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Role of Radical Cations in Aromatic Hydrocarbon Carcinogenesis

by Ercole Cavalieri* and Eleanor Rogan*

Carcinogenic activation of polycyclic aromatic hydrocarbons (PAH) involves two main pathways: one-electron oxidation and monooxygenation. One-electron oxidation produces PAH radical cations, which can react with cellular nucleophiles. Results from biochemical and biological experiments indicate that only PAH with ionization potentials below ca. 7.35 eV can be metabolically activated by one-electron oxidation. In addition, the radical cations of carcinogenic PAH must have relatively high charge localization to react effectively with macromolecules in target cells. Metabolic formation of PAH quinones proceeds through radical cation intermediates. Binding of benzo[a]pyrene (BP) to mouse skin DNA occurs predominantly at C-6, the position of highest charge localization in the BP radical cation, and binding of 6-methylBP to DNA in mouse skin yields a major adduct with the 6-methyl group bound to the 2-amino group of deoxyguanosine. Studies of carcinogenicity by direct application of PAH to rat mammary gland indicate that only PAH with ionization potentials low enough for activation by one-electron oxidation produce tumors in this target tissue. These constitute some of the results which provide evidence for the involvement of one-electron oxidation in PAH carcinogenesis.

Introduction

One concept that is basic to studies of chemical carcinogenesis is the recognition that covalent binding of chemicals to cellular macromolecules, DNA, RNA, and protein, is the first critical step in the multistage process leading to tumor formation (1,2). Most chemical carcinogens, with the exception of a few alkylating or acylating agents, require some type of metabolic activation to produce the reactive species capable of covalently binding to cellular macromolecules. These critical reactive intermediates belonging to the broad variety of different structures known as chemical carcinogens have a common unifying feature, namely their electrophilic character (*1,2*).

Metabolic activation of polycyclic aromatic hydrocarbons (PAH), as well as other chemical carcinogens, occurs by two main pathways: one-electron oxidation and two-electron oxidation, or monooxygenation (*3,4*). One-electron oxidation produces radical cations or radicals, depending on the molecule in which the oxidation occurs. A radical cation is generated by removal of a π-electron in an aromatic system, whereas one-n-electron oxidation of a phenol or amine with subsequent loss of a proton produces a radical. Two-electron oxidation, or monooxygenation, yields oxygenated metabolites. Thus the general pathways of activation and deactivation for chemical carcinogens can be summarized as presented in Figure 1.

The procarcinogen, a chemical compound requiring metabolic activation, can be oxidized by loss of an electron to produce an ultimate electrophilic intermediate. This would react with critical cellular macromolecules to initiate the process of carcinogenesis. Oxygenation of a procarcinogen can produce directly an ultimate carcinogenic metabolite or a proximate carcinogenic metabolite which requires further activation by one-electron oxidation, monooxygenation, or esterification to form the ultimate carcinogenic species. The electrophilic species produced can react with nucleophilic groups of cellular macromolecules to initiate the cancer process. Ultimate electrophilic carcinogens can more commonly bind noncritically to cellular macromolecules or sometimes decompose before reacting. The fate of a procarcinogen or proximate carcinogen also includes formation of inactive metabolites.

Thus the activating process of procarcinogens, including PAH, can occur through two main pathways, one-electron oxidation and two-electron oxidation. Study of the critical electrophiles obtained in the two pathways of activation provides information about the enzymes that catalyze these reactions.

*Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 42nd and Dewey Avenue, Omaha, NE 68105.

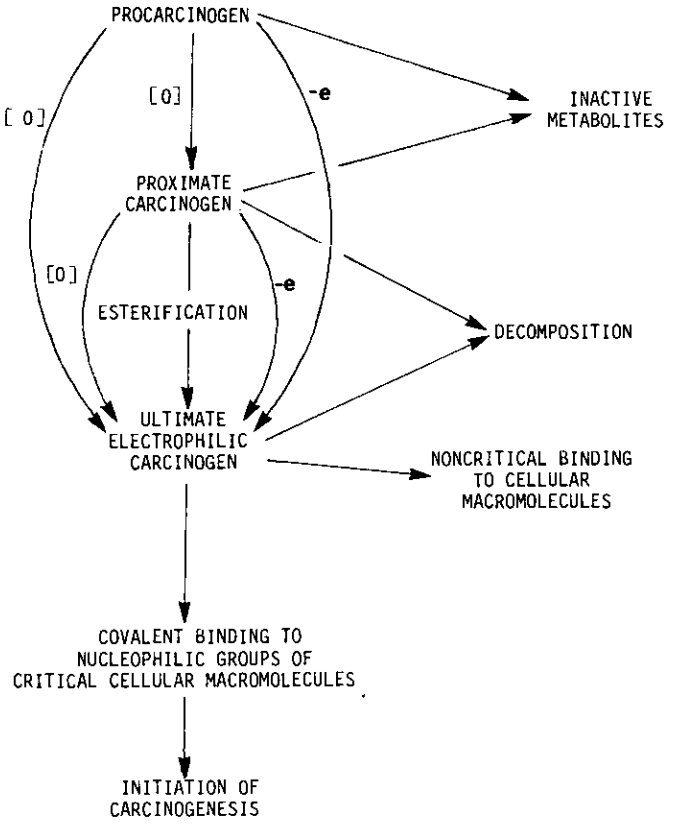


FIGURE 1. One-electron oxidation and monooxygenation in the metabolic activation of procarcinogens.

Enzymology of One-Electron and Two-Electron Oxidation

Most research on the enzymatic activation of chemical carcinogens has focused on monooxygenation by cytochrome P-450 with molecular oxygen and NADPH (*2,5,6*). This enzyme can also catalyze formation of oxygenated metabolites with hydroperoxide cofactors (*6*). Recently, activation catalyzed by cellular peroxidases, including the enzyme complex prostaglandin H synthase (PHS) (*7*), and cytochrome P-450 with NADPH (*8–13*) and hydroperoxide cofactors (*14–19*) has been investigated and observed to provide one-electron oxidation of a variety of xenobiotics, including carcinogens. Cytochrome P-450 acting as a monooxygenase with NADPH and oxygen does not in general catalyze one-electron oxidation efficiently. However, in this system, dihydropyridine (*10*) and cyclopropylamine (*8,9*) induce suicidal inactivation of cytochrome P-450 via an initial one-electron oxidation of the substrate. Similarly, sulfides and sulfoxides are oxygenated to sulfoxides and sulfones, respectively, via initial formation of a sulfinium radical intermediate (*11,12*). Cytochrome P-450 with NADPH also catalyzes one-electron oxidation of norcocaine, which plays a significant role in the hepatotoxicity of cocaine (*13*). The one-electron oxidation pathway of cytochrome P-450 is more efficiently catalyzed in the presence of hydroperoxide cofactors. This is best illustrated by the preponderant formation of benzo[a]pyrene (BP) quinones in the metabolism of BP (*18,19*). In fact we have recently demonstrated that formation of metabolites proceeds by an initial one-electron oxidation of the substrate.

Mammalian peroxidases have been observed to activate a variety of chemicals by one-electron oxidation. Mouse uterine peroxidase activates diethylstilbestrol (*20*), and rat bone marrow peroxidase activates phenol (*21*). PHS has been implicated in the activation of *N*-hydroxy-2-acetylaminofluorene in mammary cells (*22*), benzidine and 5-nitrofuran (*23–26*), diethylstilbestrol (*27–29*), tetramethylhydrazine (*30*), 2-aminofluorene (*31*), and *p*-aminophenol (*32*).

