Oxidation of 9-Alkylanthracenes by Cytochrome P450 2B1, Horseradish Peroxidase, and Iron Tetraphenylporphine/Iodosylbenzene Systems: Anaerobic and Aerobic Mechanisms†

Pavel Anzenbacher,‡,§ Toshiro Niwa,‡,|| Laren M. Tolbert,⊥ Sarath R. Sirimanne,⊥,# and F. Peter Guengerich*,‡

Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University, Nashville, Tennessee 37232-0146, and School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30322-0400

Received September 29, 1995; Revised Manuscript Received January 2, 1996⊗

ABSTRACT: Variously substituted alkylanthracenes were studied as models for polycyclic hydrocarbon oxidations. 9-Methylanthracene was oxidized to 9-(hydroxymethyl)anthracene, 10-methyl-10-hydroxy-9-anthrone, and anthraquinone in several systems, including (i) NADPH- and O_2-fortified rat liver microsomes, (ii) cytochrome P450 (P450) 2B1 supported by either iodosylbenzene (PhIO) or a mixture of NADPH−P450 reductase, NADPH, and O_2, (iii) horseradish peroxidase and either H_2O_2 or ethyl hydroperoxide, and (iv) a mixture of iron tetraphenylporphine (FeTPP) and PhIO (in anhydrous CH_2Cl_2/MeOH). The microsomal system also formed dihydrodiols from 9-methyl- and 9-ethylanthracenes. The formation of the three oxidized products by the P450/NADPH/O_2 system was dependent upon O_2, label from $^{18}O_2$ was incorporated into the products, and no label from $H_2{}^{18}O$ was incorporated. No label from $^{18}O_2$ was incorporated into the three products in the FeTPP/PhIO system. In the horseradish peroxidase/H_2O_2 system, the formation of the three products was decreased when O_2 was omitted, and label from *both* $H_2{}^{18}O$ and $^{18}O_2$ was incorporated into all three products. The results are interpreted in terms of three mechanisms. One is used by the FeTPP and P450 systems, with all oxygen transfers coming from an FeO entity. The other two pathways are utilized by horseradish peroxidase and begin with formation of a radical cation, which can undergo reactions either with H_2O or with O_2 to form the products detected here. The involvement of a 9-methylanthracene radical cation in the P450 and FeTPP pathways is a possibility, but rapid rearrangement and oxygen rebound must be invoked. Comparisons of products from various 9-alkylanthracenes do not provide evidence that one-electron oxidation is an integral part of the epoxidation process with these compounds. The significance of the lack of trapping of radical (by $H_2{}^{18}O$) in the P450 reactions to DNA adduct formation is considered.

Polycyclic aromatic hydrocarbons have long been of interest because of their ubiquitous presence in the environment and their abilities to cause cancers, at least in experimental animals (Cook *et al*., 1933). The inert nature of these compounds does not explain their binding to macromolecules, and the importance of oxidation to generation of electrophilic species capable of binding to DNA has been recognized for a number of years (Miller, 1951). The large number of oxidation products formed has posed problems in the identification of which of these are linked to mutations and cancers. Several major routes of oxidation have dominated the discussion in recent years. The first involves the subsequent processes of epoxidation, epoxide hydrolysis, and epoxidation to yield "diol epoxides", with the so-called "bay" and "fjord" regions appearing to be most deleterious. Differences in stereochemistry of these compounds appear to be critical in reactions with DNA and subsequent biological effects (Dipple *et al*., 1984; Conney, 1982). Oxidations by P450s[1] and other enzymes can also produce phenols and numerous products containing combinations of phenols, dihydrodiols, and epoxides (Conney, 1982; Lehr *et al*., 1985; Jerina, 1983; Prough *et al*., 1981). The biological actions of most of these complex products have not been investigated in detail. Some evidence has been presented that CH_2OH moieties can be added enzymatically to the polycyclics (Flesher *et al*., 1990). Another route involves one-electron oxidation to a radical cation, a proposal originally advanced by Fried and T'so and more recently by Cavalieri and Rogan (Fried & Schumm, 1967; Ts'o *et al*., 1977; Cavalieri & Rogan, 1984). Benzo[*a*]pyrene adducts bound to guanine at the N7 and C8 positions have been reported in anodic systems (RamaKrishna *et al*., 1992)

† This work was supported in part by U.S. Publich Health Service Grants CA44353 and ES00267 (F.P.G.) and CA43806 (L.M.T.). P.A. is the recipient of a Fogarty Award in the Environmental Sciences (No. FO5 TW04832). T.N. was supported by the Fujisawa Pharmaceutical Co.

‡ Vanderbilt University.

§ Present address: Institute of Experimental Biopharmaceutics, Academy of Sciences of the Czech Republic-PRO.MED.CS, 50002 Hradec Králové, Czech Republic.

|| Present address: Pharmaceutical and Pharmacokinetics Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 1-6, 2-Chome, Kashima, Yodogawa-ku, Osaka 532, Japan.

⊥ Georgia Institute of Technology.

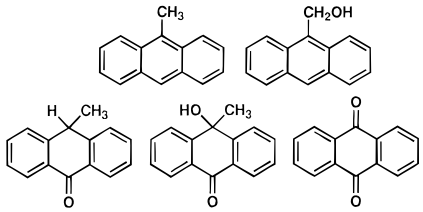
Present address: Centers for Disease Control, Atlanta, GA.

⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1996.

[1] Abbreviations: P450, cytochrome P450 [also termed "heme-thiolate protein" by the Enzyme Commission (EC 1.14.14.1) (Palmer & Reedijk, 1992)]; HRP, horseradish peroxidase (EC 1.11.11.7, donor:H_2O_2 oxidoreductase); CPO, chloroperoxidase (EC 1.11.1.10, chloride peroxidase); FeTPP, iron(III) 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine; PhIO, iodosylbenzene; HPLC, high-performance liquid chromatography; GC/MS, coupled gas chromatography−mass spectrometry; CI, chemical ionization; TMS, trimethylsilyl; Me, methyl; Et, ethyl; OH, hydroxy; CH_2OH, hydroxymethyl.

0006-2960/96/0435-2512$12.00/0

Scheme 1: Structures of 9-Me-Anthracene and the Oxidation Products 9-CH_2OH-Anthracene, 9-Me-Anthrone, 10-Me-10-OH-9-Anthrone, and Anthraquinone



and systems containing HRP (Rogan *et al*., 1988) and P450 (Cavalieri *et al*., 1990). What is often not appreciated is that one-electron oxidation is a plausible pathway in both cases, e.g., deprotonation/oxidation vs deprotonation/oxygen rebound occurring later in the sequence.

One-electron oxidations of model polycyclic hydrocarbons (i.e., anthracenes) substituted by alkyl groups at atoms 9 and/or 10 have been previously investigated as models for enzymatic oxidation. In these studies, an ethyl group was found to inhibit side-chain oxidation, presumably due to steric inhibition of the coplanarity necessary for efficient deprotonation of the putative radical cation (Tolbert *et al*., 1990). Conversely, facile cleavage of the C−Si bond in a TMS substituent was found to enhance the side-chain oxidation (Sirimanne *et al*., 1991). In later unpublished studies, Sirimanne and Tolbert discovered that side-chain cleavage of the 9-methyl group in 9-Me-anthracene led to stoichiometric formation of HCHO and anthraquinone. Despite these results, the relevance of such biomimetic approaches to the function of the purified enzymes remained an open question, particularly since conditions, enzymes, and substrates have varied greatly from laboratory to laboratory. In order to develop a model system which was subjected to enzymatic oxidation under directly comparable conditions, we reinvestigated the mechanisms involved in the FeTPP system and extended the work to the mixed-function oxidase P450 and to HRP, a prototypic peroxidase that is generally considered to utilize one-electron oxidations but not hydrogen atom abstraction or oxygenation. Mechanisms of oxidation of alkyl polycyclic hydrocarbons to quinones have been studied in some model chemical systems (Fried & Schumm, 1967; Tolbert & Khanna, 1987; Li *et al*., 1991), and some studies have been done on the oxidation of the phenols to quinones by P450s (Prough *et al*., 1981), but detailed mechanisms have not been proposed. We consider these in the FeTPP biomimetic system and with the enzymes HRP and P450.

