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HYDROGEN PEROXIDE-SUPPORTED OXIDATION OF BENZO [*a*]PYRENE BY RAT LIVER MICROSOMAL FRACTIONS*

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Abstract—In the presence of liver microsomes from phenobarbital-pretreated rats, hydrogen peroxide oxidized benzo [*a*]pyrene to a number of biologically significant products at a rate that was approximately 20 per cent as fast as that seen by us and others with NADPH and oxygen. As with NADPH-dependent reactions [J. Capdevila, R. W. Estabrook, and R. A. Prough, *Archs. Biochem. Biophys.* **200**, 186 (1980)], the hydrogen peroxide-dependent reactions resulted in the production of relatively large quantities of dihydrodiols as metabolites. This was in marked contrast to the product distribution observed when cumene hydroperoxide was utilized as a cosubstrate (foregoing reference). The formation of the various organic-soluble metabolites was dependent on the presence of functional liver microsomal cytochrome P-450 in the reaction mixture. Approximately 48 per cent of the benzo[*a*]pyrene metabolites, however, was observed to be bound to microsomal protein, and inhibition of cytochrome P-450 function, by metyrapone or *N*-octylamine did not affect the extent of covalent binding of the hydrocarbon to the microsomal protein. The differences noted during benzo[*a*]pyrene metabolism using hydrogen peroxide strongly suggest that at least two distinct mechanisms exist to account for the oxidation of the hydrocarbon, i.e. epoxidation and one-electron oxidation reactions.

Cytochrome P-450 has been shown to function as a monooxygenase requiring NADPH, molecular oxygen, and the presence of a substrate to be oxidatively metabolized [1, 2]. More recently, it has been demonstrated that cytochrome P-450 catalyzes the peroxidative metabolism of various substrates using either hydrogen peroxide or organic peroxides as cosubstrates [3–7]. These observations led to the hypothesis that several steps in the catalytic cycle for cytochrome P-450 may be common when oxidative metabolism is supported by either NADPH and oxygen or peroxides. Experimental support for this has been demonstrated for a number of *N*-demethylation reactions [4] and for the aromatic hydroxylation of aniline and phenanthrene [8, 9]. This hypothesis, however, has been challenged recently in the case of the cumene hydroperoxide-supported *N*-demethylation of aminopyrine [10] and the oxidation of benzo[*a*]pyrene [11, 12]. In these two cases, the oxidation of substrates appeared to be related, in part, to the hemoprotein catalyzed production of free radical intermediates derived from either the substrate or the organic peroxide rather than from a formal oxenoid intermediate as proposed by Hamilton [13].

It has been suggested that the metabolites of B[*a*]P* may be derived from two reaction mechanisms that include epoxidation [13–15] and one-electron oxidation [12, 16, 17]. Therefore, benzo[*a*]pyrene serves as an attractive model substrate for comparing the cytochrome P-450-dependent reactions that are supported by either NADPH and oxygen or various peroxides. In this communication, we report the product distribution and some properties of the hydrogen peroxide-supported oxidation of B[*a*]P and discuss their relationship to the NADPH- and cumene hydroperoxide-supported reactions previously reported from our laboratories [12].

MATERIALS AND METHODS

Microsomal suspensions from livers of phenobarbital-pretreated rats were prepared as described previously [18]. [7, 10-^{14}C]Benzo[*a*]pyrene was purchased from Amersham/Searle (Arlington Heights, IL). The hydrocarbon was diluted with unlabeled B[*a*]P to obtain a specific radioactivity of approximately 2 μCi/mmole and was subsequently purified

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* Abbreviations: B[*a*]P, benzo[*a*]pyrene; E.S.R., electron spin resonance; PB, phenobarbital; SDS, sodium dodecylsulfate; and h.p.l.c., high pressure liquid chromatography.

Table 1. Characteristics of the hydrogen peroxide-dependent oxidation of benzo[*a*]pyrene by hepatic microsomes from PB-treated rats*

Metabolite distribution	Denatured microsomal protein†	Native microsomal protein		
		Control	Octylamine (0.2 mM)	Metyrapone (0.5 mM)
Organic soluble	250 ± 65	1120 ± 300	289 ± 30	397 ± 55
Protein bound	191 ± 30	985 ± 165	850 ± 75	1010 ± 130
Total	441	2105	1139	1407

* Reactions were initiated by addition of hydrogen peroxide (10 mM) and were terminated after a 1-min incubation as described in Materials and Methods. The values are expressed as pmoles of product produced per mg protein per min. Each value is the average of five experiments with a standard deviation equal to 10–15 per cent of the average. The recovery of radioactivity from the h.p.l.c. column was > 93 per cent.

