Reduction of Epoxy Derivatives of Benzo(a)pyrene by Microsomal Cytochrome P-450

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ABSTRACT

Benzo(a)pyrene 4,5-oxide was rapidly reduced to benzo(a)pyrene by rat liver microsomes in the presence of reduced nicotinamide adenine dinucleotide phosphate when incubated anaerobically. The activity of benzo(a)pyrene 4,5-oxide reductase was increased about seven times by pretreatment of rats with 3-methylcholanthrene.

The addition of riboflavin to the incubation mixture resulted in the increase (up to 10-fold) in the reduced nicotinamide adenine dinucleotide phosphate-supported reduction of benzo(a)pyrene 4,5-oxide. Moreover, the xanthine oxidase-supported reduction of benzo(a)pyrene 4,5-oxide was increased up to 20-fold by riboflavin. Both stimulations by riboflavin were completely blocked by carbon monoxide.

Benzo(a)pyrene 7,8-oxide was reduced by rat liver microsomes as rapidly as was benzo(a)pyrene 4,5-oxide, but phenanthrene 9,10-oxide and trans-stilbene oxide were reduced at slower rates. No detectable reductase activities were seen when benzo(a)pyrene 7β,8α-diol-9α,10α-epoxide, benzo(a)pyrene 7,8α-diol-9β,10β-epoxide, styrene oxide, and several other epoxide compounds were used as the substrates. The activity of microsomal benzo(a)pyrene 4,5-oxide reductase was markedly depressed when incubated under an atmosphere of air. The inhibition by oxygen of the benzo(a)pyrene 4,5-oxide reductase was partially reversed by the addition of a liver 105,000 x g supernatant fraction to the incubations containing microsomes. These results suggested that epoxide metabolites formed by microsomal cytochrome P-450 were reduced to the parent hydrocarbon at significant rates in physiological environments.

INTRODUCTION

BP, a widespread environmental pollutant, is generally known to be one of the most potent carcinogens. Since BP itself is chemically inert, its carcinogenicity is due largely to the chemically activated intermediates which are formed by hepatic enzyme systems involving mixed-function oxygenase. Among a number of metabolites of BP, epoxides are thought to be prominent candidates for being the ultimate carcinogens (6, 15, 19).

BP 4,5-oxide, a K-region epoxide, has drawn much attention for it is easily synthesized, relatively stable, and more potent than the parent hydrocarbon in causing malignant transformations in cultured mammalian cells (4). Most carcinogens have been found to be mutagenic in suitable test systems either directly or after metabolic activation. Using the mutation test system, Wood et al. (25) reported that among about 30 compounds tested BP 4,5-oxide was the most potent mutagen. On the other hand, Borgen et al. (2) observed that BP 7,8-dihydrodiol, a metabolite of BP 7,8-oxide, became bound to DNA 10 times as much as did BP when incubated in the presence of NADPH and liver microsomes. The (+)-enantiomer of BP 7,8-dihydrodiol is 5- to 10-fold more tumorigenic to mouse skin than is the (-)-enantiomer (11). The latter enantiomer was found to be predominantly metabolized to BP 7β,8α-diol-9α,10α-epoxide (21, 22). These results are supported by the fact that BP 7β,8α-diol-9α,10α-epoxide is an ultimate carcinogen in newborn mice (8). Although the 7,8-diol-9,10-epoxides of BP have received much attention, it cannot be unequivocally stated that other metabolites, including BP 4,5-oxide, are not significant in causing tumors under certain experimental conditions (18, 23).

The oxidative metabolism of BP proceeds initially through the formation of reactive arene oxides which spontaneously isomerize to phenols, are hydrated to dihydrodiols by microsomal epoxide hydrase, are conjugated with glutathione by the soluble glutathione S-transferase, or are reduced to the parent hydrocarbon by microsomes (1, 5, 13, 19). Using liver microsomes and BP 4,5-oxide as a substrate (10), we demonstrated the possible involvement of cytochrome P-450 in the reduction of the reactive arene oxides to the parent hydrocarbon. This possibility was recently confirmed by us with the purified preparation of cytochrome P-450 (26).