EXPERIMENTAL PROCEDURES

Chemicals. 9-Me-anthracene, 2-Et-anthracene, 9,10-Me_2-anthracene, and 9-formyl-10-Me-anthracene were purchased from Aldrich Chemical Co. (Milwaukee, WI) (Scheme 1). 9-CH_2OH-anthracene was purchased from Lancaster Synthesis (Windham, NH). 9-Et-anthracene was synthesized in 79% yield by coupling of 9-bromoanthracene with ethyl magnesium chloride using $NiCl_2$(dppp) catalyst (Tamao *et al*., 1982). The product was recrystallized from CH_3OH to yield pale yellow prisms, mp 117−118 °C [lit. mp 63−64 °C (Liebermann, 1882); the structure was confirmed by high-resolution mass and IR spectroscopy; either the earlier lower mp is in error or the previous author was dealing with a different crystal form]. 10-Me-10-OH-9-anthrone was synthesized according to Julian *et al*. (1945) (mp 160−161 °C). 10-Methylene-9-anthrone was prepared using the procedure of Starnes (1970) (mp 146−148 °C, lit. 147−148 °C). 9-CH_2OH-10-Me-anthracene was synthesized from 9-formyl-10-Me-anthracene with $LiAlH_4$ according to Southern and Waters (1960) (mp 222−224 °C, lit. mp 222−223 °C). 9-(1-Hydroxyethyl)anthracene was synthesized by reduction of 9-acetylanthracene with $NaBH_4$ in CH_3OH. $H_2{}^{18}O$ (95−98% ^{18}O) was purchased from Cambridge Isotope Labs (Andover, MA), and $H_2{}^{18}O_2$ (90% ^{18}O) and ^{18}O (96% $^{18}O_2$) were from ICON (Mt. Marion, NY). FeTPP chloride was synthesized (by insertion of iron into tetraphenylporphine) and purified by silicic acid chromatography as described (Fleischer *et al*., 1971). PhIO was prepared from the diacetate (Aldrich) by base hydrolysis (Saltzman & Sharefkin, 1973) and stored as a solid at −20 °C; solutions (actually fine suspensions) were prepared by sonication in CH_3OH for work with FeTPP and in H_2O for work with P450.

Enzymes. Liver microsomes were prepared from phenobarbital- or 5,6-benzoflavone-treated rats (Guengerich, 1994); P450 2B1 was purified from phenobarbital-induced microsomes by modification of a previous method (Guengerich & Martin, 1980; Guengerich *et al*., 1982). NADPH−P450 reductase was purified from liver microsomes of phenobarbital-treated rabbits using the method of Yasukochi & Masters (1976) as modified (Guengerich, 1994). HRP (type IX) and *Caldariomyces fumago* CPO were purchased from Sigma Chemical Co. (St. Louis, MO).

Incubation Conditions and Sample Treatment. With microsomal systems, the reaction mixture contained 0.10 M potassium phosphate buffer (pH 7.4), 10 mM $MgCl_2$, and an NADPH-generating system. After preincubation at 37 °C for 5 min, the reaction was initiated by the addition of substrate (final concentration 100 μM). Incubation was done at 37 °C for 30 min. The reactions catalyzed by P450 2B1 were generally carried out in 1.0 mL of 0.10 M potassium phosphate buffer (pH 7.7) containing 2.0 μM P450 2B1, 2.0 μM NADPH−P450 reductase, 5 mM $MgCl_2$, 30 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, 10 mM glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase mL^{-1}, and substrate (1−100 μM). After preincubation at 37 °C for 5 min, reactions were initiated by the addition of $NADP^+$ (final concentration 0.5 mM). Incubation was done at 37 °C for 15 min. In HRP systems, reaction mixtures contained 0.10 M potassium phosphate buffer (pH 6.0), H_2O_2 (0.50 mM) or EtOOH (1.6 mM), substrate (1−100 μM), and HRP (usually 230 nM) in a final volume of 1.0 mL (Okazaki & Guengerich, 1993). After a 5 min preincubation, reaction was initiated by the addition of HRP. Incubations were done at 25 °C for 10 min.

All reactions were terminated with 5 mL of ethyl acetate or CH_2Cl_2, followed by the addition of 10 μL of 2.0 mM 2-Et-anthracene as an internal standard for HPLC. After mixing (vortex device) and centrifugation, the organic layers were transferred to another tube, and solvent was evaporated under a stream of N_2 at room temperature. Residues were dissolved in 200 μL of CH_3OH, and aliquots were analyzed by HPLC.

PhIO was used as oxidant in some experiments with P450 2B1 and FeTPP. With P450, the same concentrations of P450, buffer, and substrate were used as above. PhIO (20 μL of 10 mM PhIO suspended in H_2O by sonication prior to use) was added instead of NADPH−P450 reductase and the NADPH-generating system. Incubations were done for 60 s. In experiments with FeTPP, the metalloporphyrin

Table 1: Oxidation of Alkylanthracenes by FeTPP/PhIO[a]

compound added	amount, nmol	product	amount, nmol
9-Me-anthracene	1000	9-CH_2OH-anthracene	5−10
		10-Me-9-anthrone	28−55
		10-Me-10-OH-9-anthrone	7−15
		anthraquinone	3−10
9,10-Me_2-anthracene	1000	9-CH_2OH-10-Me-anthracene	28−49
9-CH_2TMS-anthracene	100	9-CH_2OH-anthracene	21
9-Me-10-Et-anthracene	100	9-CH_2OH-10-Et-anthracene	2
9-CH_2TMS-10-Me-anthracene	500	9-CH_2OH-10-Me-anthracene	42−47
anthracene	1000	anthraquinone	16
10-Me-9-anthrone	500	*b*	
10-methylene-9-anthrone	100	anthraquinone	7
9-CH_2OH-anthracene	1000	anthraquinone	15
10-Me-10-OH-9-anthrone	1000	*b*	
9,10-Et_2-anthracene	500	*b*	

[a] Conditions were as described in the text. [b] No products detected (<2 nmol approximate limit).

(chloride salt, 200 μM in CH_2Cl_2) and the substrate (1.0 mM) were mixed in CH_2Cl_2 (1.0 or 5 mL total volume). Reaction was initiated by addition of PhIO. Incubations were done for 60 min at room temperature. For HPLC analyses, the reaction mixture was diluted 5-fold with CH_3CN and injected directly on the column. For GC/MS, the solvent was evaporated to ~100 μL, and an aliquot of the reaction mixture was analyzed.

HPLC. HPLC analyses were performed using TSKgel ODS-80TM (5 μM, 4.6 × 150 mm; TosoHaas, Montogmeryville, PA) with a gradient of 30−100% CH_3CN in H_2O at a flow rate of 1.0 mL min^{-1}. Products were quantitated by peak height or integration. 2-Et-anthracene was used as an internal standard. (Retention times of standards and UV characteristics of all compounds are provided in the Supporting Information.)