For PAH, the two main types of ultimate carcinogenic intermediates, radical cations (*3,4*) and bay-region vicinal diol-epoxides (*5,33,34*), are formed by one-electron oxidation and two-electron oxidation, respectively. In this paper we will review the chemical, biochemical, and biological evidence indicating that radical cations play an important role in PAH carcinogenesis.

Chemical Properties of PAH Radical Cations

Radical cations are reactive intermediates obtained by removal of an electron from PAH. A few of the most common representative PAH are presented in Figure 2. It is well known that PAH radical cations can be produced in chemical systems with Fe^{3+} (*35–38*) and iodine (*35,37,39–42*). Iron-containing enzymes with the metal in the higher oxidative forms (Fe^{3+} to Fe^{5+}) are possible oxidants in biological systems. To understand the role of PAH radical cations in the mechanism of tumor initiation, we have investigated some chemical properties of these intermediates.

Trapping of Radical Cations by Nucleophiles

Radical cations have been generated in two one-electron oxidant systems: the first contains iodine as oxidant and pyridine as nucleophile and solvent (*42,43*), while the second is $Mn(OAc)_3$ in acetic acid (*44*).

$$PAH + I_2 \rightleftarrows PAH^{\cdot+} + I^- + I^{\cdot} \quad (1)$$

$$PAH + Mn(OAc)_3 \rightleftarrows PAH^{\cdot+} + Mn(OAc)_2 + {}^-OAc \quad (2)$$

In equation (1) the radical cation is trapped by pyridine to form the pyridinium iodide derivative, whereas in the second system the acetoxy derivative of the PAH is obtained. Reaction yields of nucleophilic substitution in the iodine-pyridine system for several PAH are presented in Table 1, and the ionization potentials (IP) are also reported. The compounds 5-methylchrysene and dibenz[a,h]anthracene are not oxidized because of their

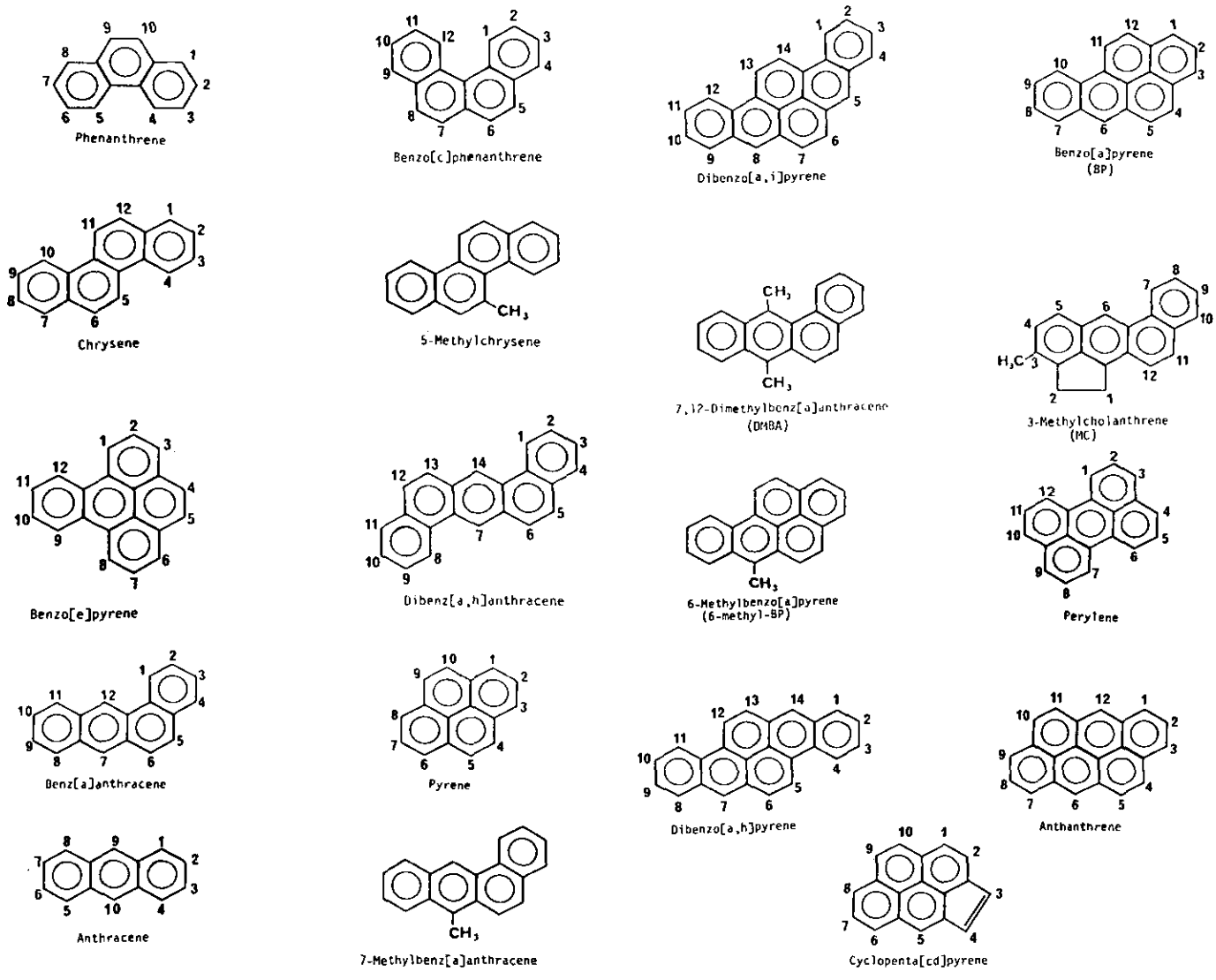


Figure 2. Structures of representative PAH.

relatively high IP. The unsubstituted PAH, benz[a]anthracene, anthracene, BP, and anthanthrene react specifically at the position of highest charge density in their radical cations. The same occurs for the 2-, 5-, 11-, and 12-monomethyl derivatives of benz[a]anthracene. When, however, some steric hindrance exists at C-7, the position of highest charge density, as in the case of 6- and 8- methylbenz[a]anthracene, the reaction takes place competitively at C-12, the position of second-highest charge density. In 7-methylbenz[a]anthracene, reaction occurs at the 7-methyl group, as well as C-12, whereas for 6-methylBP the only isolated product is the 6-methylBP pyridinium salt. In 7,12-dimethylbenz[a]anthracene, the competitive positions of substitution are C-5, as well as the 7- and 12-methyl groups. In 7-ethylbenz[a]anthracene nucleophilic substitution occurs specifically at C-12, whereas for 3-methylcholanthrene (3-MC) the substitution occurs at the 1-methylene group.