Since the epoxide reduction by microsomes is readily inhibited by atmospheric oxygen, the physiological significance of the reductive reaction is as yet unclear (10). Thus, this paper gives evidence that arene oxides, including BP 4,5-oxide, BP 7,8-oxide, and phenanthrene 9,10-oxide, are reduced by liver microsomes under an anaerobic condition and that the inhibition of BP 4,5-oxide reductase by oxygen is partially reversed by the presence of hepatic cytosol.

MATERIALS AND METHODS

Materials. BP 4,5-oxide was synthesized according to the method described by Dansette and Jerina (3). BP 7β,8α-diol-9α,10α-epoxide, BP 7β,8α-diol-9β,10β-epoxide, BP 7,8-oxide, and phenanthrene 9,10-oxide were kindly supplied by Drs. H. V. Gelboin and D. M. Jerina, NIH, Bethesda, Md. 5α,6α-Epoxycrololestone-3β-ol was kindly supplied by Dr. J. H. Weisburger, Naylor Dana Institute for Disease Prevention, Valhalla, N. Y. trans-Stilbene oxide was obtained from Aldrich Chemical Co., Milwaukee, Wis. Glucose 6-phosphate, NADP, NADPH, and glucose-6-phosphate dehydrogenase were products of...
Oriental Yeast Co., Tokyo, Japan. Xanthine oxidase was obtained from Sigma Chemical Co., St. Louis, Mo. Nitrogen gas, extra pure grade, was obtained from Yamato Sanki Co., Tokyo, Japan, and the nitrogen gas was further freed of oxygen by passage through alkali-dithionite. Other chemical reagents were the highest grade commercially available.

**Preparation of Microsomal and Soluble Fractions.** Male Wistar rats, 7 weeks old, were used throughout these experiments. The animals were obtained from Nippon Clea Inc., Tokyo, Japan. In some experiments, rats were treated with 3-MC, 40 mg/kg i.p., for 3 successive days. The last injection was given 24 hr prior to sacrifice. The livers were perfused in situ with 1.15% KCl to remove blood and were homogenized with 1.15% KCl in a motor-driven Potter homogenizer using a Teflon pestle. The homogenate was then centrifuged at 10,000 x g for 20 min, and the resultant supernatant fraction without fatty layer at the top of the centrifuge tube was recentrifuged at 105,000 x g for 1 hr in a Hitachi Model 55P-7 ultracentrifuge to separate microsomal and soluble fractions. The microsomes were resuspended in 1.15% KCl and again centrifuged at 105,000 x g for 30 min. The washed microsomes and the soluble fraction thus obtained were used.

**Measurement of BP 4,5-Oxide Reductase Activity.** The standard incubation mixture consisted of microsomes (1 mg protein), 1 μmol of NADPH, 5 μmol of glucose 6-phosphate, 1.5 IU of glucose-6-phosphate dehydrogenase, 5 μmol of MgCl₂, 0.5 μmol of 1,2 epoxy-3,3,3 trichloropropane, and 125 μmol of sodium phosphate-potassium phosphate (pH 7.4), in a final volume of 0.5 ml. The reaction was started by addition of 0.05 μmol of BP 4,5-oxide in 20 μl of acetone. Incubations were carried out at 37° for 5 min under continuous bubbling with nitrogen gas. The reaction rate was estimated by determination of BP 4,5-oxide in the incubation mixture by a Hitachi Model 203 fluorescence spectrophotometer. All determinations were conducted in duplicate, and the mean activities are presented in the tables and charts.