GC/MS. Samples were concentrated under a stream of N_2 to ~100 μL and injected directly onto a Finnigan Incos 50 GC/MS system (Finnigan, Sunnyvale, CA) equipped with a capillary column (DB-1701, 15 m; Resteck, Bellafonte, PA) with a temperature gradient of 150−300 °C at 15 °C min^{-1}. He was the carrier gas, and the positive ionization (electron impact) mode was used. Both full scan and selected ion responses were monitored. Integration of the selected ion responses was done with a Data General computer system, i.e., the M^+ at *m*/*z* 208 (unlabeled) and 210 (labeled with one ^{18}O atom) for 9-CH_2OH-anthracene, 208 (unlabeled) with 210 (labeled with one ^{18}O) and 212 (labeled with two ^{18}O atoms) for anthraquinone, and 209, 211, and 213 for 10-Me-10-OH-9-anthrone with zero, one, or two ^{18}O atoms (these ions were selected instead of the actual M^+ at *m*/*z* 224, 226, and 228 as this compound readily loses a methyl group, i.e., 15 mass units). GC/MS CI analyses of 10-Me-10-OH-9-anthrone were performed on a Nermag R-10-10C GC/MS system equipped with a CI source in the negative ionization mode, using the same capillary column as above. CH_4 was used as the CI reagent gas and He as the carrier gas. The 10-Me-10-OH-9-anthrone ions at *m*/*z* 223 (M−1), 225, and 227 were monitored, and the resulting peaks were integrated.

In all cases, the extent of ^{18}O labeling was determined by dividing the integral of the heavy isotope peak by the sum of the integrals for the M and M+2 (and M+4) ions. For the analysis of 10-Me-10-OH-9-anthrone, very similar results were obtained from electron impact and CI assays. The work with $H_2{}^{18}O$ and $^{18}O_2$ labeling with P450 2B1 and HRP was done 3 different times with rather similar results, and the work presented from the most recent analysis is presented here.

Assays. Experiments with $H_2{}^{18}O$ were done as described above. The content of the isotopically labeled water was 66% in a total volume of 2.9 mL. Anaerobic incubations and those done with $^{18}O_2$ gas utilized an evacuated system which was filled with $^{18}O_2$ for labeling experiments. The system (Burleigh *et al*., 1969) consisted of a glass manifold with three outlets (routinely used for duplicate samples and one control); the manifold was connected to a source of purified, deoxygenated Ar [using a copper catalyst at 160 °C (catalyst R3-11, Chemical Dynamics Co., Plainfield, NJ) to reduce the remaining O_2] (Guengerich, 1983) and to a vacuum pump. Samples were subjected to 10 cycles of alternate evacuation and purging with the Ar gas; reactions were started by addition of $NADP^+$ (P450/NADPH systems) or HRP (HRP/H_2O_2 systems) from an arm either in the evacuated system (for anaerobic experiments) or after introducing $^{18}O_2$ gas. The experiments were then performed as described above; to correct for the presence of unlabeled oxygen ($^{16}O_2$), control experiments with toluene as substrate for P450 2B1-catalyzed hydroxylation were done under the same conditions. The extent of labeling was shown to be 79% for benzyl alcohol as the product of toluene oxidation, taking into account the isotopic content of the gas that was used (96% atomic excess).

HCHO was measured using a 2,4-dinitrophenylhydrazine/HPLC method (Fung & Grosjean, 1981).

RESULTS

Oxidation Products of Substituted Anthracenes Formed in Various Systems. Initial studies were done with rat liver microsomes as sources of P450. These preparations also contain the enzyme epoxide hydrolase, which is known to catalyze the hydrolysis of polycyclic epoxides to dihydrodiols (Oesch *et al*., 1971). In microsomes prepared from 5,6-benzoflavone-treated rats, the major P450s present are P450 1A1 and 1A2 (Guengerich, 1987). Small amounts of 9-CH_2-OH-anthracene, 10-Me-10-OH-9-anthrone, and anthraquonine were formed, but the dominant products appeared to be the 1,2- and 3,4-dihydrodiols as judged by the similarity of their t_R values and UV spectra to the literature (LaVoie *et al*., 1985). In liver microsomes prepared from phenobarbital-treated rats, the two dihydrodiols were also formed, but 9-CH_2OH-anthracene, 10-Me-10-OH-9-anthrone, and anthraquinone constituted a larger fraction of the products. P450 2B1 is a major P450 enzyme present in such preparations

Table 2: Oxidation of Anthracenes by HRP/H_2O_2

substrate	product formed, nmol min^{-1} (nmol of HRP)$^{-1}$ [a]		
	9-alkylhydroxylation	10-Me-10-OH-9-anthrone	anthraquinone
anthracene			0.04
9-Me-anthracene	1.5 ± 0.3	4.8 ± 0.7	6.3 ± 1.2
9-CH_2TMS-anthracene	3.0 ± 0.2	<0.02	3.0 ± 1.0
9-CH_2OH-anthracene		<0.02	<0.02
9-Et-anthracene	0.013, 0.014[b]	2.1, 1.4 (Et)	0.82, 0.91
9,10-Me_2-anthracene	0.08, 0.04	<0.02	<0.02
9-CH_2TMS-10-Me-anthracene	0.34	<0.02	<0.02
9-Me-10-Et-anthracene	0.08, 0.05	<0.02	<0.02
10-Me-10-OH-9-anthrone			<0.02

[a] Anthracenes (100 μM) were incubated with HRP (230 nM) and H_2O_2 (0.50 mM) at 25 °C for 10 min. Duplicate experiments are indicated when there are two values, and "±" indicates a mean ± SD of three individual experiments. [b] Hydroxylation at α-carbon.

Table 3: Oxidation of Anthracenes by P450 2B1/NADPH/O_2

substrate	product formed, pmol min^{-1} (nmol of P450)$^{-1}$ [a]		
	9-alkylhydroxylation	10-Me-10-OH-9-anthrone	anthraquinone
anthracene			6.9, 5.6
9-Me-anthracene	54, 57	32, 25	8.8, 14
9-CH_2TMS-anthracene	6.7, 8.9	<1	33, 60
9-CH_2OH-anthracene		<1	27, 17
9-Et-anthracene	1.7, 2.1	15, 25 (Et)	132, 101
9,10-Me_2-anthracene	24 ± 6	<1	<1
9-CH_2TMS-10-Me-anthracene	18	<1	<1
9-Me-10-Et-anthracene	77 ± 28	<1	<1
10-Me-10-OH-9-anthrone			<1

[a] Anthracenes (10 or 100 μM) were incubated with P450 2B1 (2.0 μM), NADPH−P450 reductase (2.0 μM), L-α-dilauroyl-*sn*-glycero-3-phosphocholine (30 μM), and an NADPH-generating system at 37 °C for 15 min. Duplicate experiments are indicated when there are two values, and "±" indicates a mean ± SD of three individual experiments.

(Guengerich, 1987), and this purified enzyme was used in subsequent studies.

9-Me-anthracene was oxidized to 9-CH_2OH-anthracene, 10-Me-10-OH-9-anthrone, anthraquinone, and 10-Me-9-anthrone in a biomimetic system containing FeTPP and PhIO in CH_2Cl_2. The yields of the products were functions of the concentrations of both PhIO and MeOH (used to dissolve PhIO for delivery into the system). The concentrations of 5 mM PhIO and 11% MeOH (v/v) were found to be optimal, compared to several others that were tried, and were used in studies with other substituted anthracenes (Table 1). Some of the alkylanthracenes were converted only to hydroxylated products. When both the 9 and 10 positions were substituted with alkyl groups, no anthraquinone was formed. When a benzylic-type proton was replaced by a TMS group, products involving side-chain deprotonation (i.e., hydroxymethylarenes) increased in yield.

HRP formed 9-CH_2OH-anthracene, 10-Me-10-OH-9-anthrone, and anthraquinone from 9-Me-anthracene in the presence of H_2O_2 (Table 2) or EtOOH (results not shown). With some of the other alkylanthracenes, alkyl hydroxylation was observed, but no ring oxygenated products were detected with the 9,10-dialkylanthracenes. Because the results obtained with EtOOH and H_2O_2 were qualitatively similar, further HRP studies were done with H_2O_2. In other studies, we found that CPO also oxidized 9-Me-anthracene to 9-CH_2OH-anthracene, 10-Me-10-OH-9-anthrone, and anthraquinone in the presence of H_2O_2, but the rates were only ∼2% of those measured with HRP, and further studies were not done with this system.