In the $Mn(OAc)_3$–acetic acid system the weak nucleophile, acetate ion, should be more selective toward the position of highest charge localization in the radical cation. As shown in Table 2, compounds with relatively high IP, such as phenanthrene and chrysene, are not oxidized by Mn^{3+}. For PAH with lower IP, the acetate ion attack occurs specifically at the position of highest charge density on the aromatic ring (compounds III–IX, XII–XV) and/or at the methyl group blocking the position of highest charge density (compounds VIII, X–XII). Perylene and anthanthrene form monoacetoxy and diacetoxy derivatives and perylene also forms triacetoxy derivatives. The formation of diacetoxyanthanthrene indicates that the 6-acetoxyanthanthrene presumably formed first competes for one-electron oxidation with anthanthrene. In the case of perylene the larger amount of diacetoxy compared to monoacetoxyperylene and the formation of triacetoxyperylene are unexplained and suggest that mechanisms other

Table 1. One-electron oxidation of PAH by the iodine-pyridine system.[a]

Compound	Position of pyridine substitution	Yield, %[b]	Ionization potential, eV[c]
5-Methylchrysene	No reaction	0	ca. 7.7
Dibenz[a,h]anthracene	No reaction	0	7.57
Benz[a]anthracene	7	54	7.54
6-Methylbenz[a]anthracene	7	48	7.50
	12	14	
11-Methylbenz[a]anthracene	7	82	7.48
2-Methylbenz[a]anthracene	7	83	7.46
5-Methylbenz[a]anthracene	7	85	7.46
8-Methylbenz[a]anthracene	7	58	7.46
	12	14	
Anthracene	9	60	7.43
7-Ethylbenz[a]anthracene	12	68	7.39
12-Methylbenz[a]anthracene	7	78	7.38
7-Methylbenz[a]anthracene	7-CH_3	32	7.37
	12	11	
Benzo[a]pyrene	6	58	7.23
7,12-Dimethylbenz[a]anthracene	5	58	7.22
	7-CH_3	18	
	12-CH_3	15	
3-Methylcholanthrene	1	96	7.12
6-Methylbenzo[a]pyrene	6-CH_3	74	7.08
Anthanthrene	6	20	6.96

[a] Reaction at 30–35°C for 20 hr.
[b] The remainder is starting material and/or undetected minor products.
[c] Determined from maximum absorption of the charge-transfer complex of each compound with chloranil (*45*) with the exception of dibenz[a,h]anthracene, determined by polarographic oxidation (*46*).

Table 2. One-electron oxidation of PAH by the manganic acetate–acetic acid system.[a]

No.	Compound	Time	Position of acetoxy substitution	Yield, %	Starting material, %	Ionization potential, eV[b]
I	Phenanthrene	96 hr	No reaction	0	100	8.19
II	Chrysene	96 hr	No reaction	0	100	ca. 7.8
III	5-Methylchrysene	96 hr	6	28	72	ca. 7.7
IV	Benzo[e]pyrene	96 hr	1	14	66	7.62
V	Benz[a]anthracene	48 hr	7	90–100	Traces	7.54
VI	Pyrene	96 hr	1	60	24	7.50
			1,6	16		
VII	Anthracene[c]	66 hr	9,10-Dihydro-9,10-Diacetoxy	100		7.43
VIII	7-Methylbenz[a]anthracene	24 hr	7-CH_3	85	Traces	7.37
			12	10		
IX	Benzo[a]pyrene	<10 min	6	95	Traces	7.23
			quinones	5		
X	7,12-Dimethylbenz-[a]anthracene	<10 min	7-CH_3	50–60	Traces	7.22
			12-CH_3	40–50		
XI	3-Methylcholanthrene	<10 min	6-CH_3	75–80	Traces	7.12
XII	6-Methylbenzo[a]pyrene	10 min	6-CH_3	75–80	Traces	7.08
			1	15–20		
			3	15–20		
XIII	Perylene[d]	<10 min	1	22	30	7.06
			1,7-Triacetoxy	26		
				11		
XIV	Anthanthrene[d]	<10 min	6	41	26	6.96
			6,12	12		
XV	6-Methylanthanthrene[d]	5 min	12	51	37	6.85

[a] Reaction at 40°C unless otherwise specified.
[b] Determined from maximum absorption of the charge-transfer complex of each compound with chloranil (*45*).
[c] Reaction at 22°C.
[d] Reaction at 55°C.

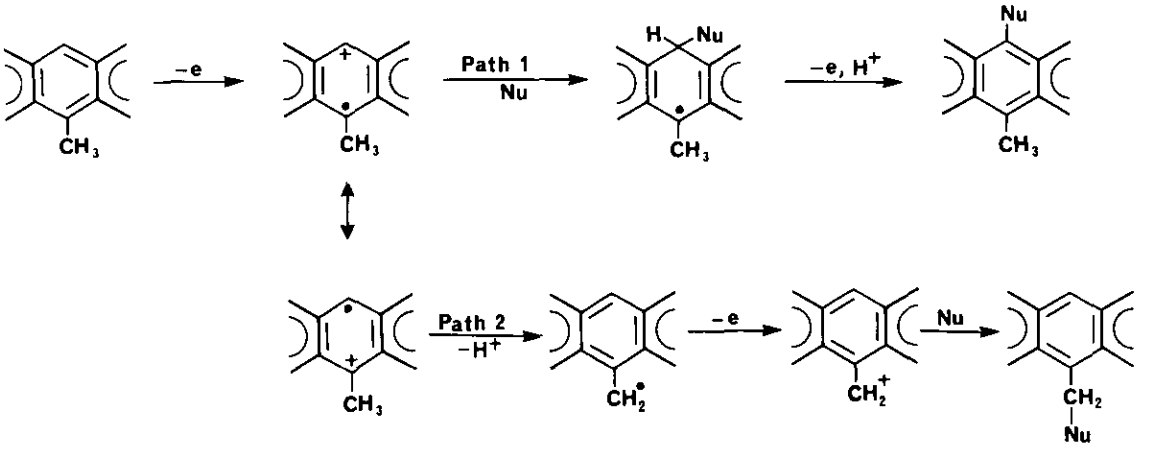


Figure 3. Nucleophilic trapping in radical cations of unsubstituted and methyl-substituted PAH.

than one-electron oxidation could occur. In the case of anthracene, reaction at room temperature forms a dihydrodiacetoxy derivative because the second acetate reacts before loss of a proton can occur. The higher selectivity in nucleophilic substitution with the weak nucleophile acetate ion when compared to pyridine in the previous oxidant system is observed for 7,12-dimethylbenz[a]anthracene in which acetoxy derivatives are formed only at the 7- and 12-methyl groups and not at C-5.

These studies show that the ability of PAH to form radical cations is related to their IP. Other important factors governing the specificity of these reactions are charge localization on one or a few carbon atoms, as well as the strength of the nucleophiles.

Synthesis of Radical Cation Perchlorates and Subsequent Substitution with Nucleophiles

The synthesis of the radical cation perchlorates of BP and 6-methylBP has been reported (*47*), following a modified method of preparation of perylene radical cation (*48,49*). More recently, the radical cation perchlorate of 6-fluoroBP has also been synthesized (*50*). Oxidation of the PAH with iodine in benzene in the presence of $AgClO_4$ instantaneously yields a black precipitate containing the radical cation perchlorate adsorbed on AgI with yields of 28, 28, and 39% for $BP^{\cdot+}ClO_4^-$, 6-methylBP$^{\cdot+}ClO_4^-$, and 6-fluoroBP$^{\cdot+}ClO_4^-$, respectively.

$$2PAH + I_2 + 2AgClO_4 \rightarrow 2PAH^{\cdot+}ClO_4^- \cdot AgI \qquad (3)$$

Reaction of the $BP^{\cdot+}ClO_4^-$ with the two strong nucleophiles NaSCN and $NaNO_2$ yields 6-thiocyanoBP and 6-nitroBP, but also derivatives at C-1, which along with C-3 is the position of second highest charge density in the radical cation after C-6. When 6-methylBP$^{\cdot+}ClO_4^-$ and 6-fluoroBP$^{\cdot+}ClO_4^-$ react with $NaNO_2$ and NaSCN, only derivatives at the 1- and/or 3-position are obtained. Substitution at the 6-methyl group or displacement of fluorine is not observed, indicating that strong nucleophiles exhibit low selectivity toward the most reactive position in the radical cation.