† Denatured microsomes were prepared by incubating microsomal protein at 90° for 10 min prior to addition of protein to the reaction mixture.

by h.p.l.c. (isocratic 80% methanol in water using a Water's Associates μBondapak C18 column, Milford, MA). The authentic B[*a*]P standards were obtained from the National Cancer Institute Carcinogenesis Research Program, Bethesda, MD. [7, 10-^{14}C]-3-Hydroxybenzo[*a*]pyrene was prepared by incubating liver microsomes, NADPH, and oxygen with [^{14}C]-B[*a*]P and collecting the radioactivity that comigrated with the 3-phenol standard by h.p.l.c. This fraction could be rechromatographed without appreciable formation of the 3,6-B[*a*]P-quinone and was considered to be relatively free from any 6-phenol.

Hydrogen peroxide (30%) was purchased from the Mallinckrodt Chemical Co. (St. Louis, MO) and diluted to an appropriate concentration prior to use. A standard reaction mixture that contained 0.5 to 2.0 mg/ml of microsomal protein, 1 mM sodium azide, 5 mM magnesium chloride, 150 mM potassium chloride, and either 0.08 mM B[*a*]P, or 0.05 mM 3-hydroxy-B[*a*]P, or 0.05 mM, 6-hydroxy-B[*a*]P, in 50 mM Tris–HCl buffer, pH 7.5, was preincubated at 37° for 2 min prior to the initiation of the reaction by the addition of hydrogen peroxide (0.5 to 40 mM final concentration). The reaction was terminated by adding chilled ethyl acetate and, following extraction, the h.p.l.c. analysis of the products was performed as described by Prough *et al.* [18]. When either 0.2 mM octylamine or 0.5 mM metyrapone was used as an inhibitor, they were added prior to the addition of the hydrocarbon. The hydrogen peroxide-dependent formation of free radicals from B[*a*]P or its 6-phenol was monitored by the E.S.R. techniques described by Capdevila *et al.* [12].

The binding of B[*a*]P or its 3-phenol to microsomal protein was analyzed as follows. After extracting the incubation mixture successively with 5 vol. of ethyl acetate, the water phases which contained denatured protein were filtered through 0.45 μm filters, washed with 5 vol. of 0.15 M sodium chloride, and dried at 50°. The radioactivity associated with the protein was retained by the filter and was measured using liquid scintillation techniques [12, 18]. The fluorescence of the hydrocarbon bound to the denatured protein was recorded using an Aminco–Bowman spectrophotofluorometer (American Instruments Co., Silver Springs, MD) after solubilization by the addition of 10% sodium dodecylsulfate.

RESULTS AND DISCUSSION

Hepatic microsomes obtained from PB-pretreated rats catalyzed the hydrogen peroxide-dependent oxidation of B[*a*]P at rates that were substantially slower than those observed with either the NADPH- or cumene hydroperoxide-supported reactions [12]. The dependence of the reaction rate on microsomal protein concentration, hydrogen peroxide concentration, and the time of incubation was determined (data not shown). The rates of B[*a*]P oxidation were linear for only the first minute of the reaction, but were linear with protein concentrations up to 1.0 mg/ml. Concentrations of hydrogen peroxide above 5 mM did not cause any further increase in the rates of B[*a*]P oxidation.

Table 2. Benzo[*a*]pyrene metabolite distribution obtained in the presence of microsomal protein from PB-treated rats and 10 mM hydrogen peroxide

	Organic-soluble products [pmoles · min^{-1} · mg^{-1}]	Per cent of total Metabolites*		
		Diols	Quinones	Phenols
Native microsomes	1120 ± 300	25 ± 8	55 ± 7	20 ± 6
Denatured microsomes	250 ± 65	7 ± 2	73 ± 11	20 ± 8
Net	850	26	49	25

* The metabolite profile shown represents the amount of radioactive product that comigrates with the following B[*a*]P metabolites: Diols, 4,5-, 7,8-, and 9,10-dihydrodiols; Quinones, 1,6-, 3,6-, and 6,12-quinones; and phenols, 3- and 9-phenols. The samples were obtained as described in Table 1, and the recovery from the h.p.l.c. columns was greater than 85 per cent for all samples run. Heat-denatured microsomes were prepared as described in the legend for Table 1. The values of the per cent of total identified metabolites are the average of five experiments and have a standard deviation equal to 10–15 per cent of the average.