The same products seen in the HRP oxidations were detected in a system involving P450 2B1, NADPH−P450 reductase, L-α-dilauroyl-*sn*-glycero-3-phosphocholine, NADPH, and O_2 (referred to as P450 2B1/NADPH/O_2 in subsequent work and contrasted with P450/PhIO) (Table 3). Qualitatively, the patterns seen with all of the anthracene derivatives were very similar to the HRP and CPO patterns in which ring oxidation products, e.g., anthrones, dominated with the singly-substituted anthracenes but side-chain oxidation products dominated with the doubly-substituted anthracenes. Again, replacement of a side-chain proton by TMS reinforced the side-chain oxidation pathway.

The formation of anthraquinone from 9-Me- or 9-CH_2-OH-anthracene requires loss of the methyl group. Previous work with inorganic model systems involving hydroxylated alkylanthracenes suggested the formation of HCHO (Camaioini, 1986; Rogan *et al*., 1979). With the P450/NADPH/O_2 system, production of HCHO was nearly stoichiometric with anthraquinone (Table 4). In the case of HRP, the level of HCHO was only about one-third that of anthraquinone. When the assays were repeated with 9-Me(C^2H_3)anthracene, the amount of HCHO was reduced by about half. (No isotope effect was observed for formation of 10-Me-10-OH-9-anthrone or anthraquinone; a large isotope effect was seen for 9-CH_2OH-anthracene production.) However, when 9-CH_2-OH was oxidized by HRP/H_2O_2, the amount of HCHO formed was very similar to that of anthraquinone.

Oxidation of 9-Et- and 9,10-Et_2-Anthracenes. The use of ethylanthracenes, which because of "flagpole" interactions cannot achieve the planarity necessary for facile deprotonation, has been shown to divert cation radical-mediated oxidation pathways to ring oxidation (Tolbert *et al*., 1990). Thus, its use would be expected to increase the lifetime of any cation radicals generated during oxidation.

The visible spectrum of 9,10-Me_2-anthracene cation radical has been reported (Masnovi *et al*., 1985) and is characterized, in part, by a broad blue band at 660 nm (in strong acid solution). The spectrum of 9,10-Et_2-anthracene should be

Table 4: Formation of HCHO from 9-Me-Anthracene and 9-CH_2OH-Anthracene

enzyme system	substrate	product, μM			
		9-CH_2OH-anthracene	10-Me-10-OH-9-anthrone	anthraquinone	HCHO
HRP/EtOOH[a]	9-Me-anthracene	1.45 ± 0.08	3.78 ± 0.46	5.05 ± 0.13	1.83 ± 0.28
	[9-C^2H_3]anthracene	0.21 ± 0.02	4.07 ± 0.80	4.21 ± 0.48	0.99 ± 0.08
	9-CH_2OH-anthracene		<0.1	2.85 ± 0.30	2.10 ± 0.22
P4502B1/NADPH/O_2	9-Me-anthracene	1.88 ± 0.29	0.43 ± 0.04	0.48 ± 0.03	0.35 ± 0.06

[a] The substrate concentration was 100 μM, the HRP concentration was 0.23 μM, the EtOOH concentration was 1.6 mM, and the incubation time was 10 min (25 °C). Results are presented as means ± SD (n = 3).

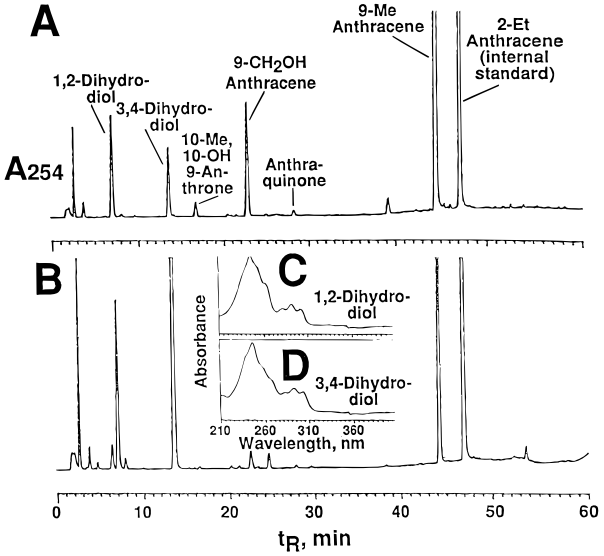


FIGURE 1: HPLC of products of 9-Me-anthracene oxidation. (A) Rat liver microsomes/NADPH/O_2 (5,6-benzoflavone-treated rats); (B) rat liver microsomes/NADPH/O_2 (phenobarbital-treated rats). The inset (parts C and D) shows the UV spectra of the two dihydrodiols (C, 1,2-dihydrodiol; D, 3,4-dihydrodiol).

indistinguishable. We attempted to observe such a spectrum when the compound was mixed with HRP and H_2O_2. Observation of the cation radical, if one indeed formed, was found to be obscured by changes in the HRP spectrum (compound I/II).

9-Et-anthracene was oxidized to 9-(1-hydroxyethyl)anthracene by both a P450 2B1/NADPH/O_2 system and an HRP/H_2O_2 system (Tables 2 and 3).

Anthracene, 9-Et-anthracene, and 9,10-Et_2-anthracene were all oxidized by microsomal preparations to products that had HPLC t_R values and UV spectra similar to those expected for dihydrodiols (Figure 1). None of these products were detected in the HRP or CPO systems or with P450 [systems devoid of epoxide hydrolase, where phenols (which were not analyzed) would be expected].

Dependence of 9-Me-Anthracene Oxidation Reactions on O_2. All reactions in the P450/NADPH/O_2 system require O_2, and the removal of O_2 using alternate vacuum/Ar cycles reduced the formation of 9-CH_2OH-anthracene, 10-OH-10-Me-9-anthrone, and anthraquinone to a low but finite level (Table 5). The sum of the products recovered in the anaerobic system appears to represent the level of O_2 remaining in the system. We avoided the addition of O_2-utilizing enzymes (e.g., glucose oxidase and catalase) because of possible artifacts due to the production and destruction of H_2O_2. The removal of O_2 from the solvent used for product extractions (CH_2Cl_2) and workup in closed systems did not affect product yields, nor did the addition of 20 μM butylated hydroxytoluene to the extraction system.

Table 5: Dependence of Anthracene Product Formation on O_2

system	product, nmol[a]		
	9-CH_2OH-anthracene	10-Me-10-OH-9-anthrone	anthraquinone
P450, NADPH, $-O_2$	3, 4	6, 7	1, 1
P450, NADPH, $+O_2$	34, 40	17, 24	3, 6
P450, PhIO, $-O_2$	88, 99	268, 370	34, 43
P450, PhIO, $+O_2$	123, 123	373, 535	48, 77
HRP, $-O_2$ (anaerobic CH_2Cl_2)	22, 34	21, 37	18, 25
HRP, $-O_2$	30, 38	39, 44	27, 27
HRP, $+O_2$	53, 53	119, 131	63, 64

[a] Results of duplicate experiments are presented.

The P450/PhIO system was relatively insensitive to the removal of O_2. The individual rates showed variability due to difficulties in controlling the short reaction time (10 s) in these experiments, which were done in the same complex glassware system under both anaerobic and aerobic conditions.

In the HRP/H_2O_2 system, the formation of all three products was decreased in the absence of O_2. However, considerable amounts of all three products were still formed, much more than the apparent limit seen for the anaerobic control for the P450/NADPH/O_2 system.

Oxygen Labeling Studies. Preliminary experiments were done to investigate rates of exchange of the oxygen atoms in the products of interest with $H_2{}^{18}O$. Mass spectrometric measurements indicated exchange of <3% for 9-CH_2OH-anthracene, 10-Me-10-OH-9-anthrone, and anthraquinone after 60 min and respective values of 0.9, 3, and 19% after 24 h at 23 °C.

No ^{18}O label from $H_2{}^{18}O_2$ was incorporated into any of the products formed by HRP (Table 6).