Reaction of BP and 6-fluoro BP radical cations with the weak nucleophile water yields a mixture of BP 1,6-, 3,6-, and 6,12- diones. These products are the result of an initial nucleophilic attack at C-6. For 6-methylBP radical cation, reaction with water affords predominantly 6-hydroxymethyl BP. When the weak nucleophile acetate ion in water is used, $BP^{\cdot+}ClO_4^-$ yields specifically 6-acetoxyBP and the three diones, which derive from reaction of the radical cation with water. For 6-fluoroBP$^{\cdot+}ClO_4^-$, the predominant products are the BP diones, whereas only traces of 6-acetoxyBP are obtained, indicating that acetate ion is sterically hindered at the 6-position in the 6-fluoroBP$^{\cdot+}ClO_4^-$. The only product of 6-methylBP$^{\cdot+}ClO_4^-$ with acetate ion is 6-hydroxymethyl BP which is formed by reaction of the radical cation with water. No 6-acetoxymethyl BP is observed.

The overall conclusion from the reaction of BP and 6-substituted BP radical cations with nucleophiles of various strengths is that weak nucleophiles display higher selectivity toward the position of highest charge localization.

Thus we can outline three important factors that determine the one-electron oxidation of PAH: (1) ease of formation of the radical cation, which is related to the IP; (2) relatively high charge localization in radical cations, which gives them specific reactivity with nucleophiles, and (3) strength of the nucleophiles, which also determines the selectivity in nucleophilic substitution.

The reaction of radical cations with nucleophiles for unsubstituted and methyl-substituted PAH can be summarized, as in Figure 3. Removal of an electron from the π-system generates a radical cation in which the positive charge can be localized mainly at an unsubstituted carbon atom (path 1) or adjacent to the methyl group (path 2). In the former case nucleophilic attack at the position of highest charge density generates an intermediate radical, which is then further oxidized to an arenonium ion with loss of a proton to complete the

substitution reaction. In path 2, the highest charge density is localized at the carbon atom adjacent to the methyl group. Loss of a methyl proton generates a benzylic radical intermediate which is rapidly oxidized to a benzylic carbonium ion with subsequent trapping by a nucleophile.

Ionization Potentials of PAH and Charge Localization in PAH Radical Cations

On the basis of the results obtained by one-electron oxidation of PAH with iodine and $Mn(OAc)_3$, we can assume that in biological systems the ability of PAH to bind covalently to cellular macromolecules should depend mainly on two factors: the ease of formation of radical cations, which is determined by their IP, and charge localization in the radical cation, which gives PAH sufficient and specific reactivity to bind to cellular nucleophiles. The IP of numerous PAH have been determined and compared to a qualitative evaluation of their carcinogenicity (*45*). Some of the most representative PAH are presented in Table 3, accompanied by the IP, a qualitative measure of carcinogenicity and the structures with arrows indicating the position(s) of high, medium, and low reactivity in the radical cation for PAH with relatively low IP. The position(s) of charge localization of the various PAH radical cations has been qualitatively determined by applying the general principle that a π-electron is predominantly removed from the position(s) in which the electronic charge is highest in the ground state, leaving that position most positively charged. Evidence on this point has been obtained by one-electron oxidation of PAH with iodine or manganic acetate. For BP, as previously presented, the positive charge is highly localized at C-6, while C-1 and C-3 have considerably less charge density. For 6-fluoroBP, C-6 remains the position of highest charge localization and when its radical cation is attacked by nucleophiles the fluorine atom is generally displaced. In 6-methylBP, the most reactive position is the 6-methyl group, as a consequence of the high charge localized on the adjacent aromatic carbon. The same applies for 7-methylbenz[a]anthracene, 7,12-dimethylbenz[a]anthracene, and 3-MC. For dibenzo[a,e]pyrene and dibenzo[a,l]pyrene, the reactivity is mainly localized at the meso-anthracenic position, whereas in dibenzo[a,i]pyrene and dibenzo[a,h]pyrene the reactivity remains highly localized at the two meso-anthracenic positions. Anthanthrene has slightly more charge localized at the two meso-anthracenic positions, but also some charge localization on four additional positions, diminishing the reactivity at the meso-anthracenic positions. The reactivity of perylene is presumably reduced because the charge location is shared equally by 4 symmetric positions.

Three lines of evidence indicate that only PAH with relatively low IP, below ca. 7.35 eV, can be activated biologically by one-electron oxidation. This evidence includes binding of PAH to DNA catalyzed by the model one-electron oxidation system, horseradish peroxidase (HRP)/H_2O_2; induction of mammary tumors by direct application of PAH to rat mammary gland; and relationship of formation of PAH quinones to IP. These three subjects are presented below.

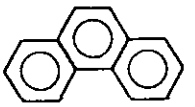
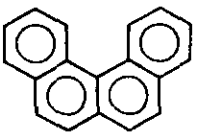
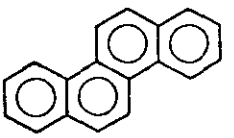
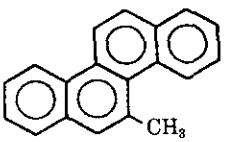
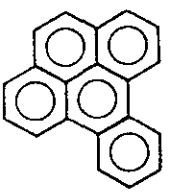
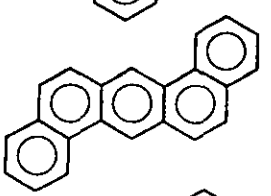
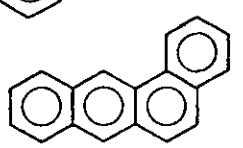
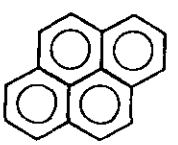
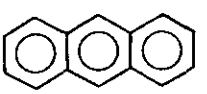
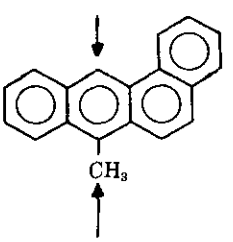
The carcinogenicity of PAH with relatively high IP, such as benzo[c]phenanthrene, benz[a]anthracene, chrysene, 5-methylchrysene, and dibenz[a,h]anthracene (Table 3), can be related to the formation of bay-region diol-epoxides, catalyzed by monooxygenase enzymes (*5*). However, the most potent carcinogenic PAH have IPs less than ca. 7.35 eV. This includes BP, 7,12-dimethylbenz[a]anthracene, 3-MC, dibenzo[a,i]pyrene, and dibenzo[a,h]pyrene, which can be activated by both one-electron oxidation and/or monooxygenation. A few PAH with low IP are inactive (Table 3), such as perylene, or weakly active, such as anthanthrene. Thus low IP is a necessary, but not sufficient factor in carcinogenic activation by one-electron oxidation. In these weakly active or inactive PAH the positive charge in the radical cation is delocalized over several aromatic carbon atoms. In contrast, the radical cations of active PAH with low IP have positive charge localized on one or two carbon atoms, rendering those positions more reactive toward nucleophiles. Thus a second critical factor in activation by one-electron oxidation is that the radical cations of carcinogenic PAH have highly localized charge.