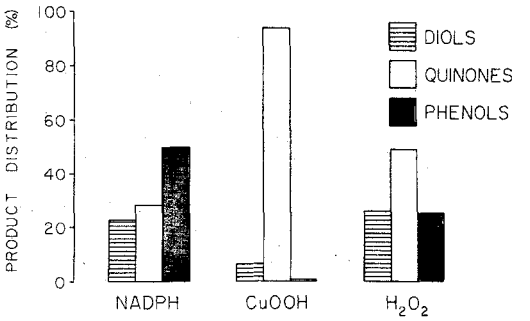

Fig. 1. Distributions of organic-soluble products generated during the oxidation of benzo[*a*]pyrene by either NADPH and oxygen or, cumene hydroperoxide, or by hydrogen peroxide. The data shown for the NADPH- and cumene hydroperoxide-supported reactions were taken from Ref. 12. The reaction mixtures containing 0.5 mg/ml of liver microsomes from PB-treated rats, 0.08 mM B[*a*]P, and 10 mM hydrogen peroxide were terminated with chilled ethyl acetate after a 1-min incubation. The ethyl acetate phases were dried under a nitrogen stream and analyzed using h.p.l.c. The values are expressed as the percent of the total identified products that partition into the ethyl acetate phase (see Materials and Methods).

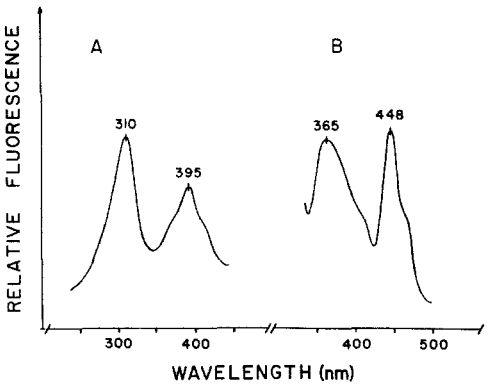

Fig. 3. Fluorescence properties of the protein-bound 3-hydroxy-benzo[*a*]pyrene metabolite(s). The reaction was performed as described in Fig. 2, and 5 ml aliquots were extracted with ethyl acetate. The denatured protein was collected by centrifigation, washed with ethanol and 0.15 M sodium chloride, and dissolved in 10% SDS. Little fluorescence was noted when hydrogen peroxide was omitted from the reaction mixture. Key: (A) uncorrected excitation spectra, λ_{em} 448 nm; (B) uncorrected emission spectra, λ_{ex} 310 nm.

As shown in Table 1, octylamine and metyrapone, known inhibitors of cytochrome P-450 function, strongly decreased the amount of organic-soluble products formed by the hydrogen peroxide-dependent oxidation of B[*a*]P. These results indicate that cytochrome P-450 was involved in the reactions. Octylamine and metyrapone, however, had little or no effect on the amount of product bound to microsomal protein. In contrast to the NADPH- or cumene hydroperoxide-dependent reactions which yielded 95–100 per cent of their metabolites as organic-soluble products [12], 48 per cent of the oxidation products of B[*a*]P formed in the presence of hydrogen peroxide and microsomal protein were recovered as protein-bound metabolites. The existence of protein-bound products could be demonstrated by the radioactivity associated with the protein from the reaction mixtures (Table 1). Attempts to characterize the fluorescence properties of the protein-bound hydrocarbon were unsuccessful due to their relatively low fluorescence compared to that obtained with SDS-solubilized microsomal protein alone. The lack of effect of metyrapone or octylamine on the amount of protein-bound hydrocarbon indicated that, although the material was formed by a process that requires the native microsomal fraction, cytochrome P-450 may not play a role in the formation of a hydrocarbon–protein adduct.