No label from $H_2{}^{18}O$ was incorporated into 9-CH_2OH-anthracene, 10-Me-10-OH-9-anthrone, or anthraquinone formed by P450. A significant level of $H_2{}^{18}O$ label was incorporated into all of these products in the HRP/H_2O_2 system, although the level in 9-CH_2OH-anthracene was considerably less than in the other two products.

The incorporation of label from $^{18}O_2$ into 9-CH_2OH-anthracene by the P450 system was nearly quantitative (Table 6). Incorporation into the two dioxygenated products was extensive but not complete. The fraction of product with two ^{18}O atoms was greater than that with one. We cannot account for the lack of complete incorporation, since no label from $H_2{}^{18}O$ was incorporated into these products in repeated experiments. If the oxygen atoms in the dioxygenated products are incorporated in a stepwise mechanism, then the values shown (for the dioxygenated products) should be divided by 0.79 for proper statistical correction and are higher. Label from $^{18}O_2$ was also incorporated into all products in the HRP/H_2O_2 system. Labeling was extensive

Table 6: Incorporation of ^{18}O into Anthracene Products

system	^{18}O source	% excess abundance ^{18}O: 9-CH_2OH-anthracene	10-OH-10-Me-9-anthrone (M+2/M+4)	anthraquinone (M+2/M+4)[a]
FeTPP, PhIO	$^{18}O_2$[b]	*e*	−/−	−/−
P450/NADPH/O_2	$H_2{}^{18}O$[c]	*e*	−/−	−/−
	$^{18}O_2$[b]	93	38/60	25/43
HRP/H_2O_2	$H_2{}^{18}O_2$[d]	*e*	−/−	−/−
	$H_2{}^{18}O$[c]	11	75/25	56/9
	$^{18}O_2$[b]	87	68/14	62/18

[a] The slash separates the values found for the M+2 and M+4 peaks. [b] A reaction was done at the same time with the P450/NADPH/O_2 system, and the conversion of toluene (100 μM) to benzyl alcohol was analyzed (GC/MS). The value for atomic excess (from selective ion monitoring GC/MS) was 79% and was used to normalize the other values for ^{18}O, as presented in the table; raw atomic excess values were divided by 0.79 (for both mono- and dioxygenated products). [c] The excess abundance in the $H_2{}^{18}O$ was 66%; this value was used to normalize the results, which are shown. [d] The $H_2{}^{18}O_2$ had 90% excess abundance. [e] A blank indicates <3% detected.

for 9-CH_2OH-anthracene. With the dioxygenated products, the major fraction of product contained one labeled oxygen, not two. The sum of labeling is nearly enough to account for all coming from the proffered $H_2{}^{18}O$ and $^{18}O_2$.

Kinetic Deuterium Isotope Effects. Preliminary studies suggested kinetic deuterium isotope effects for formation of some of the products by the P450 and HRP systems (Table 4). These were investigated in more detail; noncompetitive V_{max} (k_{cat}) and K_m values were determined, and isotope effects are presented according to the convention of Northrop (1982).

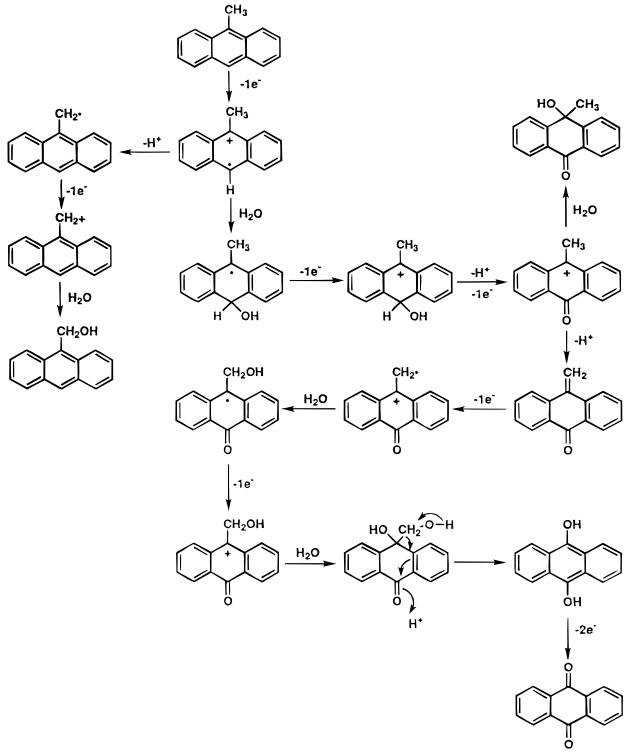
The most striking aspect of the results was the isotope effects seen for 9-Me hydroxylation by HRP. The isotope effect on V_{max} (k_{cat}), ^DV, was 5.7 ($\pm$1.3, $n = 3$, SD corrected for variability in both numerator and denominator). (The K_m, 9.2 μM, was unchanged with the deuterated substrate.) The ^DV values for the oxidation of 9-Me-anthracene to 10-Me-10-OH-anthracene and anthraquinone by HRP/H_2O_2 were 1.1 $\pm$ 0.3 and 1.3 $\pm$ 0.2, respectively. A smaller isotope effect was seen for the formation of 9-CH_2OH-anthracene by P450, $^DV = 2.8 \pm 0.8$. The other two P450-catalyzed oxidations (to 10-Me-10-OH-anthracene and anthraquinone) showed no significant isotope effects ($^DV = 1.4 \pm 0.3$ for both). In the oxidation of 9,10-Me_2-anthracene to 9-CH_2-OH-10-Me-anthracene, the ^DV for HRP was 8.3 ($\pm$3.5) and for P450 was 2.2 ($\pm$0.5), similar to the pattern seen for 9-Me-anthracene hydroxylation.

DISCUSSION

Similar products are produced in the oxidation of the model polycyclic hydrocarbon 9-Me-anthracene by three iron porphine systems, including a biomimetic model, a P450, and a peroxidase. The results of the O_2 dependence and ^{18}O labeling studies indicate that at least three mechanisms are involved in the production of the major products—9-CH_2-OH-anthracene, 10-Me-10-OH-9-anthrone, and anthraquinone—by the HRP, P450, and FeTPP/PhIO systems. Proposed mechanisms are presented in Schemes 2−4 and have some precedent in earlier inorganic oxidations of 7,12-dimethylbenz[*a*]anthracene by Fried and Schumm (Fried & Schumm, 1967; Fried, 1974).

The first mechanism involves the anaerobic oxidation of 9-Me-anthracene by HRP (Scheme 2). This pathway parallels that postulated for inorganic oxidants in aqueous systems

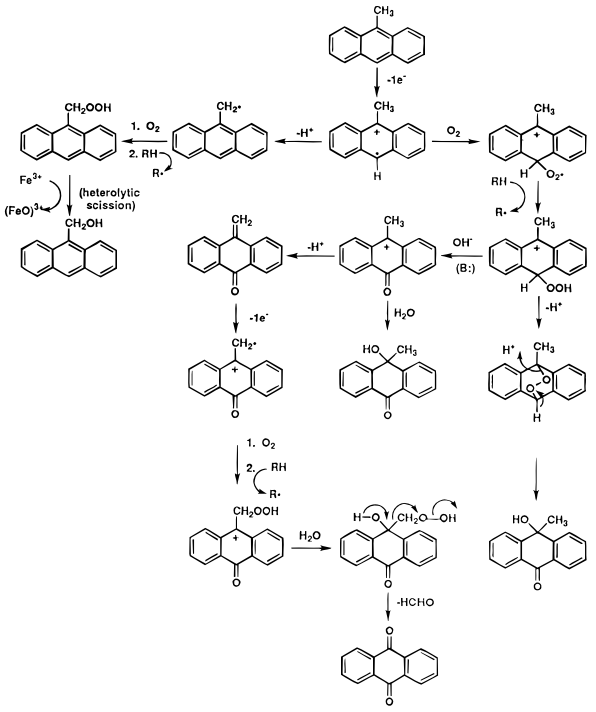
Scheme 2: Proposed Mechanism of Anaerobic Oxidation of 9-Me-Anthracene by HRP