Metabolic Formation of PAH Quinones via Radical Cation Precursors

Metabolism of BP by cytochrome P-450 monooxygenase produces three classes of products: phenols, dihydrodiols, and quinones (Figure 4). Formation of phenols and dihydrodiols is thought to proceed by an initial electrophilic attack of an enzyme-generated reactive oxygen atom. Phenols would result from direct attack at the position of substitution or rearrangement of an intermediate epoxide. Dihydrodiols are formed by chemical and/or enzymic hydrolysis of epoxides. The same pathway of activation involving an electrophilic oxygen has been postulated in the formation of quinones, although the putative 6-hydroxyBP precursor has never been isolated (*51,52*). In this mechanism, autooxidation of 6-hydroxyBP (*52*) would yield BP quinones. Substantial evidence has been obtained indicating that formation of quinones does not involve the proposed mechanism, but instead consists of an initial one-electron oxidation of BP to produce its radical cation (Figure 4).

The first line of evidence arises from the predominant or exclusive formation of quinone when metabolism of BP is conducted under peroxidatic conditions by cytochrome P-450 with cumene hydroperoxide (*18*) as cofactor or by PHS (*53*). One-electron oxidation is the

Table 3. Structure, ionization potential and carcinogenicity of selected PAH.

Compound	Structure	Ionization potential, eV[a]	Carcinogenicity[b]
Phenanthrene		8.19	—
Benzo[c]phenanthrene		7.93	+
Chrysene			±
5-Methylchrysene		ca. 7.7	+ + +
Benzo[e]pyrene		7.62	−
Dibenz[a,h]anthracene		7.57	+ + +
Benz[a]anthracene		7.54	±
Pyrene		7.50	−
Anthracene		7.43	−
7-Methylbenz[a]anthracene		7.37	+ + +

(continued)

Table 3. (Continued).

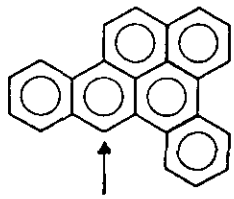
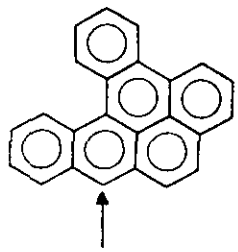
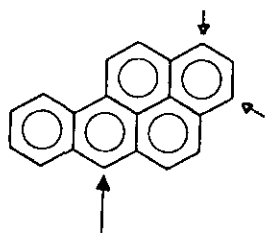
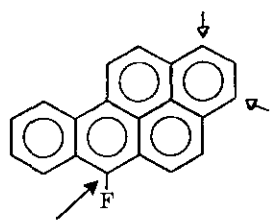
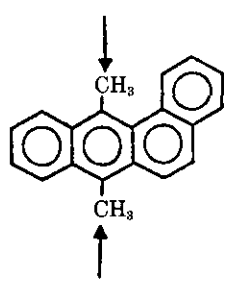
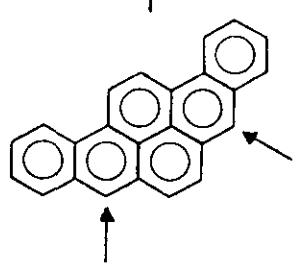
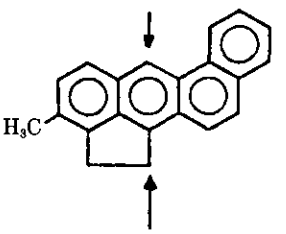
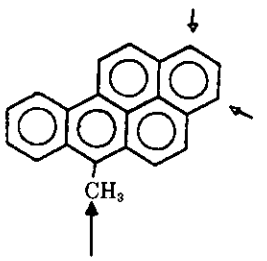
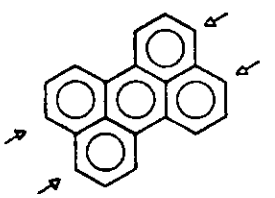
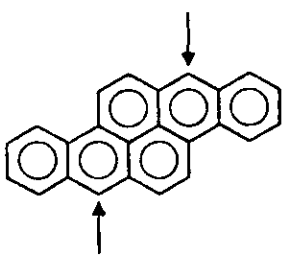
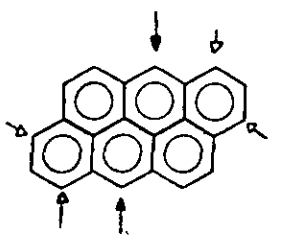
Compound	Structure	Ionization potential, eV[a]	Carcinogenicity[b]
Dibenzo[a,e]pyrene		7.35	+ + +
Dibenzo[a,l]pyrene		7.26	+ + + + +
Benzo[a]pyrene		7.23	+ + + +
6-Fluorobenzo[a]pyrene		7.23	+ +
7,12-Dimethylbenz[a]anthracene		7.22	+ + + + +
Dibenzo[a,i]pyrene		7.20	+ + + +

Table 3. (Continued).

Compound	Structure	Ionization potential eV[a]	Carcinogenicity[b]
3-Methylcholanthrene		7.12	+ + + +
6-Methylbenzo[a]pyrene		7.08	+ + +
Perylene		7.06	–
Dibenzo[a,h]pyrene		6.97	+ + + +
Anthanthrene		6.96	+

[a] Determined from absorption maximum of the charge-transfer complex of each compound with chloranil (*45*), with the exception of dibenz[a,h]anthracene determined by polarographic oxidation (*46*).

[b] Extremely active, + + + + +; very active, + + + +; active + + +; moderately active, + +; weakly active, +; very weakly active, ±; inactive, –.

[c] Arrows, ↓ , ↓ , and ↓ , indicate high, medium, and low reactivity, respectively, in the PAH radical cation.