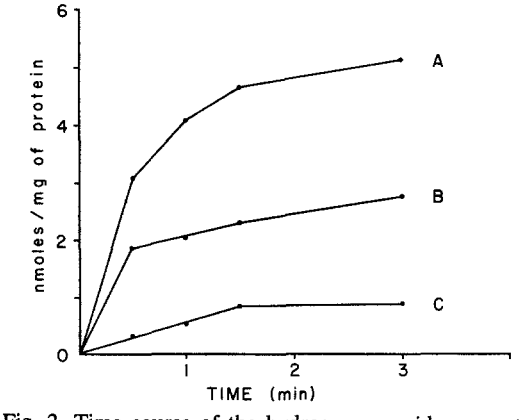

Fig. 2. Time course of the hydrogen peroxide-supported oxidation of 3-hydroxybenzo[*a*]pyrene. A reaction mixture containing 0.5 mg/ml of liver microsomes from PB-pretreated rats and 0.05 mM 3-hydroxy-B[*a*]P was incubated at 37°. After initiation of the reaction by addition of 5 mM hydrogen peroxide, 2 ml aliquots were taken at the indicated time points and the organic-soluble and protein-bound products were analyzed as indicated in Materials and Methods. The rate of metabolism was measured by h.p.l.c. as 3-hydroxy-B[*a*]P loss (A); the formation of unidentified organic-soluble, polar products was measured by h.p.l.c. (B); and protein-bound hydrocarbon was measured as described in Materials and Methods (C).

About 21 per cent of the total B[*a*]P metabolism that was supported by hydrogen peroxide might be accounted for by a non-enzymatic process having also occurred when heat-denatured microsomes were incubated with B[*a*]P and hydrogen peroxide. B[*a*]P is known to be oxidized by a mixture of hydrogen peroxide and ferrous salts, but at rates that are substantially lower than those reported here [19]. In fact, when 10 mM hydrogen peroxide was mixed with 0.05 mM B[*a*]P in a solution of 10% SDS in the absence or presence of ferrous ions, no spectral changes in the reaction mixture were detected by measurements of the absorbance spectra in the 350–500 nm region over a 30-min period, suggesting that only low rates of chemical oxidation occurred under these conditions. The non-enzymatic reaction most probably was due to the presence of the iron associated with denatured microsomal protein (cytochrome P-420).

High pressure liquid chromatography analysis of the organic-soluble products obtained with hydrogen peroxide and liver microsomes indicated that qualitatively the same three classes of B[*a*]P metabolites were produced (Table 2) as have been obtained with NADPH and oxygen [12]. An additional 5–10 per cent of the radioactivity injected onto the h.p.l.c. column eluted in the very polar region of the gradient and was unidentified. Dihydrodiols accounted for 25

per cent of the identified organic-soluble metabolites, and the 4,5-dihydrodiol was the major diol metabolite noted. The quinone fraction, which accounted for approximately 50 per cent of the products formed, was composed of the 1,6-, 3,6- and 6,12-quinones. These were present in a ratio of 1:2:2 respectively. The fact that all three quinone isomers were recovered as reaction products suggests that the 6-phenol may form as a major intermediate during the oxidation of B[*a*]P. In the phenol region of the h.l.p.c. gradient utilized, appreciable amounts of phenols that comigrated with either the peak 1 phenols (7- and 9-phenol) or the peak 2 phenols (1-, 3-, and 6-phenol) were formed. It is interesting to note that, unlike the reaction with NADPH and oxygen [12], much higher ratios of phenol 1 to phenol 2 metabolites were obtained with hydrogen peroxide. Table 2 also includes the product distribution of the organic-soluble metabolites obtained in the presence of heat-denatured microsomes under identical conditions. In this case, the oxidation of B[*a*]P resulted in the predominant formation of B[*a*]P-quinones, which are most probably generated by a one-electron oxidation process possibly involving free radicals as intermediates.

In Fig. 1, we have compared the distributions of organic-soluble metabolites obtained with liver microsomes from phenobarbital-pretreated rats and NADPH and oxygen [18], or cumene hydroperoxide [12], or hydrogen peroxide. Both the NADPH- and hydrogen peroxide-supported reactions resulted in the production of relatively large quantities of dihydrodiols (22–26 per cent of the total products formed). This suggests that, in these reactions, there may be a functional epoxidation pathway that is common for the two reactions. In the cumene hydroperoxide-supported reaction, the oxidation of B[*a*]P results in a major shift toward production of the three isomeric B[*a*]P-quinones (94 per cent of total metabolites) [12]. Only 6 per cent of the total products are recovered as B[*a*]P-dihydrodiols and, under these conditions, both the dihydrodiols and epoxide hydratase are stable to the presence of cumene hydroperoxide concentrations below 0.25 mM [12]. While the NADPH and hydrogen peroxide-dependent reactions are similar, the reaction supported by cumene hydroperoxide appears to function by a process that involves predominantly a one-electron oxidation mechanism and free radical intermediates. Similar results have been noted during the arachidonic acid-dependent oxidation of B[*a*]P catalyzed by preparations of sheep seminal vesicles containing heme or hemoproteins [19].