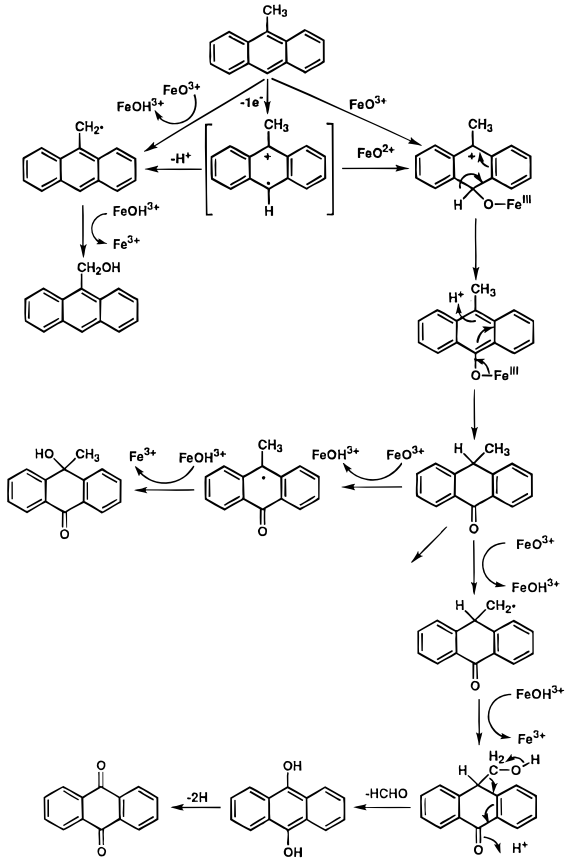
(Fried & Schumm, 1967; Fried, 1974). No label from $H_2{}^{18}O_2$ was incorporated into any of the products, ruling out any mechanism involving direct hydroxylation by the FeO entity. Because formation of the products occurred in the absence of O_2 and partial incorporation of label from $H_2{}^{18}O$ occurred (under aerobic conditions), a pathway is postulated where no O_2 is used. The initial reaction is one-electron oxidation to form the radical cation. Deprotonation of the methyl group, further one-electron oxidation, reaction with H_2O, and abstraction of a hydrogen atom yield 9-CH_2OH-anthracene. The other oxidized products are rationalized by formal addition of OH^- to the ring at the *meso* site. Further oxidation and reaction of H_2O yielded 10-Me-10-OH-9-anthrone, a terminal product. The partition between side-chain and ring oxidation is reinforced by the observation that both TMS-labeled anthracenes undergo more efficient side-chain cleavage, a known phenomenon associated with radical cation intermediates (Li *et al*., 1991). The cationic anthrone intermediate can also undergo a series of oxidations and H_2O additions to yield 9,10-dihydroxyanthracene, which would readily oxidize to anthraquinone. Variations of this mechanism can also be used to explain the oxidation of 9-CH_2-OH-anthracene to anthraquinone. There are also alternate possibilities, such as the addition of H_2O to 9-hydroxy-10-Me-10-yl radical to generate 10-Me-9-anthrone, which could be subsequently oxidized. This latter compound accumulated in the FeTPP system but was not detected in the HRP reaction and is not presented in the pathway shown in Scheme 2.

A separate aerobic HRP pathway is postulated to explain the incorporation of label from $^{18}O_2$ into products and the decreased formation of the three major products when O_2 is deleted from system (Scheme 3). The mechanism has precedent in some aspects of lipid peroxidation and peroxidase-supported epoxidations (Dix & Marnett, 1981). As in the anaerobic system, the initial event is oxidation to a radical

Scheme 3: Proposed Mechanism of Aerobic Oxidation of 9-Me-Anthracene by HRP



Scheme 4: Proposed Mechanism of Oxidation of 9-Me-Anthracene by P450 or FeTPP/PhIO



cation. Loss of a proton yields a methylene radical, which reacts with O_2 to yield the alkyl peroxyl radical. Abstraction of a hydrogen atom yields the peroxide, which can be heterolytically cleaved by HRP to generate 9-CH_2OH-anthracene.[2] The other oxidized products are postulated to result from addition of O_2 to the ring radical. Abstraction of a hydrogen atom by the peroxyl radical, deprotonation, and reaction of the peroxy anion with the carbocation yield the cyclic peroxide, which has precedent in the similar product reported by Fried for oxidation of 7,12-dimethylbenz[*a*]anthracene (Fried & Schumm, 1967; Fried, 1974). Simple heterolytic scission of the cyclic peroxide yields 10-Me-10-OH-9-anthrone. The zwitterionic peroxy anion can also rearrange to the anthrone (still with the carbocation) (Harvey *et al*., 1992). Deprotonation yields the methide/anthrone, which could undergo a further one-electron oxidation. The methylene radical formed could react with O_2 to form a peroxyl radical which could abstract a hydrogen atom and lose a proton. Reaction of the ring carbocation with H_2O yields the tetrahedral species, which could decompose to give anthrone, HCHO, and OH^-.

The two pathways presented for HRP are not mutually exclusive, and a combination of steps of each may be involved in aerobic HRP oxidations, since several of the intermediates are similar. Such a mixture of mechanisms would explain the discordance in the event of ^{18}O labeling between the three products and the incorporation of only one ^{18}O into some of the dioxygenated products recovered. HRP did catalyze the conversion of 9-CH_2OH-anthracene to anthraquinone at a slower rate (than 9-Me-anthracene) (Tables 2, 4), but it is unknown whether oxygen from O_2 or H_2O is incorporated. Mechanisms can be drawn involving one-electron oxidation of 9-CH_2OH-anthracene and addition of H_2O or O_2 at the 10 position, with steps roughly paralleling those shown in Schemes 2 and 3 to lead to anthraquinone (not presented).

The mechanisms of the FeTPP/PhIO- and P450-catalyzed oxidations appear to be quite distinct from either of the HRP reactions discussed above (Scheme 4). The FeTPP/PhIO system was devoid of H_2O, and no label from $H_2{}^{18}O$ was incorporated into any of the products in the P450 system, but label from $^{18}O_2$ was extensively incorporated.[3] No label from $^{18}O_2$ was incorporated into the products in the FeTPP/PhIO system, indicating that the oxygen should have all come from PhIO [presumably via an Fe−O complex (McMurry & Groves, 1986)]. This is implicated in all oxidations catalyzed by the system; i.e., hydrogen atom abstraction or electron abstraction/deprotonation is the first step, and rapid rebound of oxygen from the iron is involved. With the P450/NADPH/O_2 system, it is not possible to discriminate between the abstraction/oxygen rebound mechanism and the type shown in Scheme 3 for HRP. However, we did find that yields of products formed in the P450/PhIO system were not attenuated by the removal of O_2 (Table 5). This finding can be interpreted as evidence that the mechanism does not involve reaction of radical cations with O_2 of the sort shown in Scheme 3. If the Fe−O complexes formed in the P450/NADPH/O_2 and P450/PhIO systems are identical, there should be no inherent reason why the rate of oxygen rebound (to a carbon radical) should be different in the two systems,

[2] A caveat about this last proposal is that this peroxide might be too large to approach the iron of the heme of HRP, as there is evidence to support the view that it is hindered (Ortiz de Montellano, 1987; Okazaki & Guengerich, 1993). However, *tert*-butyl and cumene hydroperoxides can support HRP reactions (Kedderis & Hollenberg, 1983).

[3] Prough *et al*. (1981) examined the oxidation of 3-hydroxybenzo[*a*]pyrene to the 3,6-quinone and an unidentified oxidation product and reported extensive (but not quantitative) incorporation of label from $^{18}O_2$; labeling from $H_2{}^{18}O$ was not examined.

and aspects of Scheme 3 would be precluded. 10-Me-9-anthrone was detected as a major product in the FeTPP/PhIO reaction but not with P450/NADPH/O_2; it is indicated as an intermediate in both reactions.

