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RADICAL CATIONS IN THE HORSERADISH PEROXIDASE AND PROSTAGLANDIN H SYNTHASE MEDIATED METABOLISM AND BINDING OF BENZO[*a*]PYRENE TO DEOXYRIBONUCLEIC ACID*

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Abstract—Metabolism and DNA binding studies are used to investigate mechanisms of activation for carcinogens. In this paper we describe metabolism of benzo[*a*]pyrene (BP) and 6-fluorobenzo[*a*]pyrene (6-FBP) by two peroxidases, horseradish peroxidase (HRP) and prostaglandin H synthase (PHS), which are known to catalyze one-electron oxidation. In addition, binding of BP and BP quinones to DNA was compared in the two enzyme systems. The only metabolites formed from BP or 6-FBP by either enzyme were the quinones, BP 1,6-, 3,6- and 6,12-dione. HRP metabolized BP and 6-FBP to the same extent and produced the same proportion of each dione from both compounds, approximately 40% each of BP 1,6- and 3,6-dione and 20% BP 6,12-dione. PHS formed twice as much quinones from BP as from 6-FBP and produced relatively more BP 3,6-dione from 6-FBP (46%) compared to BP (30%) and relatively less BP 6,12-dione from 6-FBP (16%) compared to BP (33%). Removal of the fluoro substituent in the metabolism of 6-FBP is consistent only with an initial one-electron oxidation of the substrate. Since BP quinones were the only products formed in HRP- and PHS-catalyzed activation of BP, their possible binding to DNA was compared to that of BP. No significant binding of BP quinones to DNA occurred with either HRP or PHS. These results, coupled with those from other chemical and biochemical experiments, demonstrate that HRP- and PHS-catalyzed one-electron oxidation of BP to its radical cation is the mechanism of formation of quinones and binding of BP to DNA.

Benzo[*a*]pyrene (BP‡) metabolism catalyzed by cytochrome P-450 produces three classes of compounds, namely dihydrodiols, phenols and the quinones, BP 1,6-, 3,6- and 6,12-dione. It has been demonstrated that BP quinones are formed via an initial one-electron oxidation of BP to yield its radical cation [1]. The strongest evidence to prove this pathway of activation derives from the metabolism of 6-fluorobenzo[*a*]pyrene (6-FBP), which produces the same quinones as BP, with removal of the fluoro substituent [1, 2]. This can occur only by nucleophilic substitution after formation of the 6-FBP radical cation [1]. Hence, removal of fluorine from an aromatic substrate can be used as a probe for one-electron oxidation in the formation of certain metabolites.

We have extended the metabolism of BP and 6-FBP to the horseradish peroxidase (HRP) and prostaglandin H synthase (PHS) systems, since PHS has been shown to metabolize BP exclusively to BP quinones [3]. These enzymes catalyze one-electron oxidation of a variety of compounds [4–10]. Comparison of the results obtained with BP and 6-FBP allows us to determine whether these metabolites are formed by an initial one-electron oxidation of the substrate. In addition, binding of BP to DNA catalyzed by HRP and PHS has been investigated to gain more insight into the mechanism of activation involved.

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‡ Abbreviations: BP, benzo[*a*]pyrene; 6-FBP, 6-fluorobenzo[*a*]pyrene; PAH, polycyclic aromatic hydrocarbons; HRP, horseradish peroxidase; and PHS, prostaglandin H synthase.

MATERIALS AND METHODS

Materials. [^{14}C]BP (sp. act. 52 mCi/mmol) was purchased from the Amersham Corp. (Arlington Heights, IL), mixed with unlabeled BP to achieve a specific activity of 19 mCi/mmol, and used in all experiments. PHS purified from ram seminal vesicles was purchased from Oxford Biomedical Research, Inc. (Oxford, MI). Arachidonic acid (sodium salt), methemoglobin, HRP (type II) and H_2O_2 were obtained from the Sigma Chemical Co. (St. Louis, MO), and calf thymus DNA was from P-L Biochemicals (Milwaukee, WI). [^{14}C]BP quinones were prepared metabolically using 3-methylcholanthrene-induced rat liver microsomes and purified by HPLC, using the procedures described previously [11]. The mixture of quinones contained mainly BP 1,6- and 3,6-dione with a little BP 6,12-dione.

