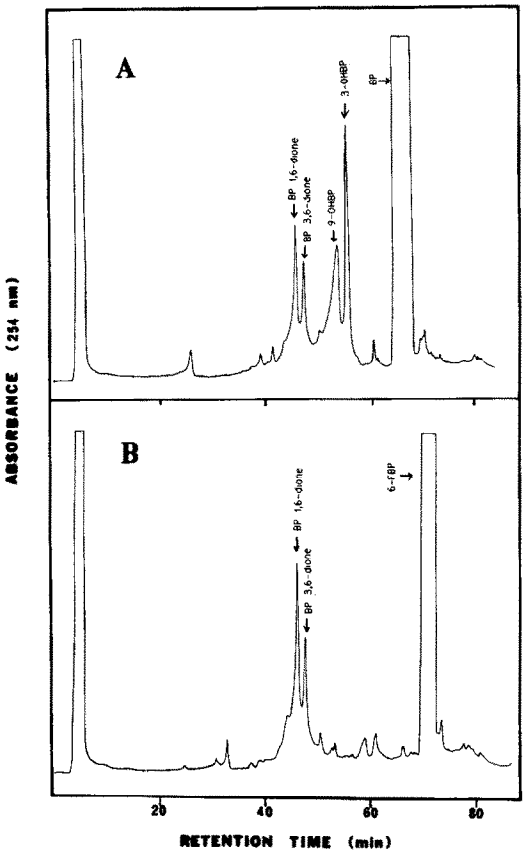

Fig. 5. CHP (0.1 mM) supported metabolism by MC-induced rat liver microsomes. Key: (A) BP; and (B) 6-FBP.

electron density after C-6 [32]. Similarly, bromination of 6-FBP with an equimolar amount of pyridinium bromide perbromide resulted in a mixture of the 1- and 3-monobromo derivatives of 6-FBP. Details of the product identification for both reactions will be presented elsewhere.* From these experimental results it is evident that fluorine can be displaced from the 6 position of BP only by one-electron oxidation.

* P. Cremonesi, E. L. Cavalieri and E. G. Rogan, manuscript in preparation.

DISCUSSION

Although the metabolic profiles of BP and 6-halogenated derivatives (Figs. 1–5) do not give a quantitative response of the various metabolites, because detection was obtained by UV absorption, it is evident that the metabolism of BP was similar to that of 6-FBP with MC-induced rat liver microsomes (Fig. 1, A and B). 6-ClBP and 6-BrBP were also metabolized similarly to BP, but to a significantly smaller extent (Fig. 1, C and D). In the metabolic profile for 6-BrBP, 1,6- and 3,6-dione were present, but these products were not obtained by Fu and Yang using MC-induced immature Sprague–Dawley rats [24]. Using uninduced microsomes in the NADPH-dependent metabolism of BP (Fig. 2A), the same spectrum of metabolites was obtained as with induced microsomes, but the level was lower. With 6-FBP (Fig. 2B), the only significant metabolites were the quinones, whereas 6-ClBP and 6-BrBP did not show any metabolic products (Fig. 2, C and D). The metabolic profile obtained by Buhler *et al.* [17] for 6-FBP resembled their profile for BP, whereas in our study only quinones were obtained from 6-FBP.

In the CHP-dependent metabolism of BP and 6-FBP by induced and uninduced microsomes, the constitutive isozyme(s) of cytochrome P-450 afforded almost exclusively BP quinones as metabolites (Figs. 3A, 3B, 4A and 4B). The only noticeable difference occurred in the metabolism with 0.1 mM CHP (Fig. 5), in which BP produced quinones and phenols [29, 30] and 6-FBP yielded virtually only quinones. With either uninduced or induced microsomes, the constitutive isozyme(s) selected by CHP was unable to catalyze the metabolism of 6-ClBP and 6-BrBP. These metabolic studies with induced and uninduced rat liver microsomes supported by NADPH or CHP reveal a similarity between the metabolism of BP and 6-FBP and between 6-ClBP and 6-BrBP. This similarity is also observed in the radical cation chemistry and the carcinogenic activity of these compounds in mouse skin and rat mammary gland [33].

The fact that the same BP quinones were formed in the metabolism of BP and 6-FBP and, to a much smaller extent, in the NADPH-dependent metabolism of 6-ClBP and 6-BrBP by MC-induced microsomes suggests that these products are formed by an initial attack of a nucleophilic oxygen at C-6 in the 6-FBP$^{+\cdot}$ with displacement of fluorine.