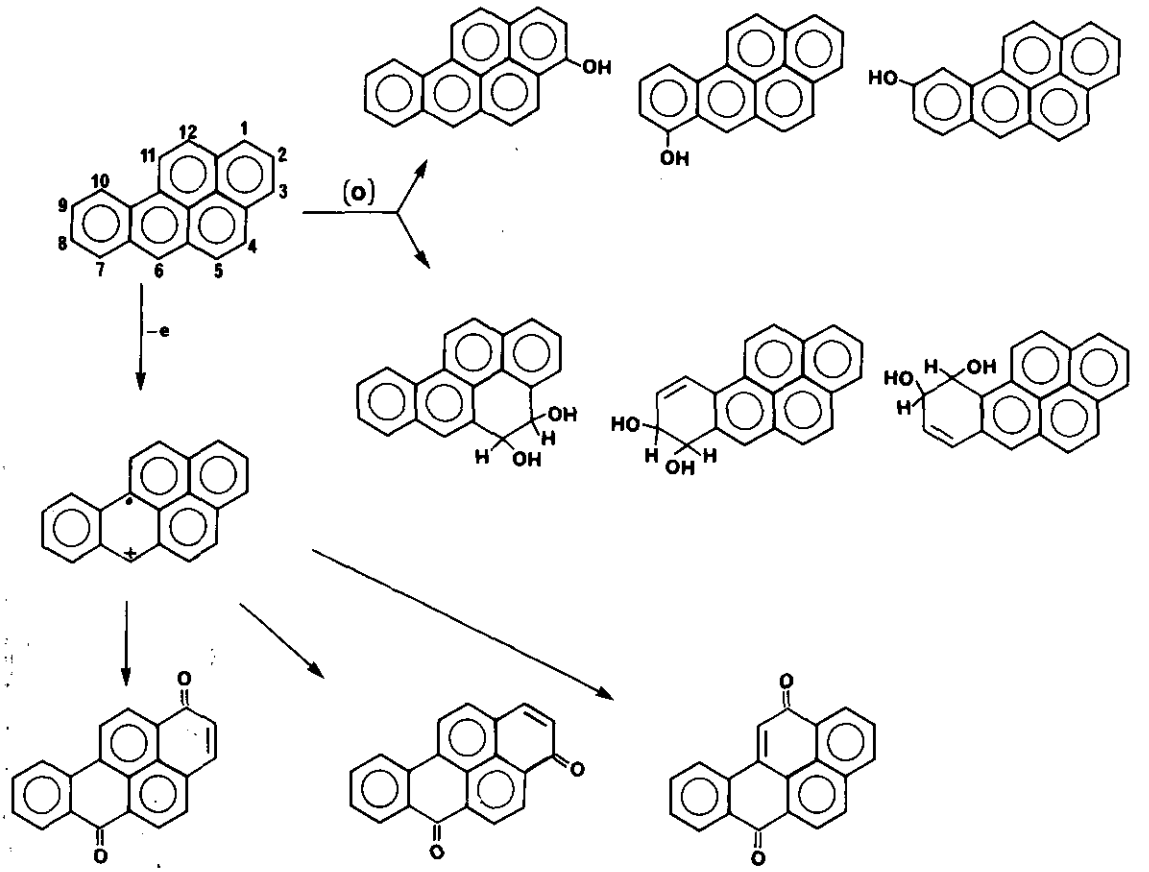


Figure 4. Metabolic products of BP: phenols, dihydrodiols, and quinones.

predominant mechanism of activation under these conditions.

In addition, the same BP quinones obtained in the metabolism of BP are formed when 6-fluoroBP is metabolized by the cytochrome P-450 monooxygenase (*54*). This suggests that BP quinones are produced by an initial attack of a nucleophilic oxygen at C-6 in the 6-fluoroBP radical cation with displacement of fluorine. The reaction of 6-fluoroBP with $Mn(OAc)_3$, in which the major products obtained are 6-acetoxyBP and a mixture of 1,6- and 3,6- diacetoxyBP (*50*) corroborates this point. Attack of acetate ion takes place at C-6 after formation of the 6-fluoroBP radical cation. Conversely, electrophilic substitution with bromine or deuterium ion shows that substitution occurs at C-1 and/or C-3 with retention of the fluoro substituent. These results indicate that the formation of quinones from 6-fluoroBP is consistent only with an initial one-electron oxidation of the compound to form 6-fluoroBP^{+}.

The third type of evidence is related to the metabolism of a series of PAH with high and low IP. Aroclor-induced rat liver microsomes with NADPH or cumene hydroperoxide as cofactor are used in these studies. With NADPH as cofactor, benz[a]anthracene and dibenz[a,h]anthracene do not produce quinones (Table 4), whereas with cumene hydroperoxide a trace of benz[a]anthracene quinone is observed. For the PAH with relatively low IP, dibenzo[a,i]pyrene, BP, dibenzo[a,h]pyrene, and anthanthrene, quinones are formed in the presence of either cofactor and become the predominant metabolic product in the presence of cumene hydroperoxide. Thus the relationship between IP and formation of quinones constitutes an additional piece of evidence that these metabolites are formed via an intermediate radical cation.

As shown in Figure 5 for BP, but applicable to some other PAH, the initial step involves an electron transfer from the PAH to the activated cytochrome P-450–oxygen complex with Fe in a highly oxidized form, but not necessarily the perferryl oxygen complex presented in the reaction scheme. The reduced cytochrome P-450 oxygen complex formed renders the oxygen atom more nucleophilic, thereby reacting at C-6 of BP radical cation in which the positive charge is appreciably localized. The 6-oxyBP radical formed would then dissociate to leave the Fe of cytochrome P-450 in the normal ferric state. Autoxidation of the 6-oxyBP radical, in which the spin density is mainly localized on oxygen, C-1, C-3, and C-12 (*51,52*), would form the three BP diones. The same mechanism of activation has been proposed in the metabolic formation of sulfoxides and sulfones from sulfides and sulfoxides, respectively (*11,12*).

Table 4. Metabolic formation of quinones for PAH of various ionization potentials.

Compound	Ionization potential, eV[a]	Formation of quinone by Aroclor-induced rat liver microsomes with	
		NADPH	Cumene hydroperoxide
Dibenz[a,h]anthracene	7.57	–	–
Benz[a]anthracene	7.54	–	±[b]
Benzo[a]pyrene	7.23	+	+
Dibenzo[a,i]pyrene	7.20	+	+
Dibenzo[a,h]pyrene	6.97	+	+
Anthanthrene	6.96	±[b]	+

[a] Determined from absorption maximum of the charge-transfer complex of each compound with chloranil (*45*), with the exception of dibenz[a,h]anthracene, which was determined by polarographic oxidation (*46*).
[b] ± indicates formation of a trace amount of quinone.

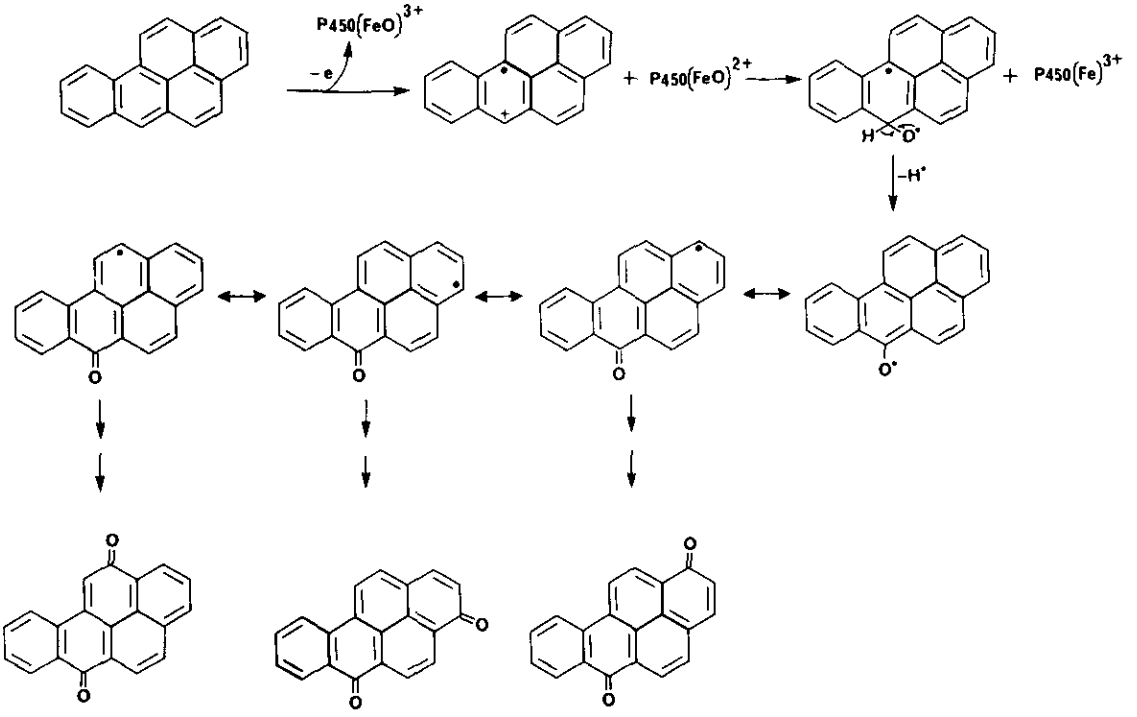


Figure 5. Proposed mechanism of metabolic formation of BP quinones.