To gain information related to the mechanism of quinone formation during the hydrogen peroxide-supported oxidation of B[*a*]P, the metabolism of 3- and 6-hydroxy-B[*a*]P was studied using h.p.l.c. and spectrophotometric techniques [12, 18]. The addition of 5 mM hydrogen peroxide to an incubation mixture containing microsomal protein and 6-hydroxy-B[*a*]P resulted in the rapid oxidation of the phenol to three products, i.e. the 1,6-, 3,6- and 6,12-quinones, in a 1:1:1 ratio as determined by h.p.l.c. Both the rate of 6-phenol oxidation and the product ratio obtained when heat-denatured liver microsomes were used in the reaction were similar to those observed with native liver microsomes. No E.S.R. signal similar to that of the 6-oxo radical of B[*a*]P [12] could be detected, however, when either B[*a*]P or its 6-phenol was incubated with 0.5 mg/ml of liver microsomes and 10 mM hydrogen peroxide. This indicates that, in the presence of hydrogen peroxide, either no substrate-derived free radicals, such as the 6-oxo radical, were formed or that their steady-state concentration, if formed, was low.

Although the 3-phenol has been shown to be converted to the 3,6-quinone in the presence of liver microsomes and either NADPH and oxygen or cumene hydroperoxide, in the present experiment, no 3,6-B[*a*]P-quinone could be detected by h.p.l.c. analysis of the ethyl acetate soluble metabolites obtained by extracting reaction mixtures containing 0.5 mg/ml of liver microsomes, 5 mM hydrogen peroxide, and 0.05 mM 3-hydroxy-B[*a*]P with ethyl acetate. The 3-phenol, however, was consumed rapidly during the first 30 sec of the reaction and the reaction rate decreased beyond this time period, possibly due to the massive amount of heme destruction noted (Fig. 2). During this time period, 60–70 per cent of the total phenol metabolized could be recovered bound to microsomal protein, and 10–20 per cent was recovered as unidentified organic-soluble products migrating in the polar region of the h.p.l.c. gradient utilized. Only small amounts (< 5 per cent) of water-soluble material were detected other than that bound to protein.

The radioactivity bound to protein could not be removed by extensive extraction with ethyl acetate or 0.15 M sodium chloride. When the protein samples were solubilized with 10% SDS, there was significant fluorescence noted in the samples above that expected from SDS-solubilized microsomal protein alone (Fig. 3). The excitation and emission maxima shown are consistent with the existence of an intact benzo[*a*]pyrene ring bound to the protein and suggest that the protein-bound metabolite(s) may have been derived from a reactive intermediate that had fluorescence characteristics similar to those of mono- and dihydroxy-B[*a*]P derivatives. Therefore, it seems likely that the oxidation of B[*a*]P phenols by hydrogen peroxide might, in part, account for the significant amount of protein-bound hydrocarbon formed from B[*a*]P.

Incubation mixtures containing heat-denatured microsomes oxidized the 3-phenol at one-fifth the rate seen with intact microsomes, and the organic-soluble product appeared to be exclusively the 3,6-quinone. Considerable protein binding occurred under these conditions. Repetitive scan spectral analysis of solutions of the 3-phenol (3.6 μM) in 10% SDS solution at 37° indicated that the phenol was stable in the presence of 5 mM hydrogen peroxide (Fig. 4 A and B). Only upon addition of ferrous salts and adjustment of the pH to 9–10 could one see the rapid oxidation of the phenol, suggesting that the non-enzymatic reaction may have involved a Fe^{+}-H_2O_2-dependent process (Fig. 4C).