HRP-catalyzed metabolism. Reaction mixtures (1 ml) containing 0.067 M sodium–potassium phos-

phate, pH 7.0, 120 nmol [^{14}C]BP or 6-FBP and 250 μg HRP were preincubated for 3 min at 37°. The reactions were initiated by addition of 0.5 mM H_2O_2, and the incubations were continued for 30 min. Control reactions omitted HRP or H_2O_2. The reactions were terminated by addition of 1 ml acetone, and the metabolites were extracted twice with 2 ml ethyl acetate.

PHS-catalyzed metabolism. Reaction mixtures (1 ml) containing 0.08 M potassium phosphate, pH 7.8, 2.2 μM methemoglobin, 120 nmol [^{14}C]BP or 6-FBP and 15 μg (1000 units) PHS were preincubated for 3 min at 37°. The reaction was initiated by the addition of 1.8 mM arachidonic acid, and the incubation was continued for 30 min. The reaction was terminated by addition of 1 ml acetone, and the metabolites were extracted twice with 2 ml ethyl acetate. Methemoglobin and arachidonic acid were omitted in the control reactions.

Analysis of metabolites by HPLC. Ethyl acetate extracts were evaporated to dryness under argon and metabolites were redissolved in 0.2 ml methanol–dimethyl sulfoxide (1:1). Samples were analyzed by HPLC using a Spectra Physics 8700 system and an Altex Ultrasphere 5 μm ODS 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 UV absorbance at 254 nm (Kratos Spectroflow monitor) and for radioactivity in the case of [^{14}C]BP with a continuous flow system using a RAMONA radiometric detector (IN/US, Fairfield, NJ) with a 2-ml liquid cell. Radioactivity data were processed with an automatic data integration system. BP quinones were identified by comparison with authentic samples. The quinones were quantitated by correlating the UV absorbance tracings with radioactivity data for BP.

Binding of BP and BP quinones to DNA. Reaction mixtures (1 ml) for the HRP/H_2O_2-catalyzed binding of BP or BP quinones contained 2.3 mM calf thymus DNA, 100 μM [^{14}C]BP or 10 μM [^{14}C]BP quinones, 0.5 mM H_2O_2 and 100 μg HRP in 0.067 M sodium–potassium phosphate, pH 7.0. The mixtures were incubated for 30 min at 37°. Control experiments were carried out in the absence of H_2O_2. Reaction mixtures (1 ml) for the PHS/arachidonic acid-catalyzed reaction contained 2.3 mM calf thymus DNA, 2.2 μM methemoglobin, 100 μM [^{14}C]BP or 10 μM [^{14}C]BP quinones, 3 mM arachidonic acid, and 200 units of PHS in 0.075 M potassium phosphate, pH 7.8. The reactions were initiated by addition of arachidonic acid and were carried out for 30 min at 37°. DNA was purified to constant specific radioactivity as previously described [12]. In both systems 1 to 1.5 mmol DNA was recovered and analyzed.