HCHO was produced in oxidations catalyzed by the P450/NADPH/O_2 and HRP/H_2O_2 systems (Table 4). In the case of P450, the amount of HCHO was nearly stoichiometric with that of anthraquinone, and Scheme 4 presents a reasonable explanation. In the (aerobic) oxidation of 9-CH_2-OH-anthracene by HRP, equimolar stoichiometry was also observed (Table 4). However, the yield of HCHO is considerably less than that of anthraquinone in the HRP-catalyzed oxidation of 9-Me-anthracene (Table 4). A possible explanation is that the 9-methyl moiety is removed as a different entity than HCHO in the aerobic systems (Scheme 3).

A substantial kinetic deuterium isotope effect was seen for methyl hydroxylation by HRP with both 9-Me- and 9,10-Me_2-anthracene. These isotope effects were not seen for oxidation of 9-Me-anthracene to other products by HRP. An explanation for the high isotope effects comes from Schemes 2 and 3, with the rate-limiting step being deprotonation of the cation radical to a form with the resulting methylene bearing a radical. The lower kinetic isotope effects observed with P450 are consistent with other P450-catalyzed carbon hydroxylations, which usually have large intrinsic hydrogen isotope effects and small intermolecular isotope effects (Groves *et al*., 1978; Ullrich, 1969; Ortiz de Montellano, 1986).

In the oxidations catalyzed by P450 and FeTPP/PhIO, we cannot unambiguously distinguish between hydrogen abstraction and one-electron oxidation/rearrangement (preceding oxygen rebound) on the basis of the available evidence. The oxidation−reduction potentials of the alkylanthracenes are certainly low enough for an Fe−O complex of FeTPP, HRP, or P450 to carry out one-electron oxidations (Lee *et al*., 1985; Hayashi & Yamazaki, 1979; Macdonald *et al*., 1989). The distinction between HRP and P450 in labeling from $H_2{}^{18}O$ (Table 6) and kinetic deuterium isotope effects might argue that the radical cation should not be an intermediate in both systems, but it is conceivable that a P450 hypervalent complex (but not HRP) might catalyze the rearrangement of a cation radical [as evidence has been provided in the case of aminium radicals (Okazaki & Guengerich, 1993)]. Also, the difference in kinetic isotope effects may be the result of the slower nature of P450 oxygen activation. Discrimination between hydrogen and one-electron abstraction by P450s in the oxidation of polycyclic hydrocarbons is the subject of further research in our laboratories.

An initial interest in this research project was the question of whether epoxidation is necessarily linked to one-electron oxidation of polycyclic hydrocarbons. The trend observed in the model systems has been that a side-chain ethyl group inhibits side-chain oxidation while a side-chain TMS group promotes side-chain oxidation, in mechanisms for which cation radicals are implicated. It is curious that this motif seems to apply here with HRP but not with P450. In the P450 case, however, anthraquinones dominate the oxygenated products and may obscure intermediate oxidized forms. As in the case of P450, the sluggishly-deprotonated ethylanthracenes exhibit significant dihydrodiol formation with microsomes. However, this formation is not significantly enhanced relative to the same reaction with 9,10-Me_2-anthracene. What is clearly not consonant with the usual cation radical-mediated mechanistic scheme is the nucleophilic attack of an intermediate anthracenylmethyl cation, following deprotonation/oxidation. In such a case, ^{18}O incorporation in the presence of $H_2{}^{18}O$ would have predominated. Thus, these results fail to provide evidence for one-electron oxidation in the epoxidation pathway, and an alternate mechanistic explanation is in order. Numerous possibilities have been proposed, but we currently favor a σ complex (possibly preceded by a π-complex) that rearranges to a phenol or epoxide (Ostovic & Bruice, 1989; Guengerich & Macdonald, 1990).

Finally, the relevance of this work to studies on oxidation of polycyclic hydrocarbons and DNA adduct formation is considered. The oxidation potentials of the alkylanthracenes considered here are similar to carcinogenic polycyclic hydrocarbons (Cremonesi *et al*., 1992) and accessible to FeO complexes of FeTPP (Groves & Gilbert, 1986), HRP (Hayashi & Yamazaki, 1979), and P450 (Macdonald *et al*., 1989). Electrochemical oxidation of 6-substituted benzo[*a*]pyrenes in the presence of nucleosides yields guanyl N^7 and C^8 adducts (RamaKrishna *et al*., 1992, 1993), which are also detected as products following incubation of DNA with HRP/H_2O_2 (Cavalieri *et al*., 1988; Rogan *et al*., 1988) and microsomal systems containing P450s (and NADPH and O_2) (Devanesan *et al*., 1992; Cavalieri *et al*., 1990). These reports are all consistent with the view that HRP and P450s produce cation radicals that are capable of forming these adducts. However, we did not find any evidence of incorporation of H_2O into 9-Me-anthracene oxidation products formed by P450 2B1, in contrast to HRP, and attempts at direct observation of polycyclic cation radicals by P450 (and HRP) have been unsuccessful to date. How can the results be reconciled? There are several possibilities: (i) Benzo[*a*]pyrene forms a more stable radical than does 9-Me-anthracene, and the latter is not a good model for the former. It is conceivable that rearrangement and oxygen rebound could be faster with 9-Me-anthracene, or even that P450 2B1 is more effective in this regard than the other P450s in enzyme preparations examined to date. (ii) The DNA adducts identified in the work of Cavalieri and Rogan are consistent with reactions involving radical cations but alternative mechanisms occur (e.g., reaction of electrophilic products other than radicals). (iii) Guanyl residues are more effective at reacting with radical cations than is H_2O. None of these possibilities is completely satisfactory but neither can any be dismissed at this time. We began these studies with the expectation that careful examination of identical substrates under identical conditions, using purified enzymes, would minimize controversy surrounding mixed-function oxidases and the mechanism of aromatic oxidation. Our results have narrowed the scope of possible mechanisms and have provided some insight into the rate of cation radicals, at least in the case of HRP oxidations. Further study of polycyclic cation radicals in P450 and peroxidase reactions is in order in light of the questions raised here.

ACKNOWLEDGMENT

We thank M. V. Martin for preparing P450 2B1, C. G. Turvy for preparing NADPH−P450 reductase, and B. Fox and B. Nobes for technical assistance with aspects of GC/MS.

SUPPORTING INFORMATION AVAILABLE

Supplementary information is available (5 pages) and includes HPLC t_R parameters and UV λ_{max} values for all compounds used in this work, plus mass spectra and selected ion monitoring traces for the formation of 9-CH_2OH-anthracene, 10-Me-10-OH-9-anthrone, and anthraquinone in the presence of HRP/H_2O_2 and $^{18}O_2$. Ordering information is given on any current masthead page.

REFERENCES

Burleigh, B. D., Jr., Foust, G. P., & Williams, C. H., Jr. (1969) *Anal. Biochem. 27*, 536−544.

Camaioini, D. M. (1986) in *Report No. GRI-86/0100*, Gas Research Institute, Chicago, IL.

Cavalieri, E. L., & Rogan, E. G. (1984) in *Free Radicals in Biology*, pp 323−369, Academic Press, Inc., New York.

Cavalieri, E. L., Devanesan, P. D., & Rogan, E. G. (1988) *Biochem. Pharamcol. 37*, 2183−2187.

Cavalieri, E. L., Rogan, E. G., Devanesan, P. D., Cremonesi, P., Cerny, R. L., Gross, M. L., & Bodell, W. J. (1990) *Biochemistry 29*, 4820−4827.

Conney, A. H. (1982) *Cancer Res. 42*, 4875−4917.

Cook, J. W., Hewett, C. L., & Hieger, I. (1933) *J. Chem. Soc.*, 394−405.