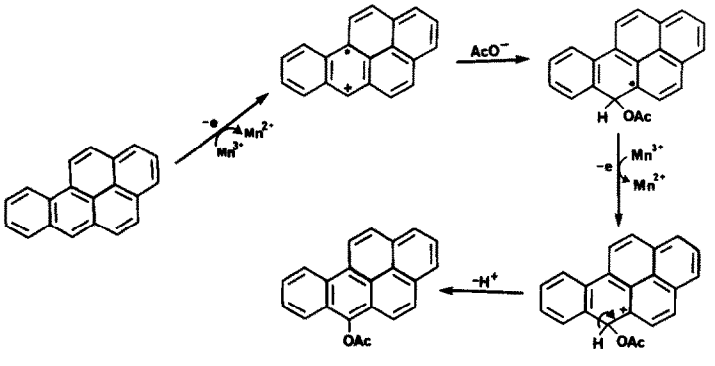

Fig. 6. Mechanism of one-electron oxidation of BP by $Mn(OAc)_3$.

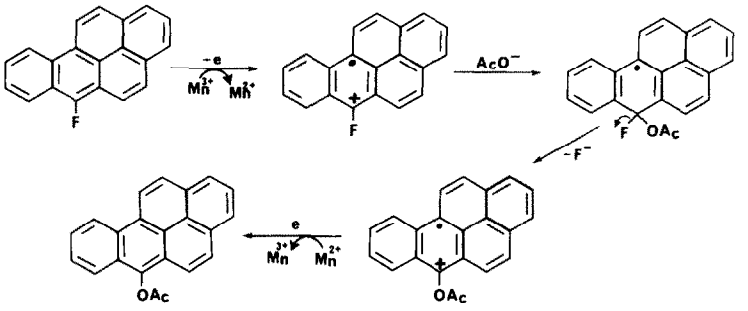

Fig. 7. Mechanism of one-electron oxidation of 6-FBP by $Mn(OAc)_3$.

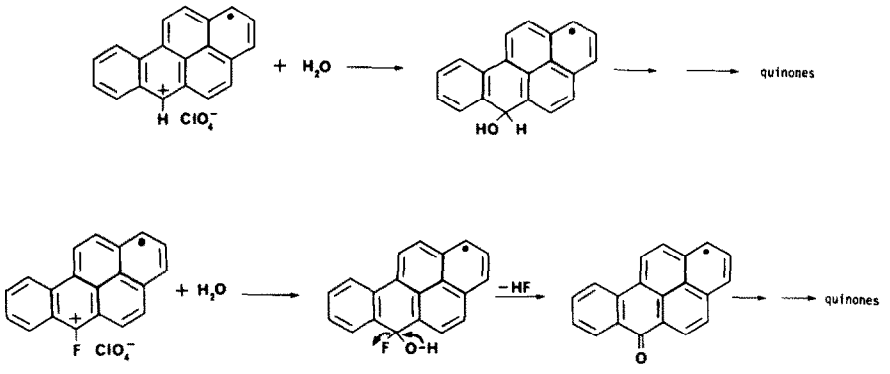

Fig. 8. Reaction of $BP^{+}ClO_4^-$ and $6\text{-}FBP^{+}ClO_4^-$ with water.