One-Electron Oxidation in the Covalent Binding of Benzo[a]pyrene and 6-Methylbenzo[a]pyrene to DNA

Evidence for one-electron oxidation of PAH has been gathered by studying the binding of BP and 6-methylBP to DNA *in vitro* and *in vivo*. By using the HRP/H_2O_2 system, which catalyzes one-electron oxidation of a variety of chemicals (*20,21,26,27,32,55–66*), we have demonstrated that only PAH with an IP value below ca. 7.35 eV bind to DNA at significant levels (Table 5) (*45*). For both BP (*67,68*) and 6-methylBP (*69*), clear evidence has been obtained confirming one-electron oxidation as the mechanism of activation for the HRP/H_2O_2 system. A DNA adduct has been identified in which the 6-methyl group of 6-methylBP is covalently bound to the 2-amino group of deoxyguanosine (*69*). This adduct is also found in DNA isolated from mouse skin treated with 6-methylBP, providing the first evidence for one-electron oxidation of PAH in a target tissue (*69*).

Preliminary evidence for activation of BP by one-electron oxidation in both the HRP/H_2O_2 system and mouse skin was obtained using double-labeled [^{3}H,^{14}C]BP (*67,70*). The strategy of double-labeling experiments can be applied because one-electron oxidation involves a substitution reaction and tritium is lost from the position participating in the covalent bond between BP and DNA. Nucleophilic trapping in chemical experiments proceeds almost exclusively at C-6 in the BP radical cation (Tables 1 and 2) (*37,39,41,43,44,71,72*). Indeed almost 80% of the tritium is lost from C-6 when

Table 5. Ionization potentials and horseradish peroxidase/H_2O_2-catalyzed binding of PAH to DNA.

Compound	Ionization potential, eV[a]	DNA-bound[^{14}C] or [^{3}H]PAH, μmole/mole DNA[b]
Phenanthrene	8.19	3.8 ± 0.8 (11)
5-Methylchrysene	ca. 7.7	1.4 ± 0.5 (8)
Benzo[e]pyrene	7.62	5.1 ± 0.9 (5)
Dibenz[a,h]anthracene	7.57	4.3 ± 1.0 (10)
Benz[a]anthracene	7.54	4.0 ± 0.5 (12)
Pyrene	7.50	2.8 ± 1.4 (4)
Anthracene	7.43	8.8 ± 1.6 (9)
7-Methylbenz[a]anthracene	7.37	5.6 ± 0.6 (6)
Benzo[a]pyrene	7.23	89.2 ± 5.6 (8)
7,12-Dimethylbenz[a]anthracene	7.22	63.9 ± 4.6 (12)
3-Methylcholanthrene	7.12	60.6 ± 4.1 (10)
6-Methylbenzo[a]pyrene	7.08	39.8 ± 5.3 (9)
Anthanthrene	6.96	27.0 ± 7.1 (8)
6,12-Dimethylanthanthrene	6.68	62.0 ± 13 (5)

[a] The IP were calculated from absorption maximum of the charge-transfer complex of each compound with chloranil (*45*) with the exception of dibenz[a,h]anthracene determined by polarographic oxidation (*46*).

[b] Control levels of binding have been subtracted from these levels, which are presented as average ± standard error of measurement. Number in parentheses corresponds to number of determinations.

BP is bound to DNA in mouse skin and 94% from C-6 in the HRP/H_2O_2-catalyzed binding of BP to DNA. Although these results suggest that C-6 of BP is involved in the covalent bond to DNA, determination of the structure of BP-DNA adducts is necessary to substantiate this evidence.

We are currently examining some of the BP-DNA adducts formed in mouse skin by one-electron oxidation and comparing them to model adducts prepared by electrochemical anodic oxidation of BP in the presence of deoxyguanosine. After mouse skin has been treated for 4 hr with [^{14}C]BP, the skin is excised and the DNA purified and enzymically digested to mononucleosides. DNA adducts are separated by reverse phase high-pressure liquid chromatography as shown in Figure 6. While peak C contains BP diol-epoxide adducts, peaks D–F correspond to model adducts formed by reaction of BP^{+} with deoxyguanosine. Peak D has been analyzed further and found to co-chromatograph with a model adduct having a molecular weight of 517, as expected from reaction of BP^{+} with deoxyguanosine. This model adduct also exhibits a UV absorption spectrum (Figure 7*a*) similar to that of 6-methylBP (Figure 7*b*), having a red shift of 8 to 10 nm for each maximum when compared to BP (Figure 7*c*). Since alkylation at C-6 produces a red shift larger than any other position, the spectrum of the adduct suggests that binding of deoxyguanosine to BP occurs at C-6. Complete determination of the structure of the adducts resides in obtaining their proton NMR spectra. Although identification of the DNA adducts formed by one-electron oxidation provides evidence that this mechanism of activation takes place in target tissues, this does not prove that it is responsible for initiating the tumorigenic process.

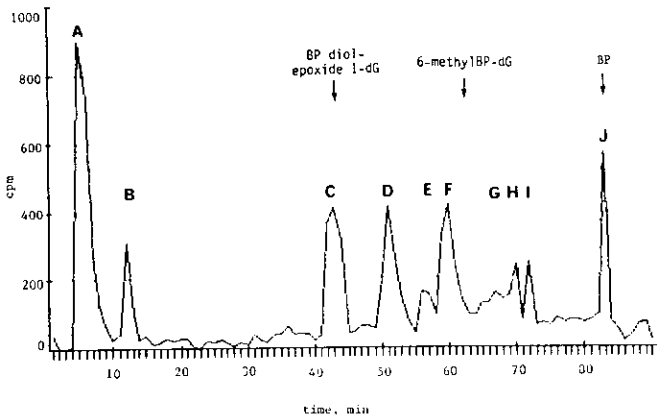


Figure 6. High-pressure liquid chromatography (HPLC) profile of BP adducts obtained from mouse skin DNA hydrolyzed enzymically to mononucleosides.

Comparative Carcinogenicity Studies in Rat Mammary Gland and Mouse Skin

Multiple mechanisms of PAH activation appear to occur in the target tissue mouse skin, since studies of PAH binding to mouse skin DNA reveal that both diol-epoxide (*5*) and radical cation (*68–70*) intermediates are formed and could play a role in carcinogenesis. We have therefore chosen to study PAH carcinogenesis in rat mammary gland because two lines of evidence suggest that one-electron oxidation is the predominant mechanism of activation in this organ: first, *N*-hydroxy-2-acetylaminofluorene is activated in rat mammary cells by one-electron oxidation (*22,73*); secondly, only PAH with IP below ca. 7.35 eV have been observed to be carcinogenic therein. The carcinogenicity of 14 PAH has been examined in 50-day-old female Sprague-Dawley rats by direct application of the compounds to the mammary gland (*74–76*). In Table 6 the results of these experi-