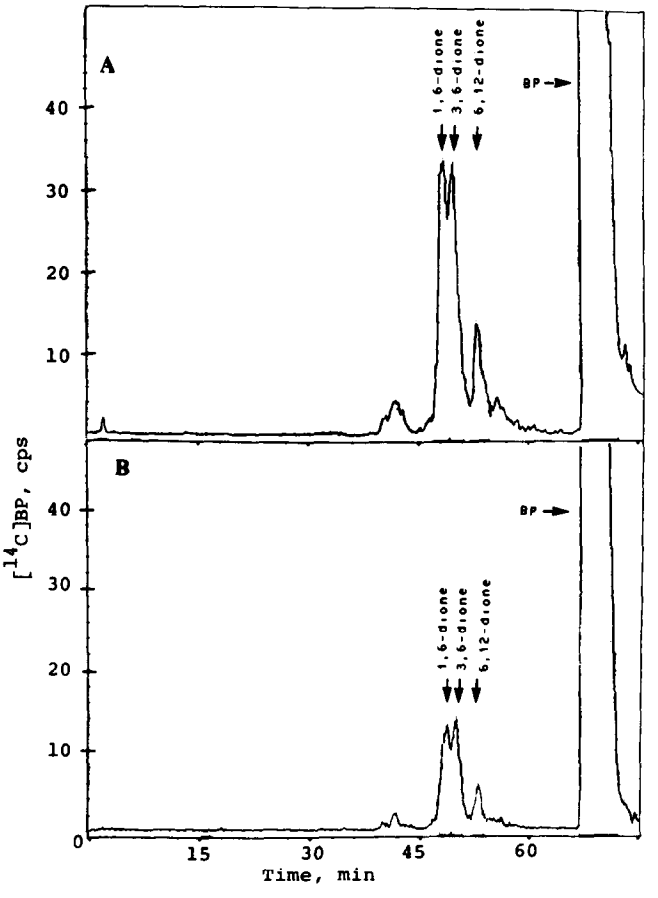

Fig. 1. Profile of BP metabolites formed in HRP-catalyzed reactions. Key: (A) complete reaction mixture; and (B) control reaction mixture without enzyme.

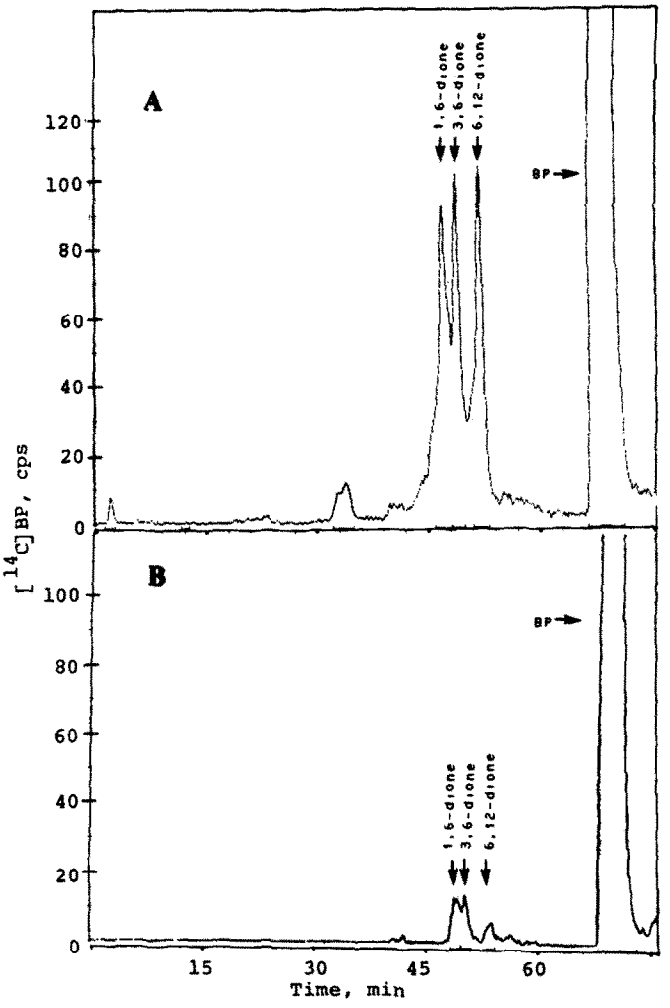

Fig. 2. Profile of BP metabolites formed in PHS-catalyzed reactions. Key: (A) complete reaction mixture; and (B) control reaction mixture without methemoglobin and arachidonic acid.