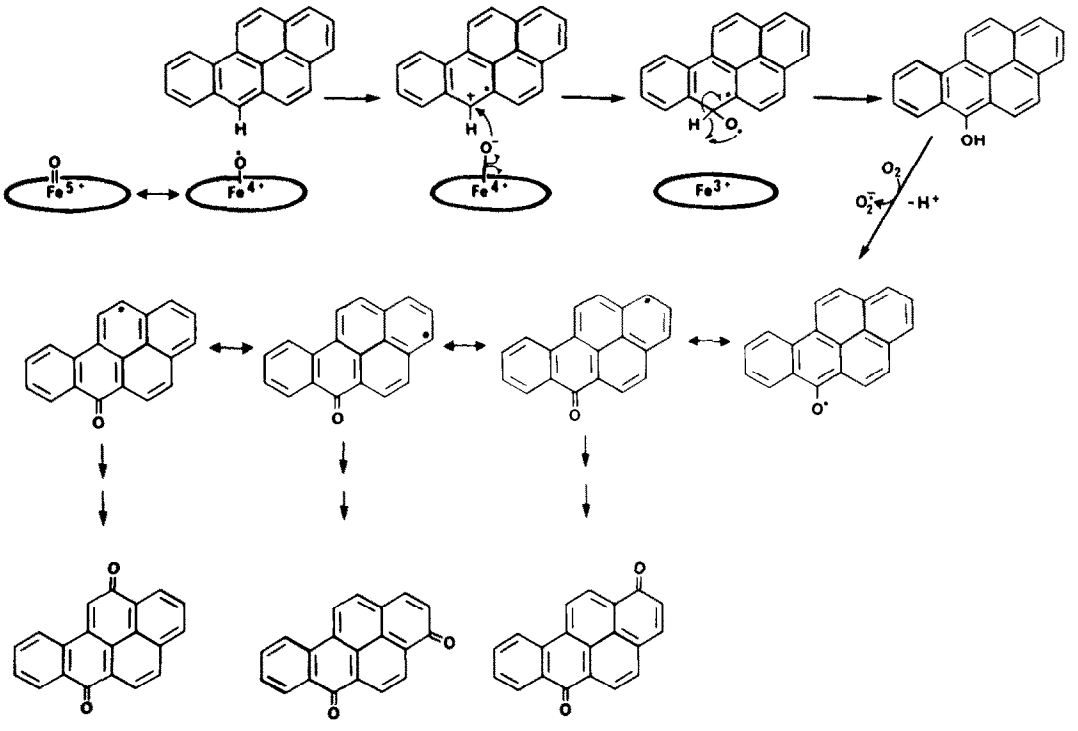

Fig. 9. Proposed mechanism of cytochrome P-450-catalyzed one-electron oxidation of BP to form BP quinones.

Nucleophilic substitution of BP radical cation generated by chemical oxidation proceeds almost exclusively at position 6 [34–42]. In the experiments reported here, one-election oxidation of BP by $Mn(OAc)_3$ proceeded exclusively at C-6 with predominant formation of 6-OAcBP and small amounts of BP quinones. In the one-electron oxidation of 6-FBP by $Mn(OAc)_3$, the major products obtained were 6-OAcBP, a mixture of 1,6- and 3,6-$(OAc)_2$BP and BP quinones. These results indicate that nucelophilic attack in the radical cation occurs exclusively at C-6. Reaction of the synthesized BP and 6-FBP radical cation perchlorates with water produced the same BP quinones. Conversely, electrophilic substitution of 6-FBP with bromine or deuterium ion afforded the C-1 and/or C-3 derivatives with retention of the fluoro substituent at C-6. These results indicate that metabolic formation of quinones from 6-FBP is consistent only with an initial one-electron oxidation to form 6-FBP$^{+\cdot}$.

On the basis of these results, transfer of the activated oxygen by cytochrome P-450 to BP is proposed to occur as shown in Fig. 9. The activated oxygen in NADPH-supported metabolism derives from molecular oxygen, as demonstrated by $^{17}O_2$ incorporation into the 6-oxyBP radical [43]. The initial metabolic step involves an electron transfer from BP to the activated cytochrome P-450–oxygen complex, with iron in a highly oxidized form, namely the perferryl oxygen complex. This generates a formal Fe^{4+} species and a nucleophilic oxygen atom, which would react at C-6 of BP$^{+\cdot}$, where the positive charge is appreciably localized. The dissociation of this complex would leave the iron of cytochrome P-450 in the normal Fe^{3+} state and form 6-hydroxyBP. Autooxidation of 6-hydroxyBP yields 6-oxyBP radical, in which the spin density is mainly localized on oxygen, C-1, C-3 and C-12 [14, 15]. Subsequent one-electron oxidation steps generate the three BP diones.

Metabolic formation of BP quinones from 6-ClBP and 6-BrBP was limited only to NADPH-dependent metabolism with MC-induced microsomes. This presumably indicates that the initial attack of the activated oxygen atom cannot occur due to an increased hindrance created by the bulky Cl or Br substituent. These results are in agreement with the metabolism of 4-Cl- and 4-Br-aniline, in which the metabolic formation of the 4-hydroxy product virtually does not occur, as reported by Daly *et al.* [44]. However, 4-F-aniline yielded 4-hydroxyaniline, similarly to aniline itself. Replacement of fluorine has also been observed in the metabolism of 7-F-2-acetamidofluorene [45], in the enzymatic conversion of 4-F phenylalanine to tyrosine [46], and in conversion of 2-F-estradiol to 2-hydroxyestradiol [47]. The results suggest that these metabolic products are formed by an initial electron transfer to the cytochrome P-450–oxygen complex. Therefore, displacement of fluorine from a position undergoing metabolic oxygenation in an aromatic compound provides a powerful tool for demonstrating that the metabolic product arises via an initial one-electron oxidation of the substrate.

Recent experimental results concerning the BP radical cation indicate that the positions with the highest charge density are 6, 1, 3, 9 and 12 in decreasing order [31]. Positions 1 and 12 are virtually not involved in the metabolism of BP by cytochrome P-450 [48, 49], because of presumable restrictions imposed by the binding site of the enzyme [48]. Thus, it is plausible to postulate that the formation of 3- and 9-hydroxyBP can occur via an initial one-electron oxidation of BP, similarly to the first step in the formation of BP quinones.

Acknowledgements—This research was supported by U.S. Public Health Service Grants 1RO1 CA32376, CA25176 and CA36727 from the National Cancer Institute.

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