This report provides evidence indicating that liver microsomes obtained from phenobarbital-pretreated rats can catalyze the hydrogen peroxide-dependent aromatic hydroxylation of B[*a*]P. The product distribution of organic-soluble metabolites obtained

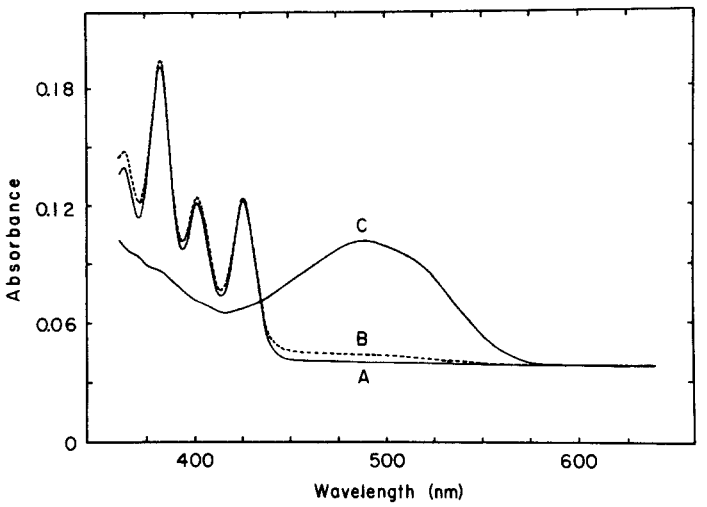

Fig. 4. Difference spectral analysis of the non-enzymatic oxidation of 3-hydroxy-benzo[*a*]pyrene by hydrogen peroxide. The curves shown are (A) a solution of 3.6 μM 3-hydroxy-B[*a*]P and 5 mM hydrogen peroxide in 10% SDS at neutral pH immediately after addition of the aromatic phenol; (B) a solution of 3.6 μM 3-hydroxy-B[*a*]P and 5 mM hydrogen peroxide in 10% SDS at neutral pH after incubation at 37° for 30 min; and (C) a solution of 3.6 μM 3-hydroxy-B[*a*]P, 5 mM hydrogen peroxide, and 0.1 mM ferrous ammonium sulfate in 10% SDS at pH 9.5 incubated at 37° for 30 min. The increase of absorbance in the 480 nm region was continuous during the 20-min period for spectra C.

with hydrogen peroxide as an oxidant resembles that which has been obtained during the NADPH- and oxygen-supported reaction. The B[*a*]P-quinones most likely are formed by one-electron oxidation processes involving cytochrome P-450 and the 6-phenol of B[*a*]P. The pronounced differences, observed, however, compared with the NADPH and oxygen- or cumene hydroperoxide-dependent reactions [12] support the suggestion that these three reactions, most likely do not uniformly proceed by one common mechanism involving a compound I type intermediate form of cytochrome P-450. The differences in product distributions are probably due to the existence of at least two chemical oxidation processes involving either an epoxidation pathway or a one-electron oxidation pathway, as postulated in Fig. 5.

Although it has been established that dihydrodiols of polycyclic aromatic hydrocarbons are formed by epoxidation and hydration steps [14, 15], there exist several possible chemical mechanisms for phenol and quinone formation [12, 16, 17]. Two possible mechanisms for hydroxylation of B[*a*]P have been suggested: an epoxidation step followed by rearrangement of the epoxide to a phenol or a direct hydroxylation step [20]. In addition, one route of quinone formation might involve hydroxylation (epoxidation and rearrangement or direct hydroxylation) to yield an easily air-oxidized dihydroxy derivative of B[*a*]P [21–23]. Nagata *et al.* [16] and Lesko *et al.* [17], however, have provided evidence suggesting that quinone formation probably results from a one-electron oxidation reaction that gives rise to a number of free radical intermediates. The results of the present report on the hydrogen peroxide-dependent oxidation of B[*a*]P and of those published on the cumene hydroperoxide-dependent oxidation of B[*a*]P by liver microsomes [11, 12] strongly suggest that the conversion of B[*a*]P to phenols and quinones may proceed, in part, by a one-electron oxidation reaction as postulated in Fig. 5. Although no direct evidence is available to test the relative participation of the epoxidation or one-electron oxidation pathways in B[*a*]P metabolism in the presence of NADPH and oxygen, the possibility that both chemical oxidation reactions function in the presence of the physiological electron donor, NADPH, remains a viable explanation for the metabolic formation of the B[*a*]P quinones.

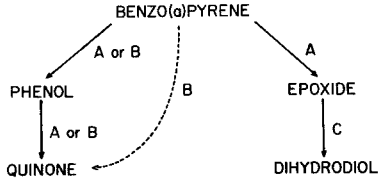

Fig. 5. Postulated reaction scheme for the oxidation of benzo[*a*]pyrene by either NADPH and oxygen, hydrogen peroxide, or cumene hydroperoxide. Key: (A) epoxidation (or epoxidation and rearrangement) reactions; (B) one-electron oxidation reactions; and (C) hydration reactions.

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