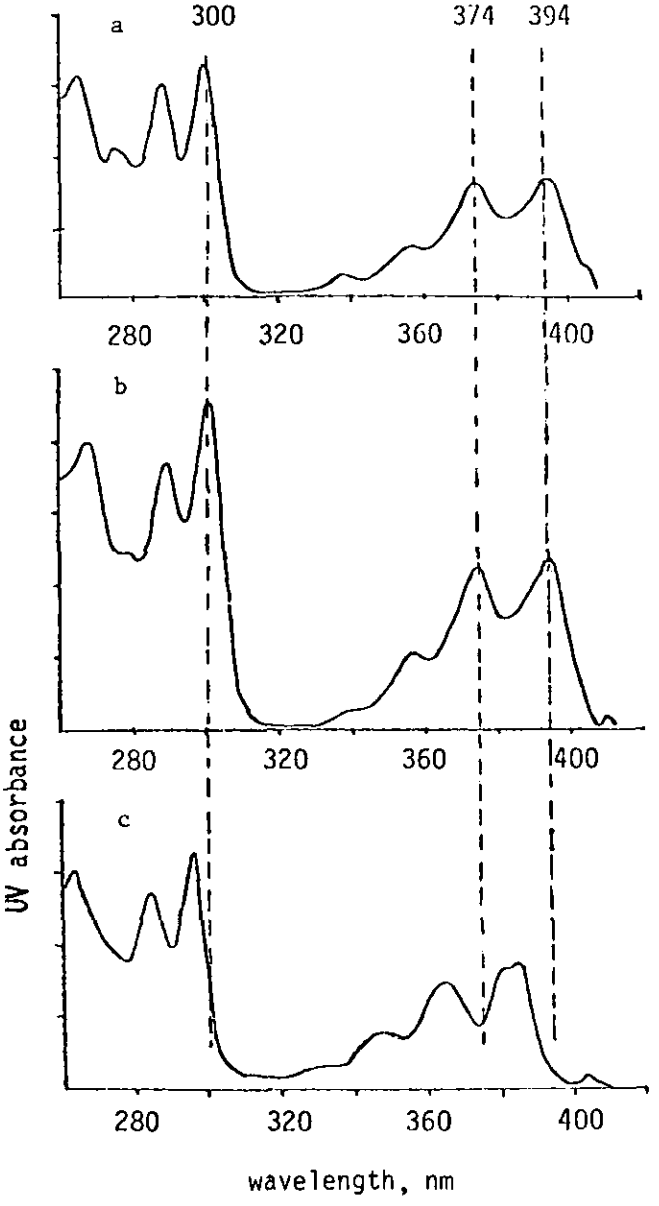


Figure 7. UV absorbance spectra of (a) BP-dG adduct; (b) 6-methylBP; (c) BP.

ments are presented and compared to the carcinogenicity of PAH in mouse skin by repeated application obtained in our laboratory and others. Based on the hypothesis that compounds with relatively high IP cannot be activated by one-electron oxidation, PAH were selected because they were or were not expected to be activated by this mechanism. Some additional PAH were chosen in which activation by monooxygenation or one-electron oxidation was blocked.

Compounds are generally carcinogenic in both mouse skin and rat mammary gland if they have low IP and the radical cation has sufficient charge localization. These include 7-methylbenz[a]anthracene, BP, 7,12-dimethylbenz[a]anthracene, 10-fluoro-3-MC, 8-fluoro-3-MC, 2,3- dimethylcholanthrene, 3-MC and 6-methylBP. In contrast 1,3- dimethylcholanthrene, which has a low IP, is active only in mouse skin, presumably because steric hindrance at C-1, the position of nucleophilic substitution in the 3-MC radical cation, prevents activation by one-electron oxidation in the mammary gland, while activation by monooxygenation can occur in mouse skin. The activity of 2,3-dimethylcholanthrene in both tissues suggests that the methyl substituent at C-2 does not prevent nucleophilic substitution at C-1 in the radical cation. Both dibenz[a,h]anthracene and 5-methylchrysene, which have relatively high IP, are not carcinogenic when applied directly to rat mammary gland. In mouse skin, the carcinogenicity of 5-methylchrysene has been shown to occur via formation of the diol-epoxide intermediate (*77*), and the potent activity of dibenz[a,h]anthracene (*5*) presumably proceeds through the same mechanism. The inactivity of these two skin carcinogens suggests that diol-epoxides are not formed in the mammary gland. Furthermore, no carcinogenic activity is observed in this tissue for the mouse skin carcinogens BP 7,8-dihydrodiol (5,33) and cyclopenta[cd]pyrene (*78*), both of which require only a simple epoxidation for activity.

There are three main conclusions which can be drawn from these experiments: (1) oxygenation of PAH by cytochrome P-450 monooxygenase does not appear to elicit carcinogenicity in rat mammary gland; (2) the results from these experiments support the hypothesis that one-electron oxidation might be the predominant mechanism of activation in this tissue; and (3) multiple mechanisms of activation seem to occur in mouse skin, although these experiments do not provide evidence on this point.

Conclusions

Based on present knowledge radical cations of PAH play an important role in the carcinogenesis and metabolism of these compounds. Metabolic formation of quinones in unsubstituted PAH occurs via an intermediate radical cation. For PAH which have an IP below ca. 7.35 eV (Table 3) (*3,4*), the formation of radical cations can occur in biological systems. Thus the carcinogenicity of compounds with relatively high IP, such as chrysene, 5-methylchrysene and dibenz[a,h]anthracene, proceeds by monooxygenation with formation of bay-region vicinal diol-epoxides (*5*). Most potent PAH, however, have IP below ca. 7.35 eV. These include BP, 3-MC, 7,12- dimethylbenz[a]anthracene, dibenzo[a,i]pyrene and dibenzo[a,h]pyrene. These PAH can be activated by one-electron oxidation and monooxygenation, depending on the type of enzymes present in the tissue in which activation occurs. The ubiquity of peroxidases, in particular PHS, in extrahepatic tissues responsive to PAH carcinogenesis suggests that one-electron oxidation may be a major pathway of activation in most target tissues. In addition the ability of cytochrome P-450 acting as a peroxidase to catalyze one-

Table 6. Comparative carcinogenicity of PAH in mouse skin and rat mammary gland.

Compound	Ionization potential, eV[a]	Carcinogenicity[b]	
		In mouse skin	In rat mammary gland
Cyclopenta[cd]pyrene		+ +	–
Benzo[a]pyrene 7,8-dihydrodiol		+ + + +	–
5-Methylchrysene	ca. 7.7	+ + +	–
Dibenz[a,h]anthracene	7.57	+ + +	–
Benz[a]anthracene	7.54	±	–
7-Methylbenz[a]anthracene	7.37	+ + +	+
Benzo[a]pyrene	7.23	+ + + +	+ + +
7,12-Dimethylbenz[a]anthracene	7.22	+ + + + +	+ + + + +
10-Fluoro-3-methylcholanthrene	7.17	NT[c]	+ +
1,3-Dimethylcholanthrene	7.15	+ +	–
8-Fluoro-3-methylcholanthrene	7.14	NT	+ +
2,3-Dimethylcholanthrene	7.13	NT	+ +
3-Methylcholanthrene	7.12	+ + + +	+ + + +
6-Methylbenzo[a]pyrene	7.08	+ + +	+

[a] Determined from absorption maximum of the charge-transfer complex of each compound with chloranil (*45*), with the exception of dibenz[a,h]anthracene determined by polarographic oxidation (*46*).

[b] Extremely active, + + + + +; very active, + + + +; active + + +; moderately active, + +; weakly active, +; very weakly active, ±; *inactive*, –.

[c] NT = not tested.

electron oxidation efficiently may also be responsible for carcinogenic activation of PAH. The role of different mechanisms of PAH carcinogenesis in a certain target organ will be determined by combined studies of enzymology, carcinogenicity and binding to cellular macromolecules.

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