RESULTS

Metabolism of BP and 6-FBP. Metabolism of BP and 6-FBP catalyzed by HRP/H_2O_2 and PHS/arachidonic acid produced exclusively a mixture of BP 1,6-, 3,6- and 6,12-dione (Figs. 1 and 2). This confirms the results obtained for BP with PHS by Marnett and Reed [3]. The relative amounts of the three quinones obtained from BP and 6-FBP metabolism in HRP/H_2O_2-catalyzed reactions are shown in Table 1. For both compounds, the total amount of BP quinones was similar, and the distribution of

Table 1. HRP-catalyzed metabolism

Substrate	Amount of quinones formed (nmol)			Total metabolites (nmol)
	1,6-dione	3,6-dione	6,12-dione	
BP				
Complete system	0.420 (43)*	0.372 (38)	0.181 (19)	0.973
Minus H_2O_2	0.204 (45)	0.180 (39)	0.072 (16)	0.456
Minus HRP	0.168 (45)	0.144 (39)	0.060 (16)	0.372
6-FBP				
Complete system	0.396 (41)	0.396 (41)	0.182 (19)	0.974
Minus H_2O_2	0.276 (45)	0.235 (39)	0.096 (16)	0.607
Minus HRP	0.270 (43)	0.252 (40)	0.106 (17)	0.628

* Numbers in parentheses indicate percentage of total metabolites.

Table 2. PHS-catalyzed metabolism

Substrate	Amount of quinones formed (nmol)			Total metabolites (nmol)
	1,6-dione	3,6-dione	6,12-dione	
BP				
Complete system	1.464 (37)*	1.188 (30)	1.284 (33)	3.936
Minus methemoglobin and arachidonic acid	0.192 (44)	0.156 (36)	0.084 (19)	0.432
6-FBP				
Complete system	0.684 (38)	0.828 (46)	0.300 (16)	1.812
Minus methemoglobin and arachidonic acid	0.208 (46)	0.168 (38)	0.072 (16)	0.448

* Numbers in parentheses indicate percentage of total metabolites.

Table 3. Binding of BP and BP quinones to DNA

Substrate	Binding to DNA (μmol/mol DNA-P)
HRP/H_2O_2	
[^{14}C]BP	
Complete system	80
Minus H_2O_2	4.0
[^{14}C]BP quinones	
Complete system	3.5
Minus H_2O_2	2.9
PHS/arachidonic acid	
[^{14}C]BP	
Complete system	290
Minus methemoglobin and arachidonic acid	6
[^{14}C]BP quinones	
Complete system	12
Minus methemoglobin and arachidonic acid	6

the three quinones was about the same. Although the amount of metabolites was low, the results were reproducible.

In the PHS/arachidonic acid-catalyzed metabolism of BP and 6-FBP (Table 2), a larger amount of quinones was produced from BP than from 6-FBP. BP afforded relatively more 6,12-dione (33%) and less 3,6-dione (30%) compared to the profile obtained with 6-FBP (16 and 46% respectively).

Binding of BP and BP quinones to DNA. The binding of [^{14}C]BP to DNA was compared to that of [^{14}C]BP quinones in the HRP/H_2O_2 and PHS/arachidonic acid systems (Table 3). The binding of BP to DNA in both systems corresponded to 5–10% of the level of metabolites formed and was similar to previously obtained values [13, 14]. In contrast, the binding of BP quinones, present at a concentration several-fold above that produced from BP by the enzymes, was minimal in both systems. Thus, the binding of BP to DNA observed with HRP and PHS cannot arise from BP quinones and must arise from their metabolic precursor.

DISCUSSION

The results presented in this paper (Tables 1 and 2) demonstrate that both HRP and PHS produced BP quinones from both BP and 6-FBP. Formation of BP quinones not only in the metabolism of BP but also in that of 6-FBP indicates that these metabolites arise via an initial one-electron oxidation of the substrate to its radical cation [1]. These results are analogous to those obtained in the metabolism of BP and 6-FBP by cytochrome P-450 [1, 2]. Since BP quinones did not mediate the binding of BP to DNA (Table 3), a reactive precursor radical cation must be involved. In fact, in the HRP-catalyzed binding to DNA, BP is linked at C-6 to the C-8 and N-7 positions of guanine [15]. These adducts arise by enzymic activation of BP to its radical cation, which has the positive charge localized at C-6. Thus, one-electron oxidation of BP catalyzed by HRP and PHS is the mechanism of formation of quinones, the only metabolites, and binding of BP to DNA.

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