Cremonesi, P., Rogan, E., & Cavalieri, E. (1992) *Chem. Res. Toxicol. 5*, 346−355.

Devanesan, P. D., RamaKrishna, N. V. S., Todorovic, R., Rogan, E. G., Cavalieri, E. L., Jeong, H., Jankowiak, R., & Small, G. J. (1992) *Chem. Res. Toxicol. 5*, 302−309.

Dipple, A., Moschel, R. C., & Bigger, C. A. H. (1984) in *Chemical Carcinogens (ACS Monograph 182)* (Searle, C. E., Ed.) pp 41−163, American Chemical Society, Washington, D.C.

Dix, T. A., & Marnett, L. J. (1981) *J. Am. Chem. Soc. 103*, 6744−6746.

Fleischer, E. B., Palmer, J. M., Srivastava, T. S., & Chatterjee, A. (1971) *J. Am. Chem. Soc. 93*, 3162−3167.

Flesher, J. W., Myers, S. R., & Stansbury, K. H. (1990) *Carcinogenesis 11*, 493−496.

Fried, J. (1974) in *Chemical Carcinogenesis, Part A* (Ts'o, P. O., & DiPaolo, J. A., Eds.) pp 197−215, Marcel Dekker, New York.

Fried, J., & Schumm, D. E. (1967) *J. Am. Chem. Soc. 89*, 5508−5509.

Fung, K., & Grosjean, D. (1981) *Anal. Chem. 53*, 168−171.

Groves, J. T., & Gilbert, J. A. (1986) *Inorg. Chem. 25*, 123−125.

Groves, J. T., McClusky, G. A., White, R. E., & Coon, M. J. (1978) *Biochem. Biophys. Res. Commun. 81*, 154−160.

Guengerich, F. P. (1983) *Biochemistry 22*, 2811−2820.

Guengerich, F. P. (1987) in *Mammalian Cytochromes P-450* (Guengerich, F. P., Ed.) Vol. 1, pp 1−54, CRC Press, Boca Raton, FL.

Guengerich, F. P. (1994) in *Principles and Methods of Toxicology* (Hayes, A. W., Ed.) pp 1259−1313, Raven Press, Ltd., New York.

Guengerich, F. P., & Martin, M. V. (1980) *Arch. Biochem. Biophys. 205*, 365−379.

Guengerich, F. P., & Macdonald, T. L. (1990) *FASEB J. 4*, 2453−2459.

Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., & Kaminsky, L. S. (1982) *Biochemistry 21*, 6019−6030.

Harvey, R. G., Abu-Shqara, E., & Yang, C. X. (1992) *J. Org. Chem. 57*, 6313−6317.

Hayashi, Y., & Yamazaki, I. (1979) *J. Biol. Chem. 254*, 9101−9106.

Jerina, D. M. (1983) *Drug Metab. Dispos. 11*, 1−4.

Julian, P. L., Cole, W., & Diemer, G. J. (1945) *J. Am. Chem. Soc. 67*, 1721.

Kedderis, G. L., & Hollenberg, P. F. (1983) *J. Biol. Chem. 258*, 8129−8138.

LaVoie, E. J., Coleman, D. T., Rice, J. E., Geddie, N. G., & Hoffman, D. (1985) *Carcinogenesis 6*, 1483−1488.

Lee, W. A., Calderwood, T. S., & Bruice, T. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4301−4305.

Lehr, R. E., Kumar, S., Levin, W., Wood, A. W., Chang, R. L., Conney, A. H., Yagi, H., Sayer, J. M., & Jerina, D. M. (1985) in *Polycyclic Hydrocarbons and Carcinogenesis, ACS Symposium Series 283* (Harvey, R. G., Ed.) pp 63−84, American Chemical Society, Washington, D.C.

Li, Z., Sirimanne, S. R., VanderVeer, D. R., & Tolbert, L. M. (1991) *J. Am. Chem. Soc. 113*, 1766−1769.

Liebermann, C. (1882) *J. Liebigs Ann. Chem. 212*, 109−121.

Macdonald, T. L., Gutheim, W. G., Martin, R. B., & Guengerich, F. P. (1989) *Biochemistry 28*, 2071−2077.

Masnovi, J. M., Kochi, J. K., Hilinski, E. F., & Rentzepis, P. M. (1985) *J. Phys. Chem. 89*, 5387.

McMurry, T. J., & Groves, J. T. (1986) in *Cytochrome P-450* (Ortiz de Montellano, P. R., Ed.) pp 1−28, Plenum Press, New York.

Miller, E. C. (1951) *Cancer Res. 11*, 100−108.

Northrop, D. B. (1982) *Methods Enzymol. 87*, 607−625.

Oesch, F., Kaubisch, N., Jerina, D. M., & Daly, J. W. (1971) *Biochemistry 10*, 4858−4866.

Okazaki, O., & Guengerich, F. P. (1993) *J. Biol. Chem. 268*, 1546−1552.

Ortiz de Montellano, P. R. (1986) in *Cytochrome P-450* (Ortiz de Montellano, P. R., Ed.) pp 217−271, Plenum Press, New York.

Ortiz de Montellano, P. R. (1987) *Acc. Chem. Res. 20*, 289−294.

Ostovic, D., & Bruice, T. C. (1989) *J. Am. Chem. Soc. 111*, 6511−6517.

Palmer, G., & Reedijk, J. (1992) *J. Biol. Chem. 267*, 665−677.

Prough, R. A., Saeki, Y., & Capdevila, J. (1981) *Arch. Biochem. Biophys. 212*, 136−146.

RamaKrishna, N. V. S., Gao, F., Padmavathi, N. S., Cavalieri, E. L., Rogan, E. G., Cerny, R. L., & Gross, M. L. (1992) *Chem. Res. Toxicol. 5*, 293−302.

RamaKrishna, N. V. S., Li, K. M., Rogan, E. G., Cavalieri, E. L., George, M., Cerny, R. L., & Gross, M. L. (1993) *Chem. Res. Toxicol. 6*, 837−845.

Rogan, E. G., Roth, R. G., & Cavalieri, E. (1979) in *Polynuclear Aromatic Hydrocarbons*: *Chemistry & Biological Effects* (Bjorseth, A., & Dennis, A. J., Eds.) pp 259, Battelle Press, Columbus, OH.

Rogan, E. G., Cavalieri, E. L., Tibbels, S. R., Cremonesi, P., Warner, C. D., Nagel, D. L., Tomer, K. B., Cerny, R. L., & Gross, M. L. (1988) *J. Am. Chem. Soc. 110*, 4023−4029.

Saltzman, H., & Sharefkin, J. G. (1973) *Org. Synth. Coll. Vol. 5*, 658−659.

Sirimanne, S. R., Li, Z., VanderVeer, D. R., & Tolbert, L. M. (1991) *J. Am. Chem. Soc. 113*, 1766.

Southern, P. F., & Waters, W. A. (1960) *J. Chem. Soc.*, 4340.

Starnes, W. H., Jr. (1970) *J. Org. Chem. 35*, 1974−1978.

Tamao, K., Kodama, S., Nakajima, I., Kumada, M., Minato, A., & Suzuki, K. (1982) *Tetrahedron 38*, 3347.

Tolbert, L. M., & Khanna, R. K. (1987) *J. Am. Chem. Soc. 109*, 3477−3478.

Tolbert, L. M., Khanna, R. K., Popp, A. E., Gelbaum, L., & Bottomley, L. A. (1990) *J. Am. Chem. Soc. 112*, 2373−2378.

Ts'o, P. O., Caspary, W. J., & Lorentzen, R. J. (1977) in *Free Radicals in Biology* (Pryor, W. A., Ed.) Vol. III, pp 251−303, Academic Press, New York.

Ullrich, V. (1969) *Hoppe-Seyler's Z. Physiol. Chem. 350*, 357−365.

Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337−5344.

BI952330F