**Spectrophotometric Measurements.** Spectrophotometric measurements were carried out at 37° in a cuvet sealed with a rubber cap (9). The reaction mixture was bubbled through a needle with oxygen-free nitrogen gas. All solutions added to the mixture were also freed of oxygen by bubbling with nitrogen. When the rate of reoxidation of NADPH-reduced riboflavin associated with the reduction of BP 4,5-oxide was measured, the incubation mixture (2.5 ml) consisting of 2.5 mg of protein of liver microsomes from 3-MC-treated rats, 125 nmol of riboflavin, and 500 μmol of sodium phosphate-potassium phosphate (pH 7.4) was placed in the cuvet and sealed; then NADPH (2.5 μmol) and BP 4,5-oxide (0.1 ml of acetone) were added through a syringe at the indicated times. The change in the absorbance at 450 nm due to the reduction and oxidation of riboflavin was recorded. The reoxidation of riboflavin by BP 4,5-oxide was also determined using a hypoxanthine-xanthine oxidase system as an electron donor. For this experiment, the reaction mixture (2.5 ml) containing 1.25 mg of protein of liver microsomes from 3-MC-treated rats, 125 nmol of riboflavin, 1.25 units of xanthine oxidase, and 500 μmol of sodium phosphate-potassium phosphate (pH 7.4) was utilized. To this mixture were added hypoxanthine (10 μmol) and BP 4,5-oxide (0.1 ml of acetone) at the indicated times. The change in the absorbance at 450 nm was also recorded.

**Other Determinations.** Cytochrome P-450 was determined according to the method of Omura and Sato (17) except that 20% glycerol and 0.2% Emulgen 913, a non-ionic detergent kindly supplied by Kao-Atlas Co., Tokyo, Japan, were added in all determinations. Protein was determined by the method of Lowry et al. (12), using bovine serum albumin as the standard.

**RESULTS**

**Effect of Oxygen on Microsomal BP 4,5-Oxide Reductase Activity.** Reduction of BP 4,5-oxide is catalyzed by cytochrome P-450 in liver microsomes as described in a previous paper (26). Since the epoxide reductase activity is inhibited by oxygen, the incubations were conducted under anaerobic conditions to measure the accurate activity. Thus, to exclude the possibility that the reductase activity may be inhibited by residual oxygen in our incubation mixture, the effect of addition of the glucose-glucose oxidase system, which consumes oxygen in the incubation mixture, was examined. As shown in Table 1, addition of glucose-glucose oxidase did not result in an appreciable increase in reductase activity. Therefore, it was confirmed that the incubation medium used in this study was sufficiently anaerobic. Treatment of rats with 3-MC induced the reductase activity by about 7-fold. The induced activity was not considerably enhanced by the addition of the glucose-glucose oxidase system. Michaelis-Menten kinetic analysis of NADPH-supported BP 4,5-oxide reductase activity of liver microsomes from 3-MC-treated rats showed a Kₘ of 0.1 mM and a Vₘₐₓ of 3.1 nmol per mg protein per min.

To know the extent of inhibition by oxygen of the activity of BP 4,5-oxide reductase, the effect of aerobic incubation on the activity of BP 4,5-oxide reductase was examined using a 10,000 x g supernatant fraction prepared from liver homogenate of 3-MC-treated rats. The activity of BP 4,5-oxide reductase in the 10,000 x g supernatant fraction was 1.84 nmol per 0.1 g liver per min, and the activity was inhibited about 45% by aerobic incubation. Furthermore, the addition of 50 μM 7,8-benzoflavone, a specific inhibitor of a 3-MC-inducible cytochrome P-450, to the mixtures incubated anaerobically and aerobically resulted in 64 and 72% inhibition of the activities, respectively, suggesting that the same cytochrome P-450 species is responsible for the reductase activity irrespective of aerobic and incubations (14, 24).

**BP 4,5-Oxide Reductase Activity Supported by Reduced Riboflavin in 3-MC-treated Rat Liver Microsomes.** The addition of riboflavin (0.01 to 0.5 μM) to the incubation mixture supported BP 4,5-oxide reductase activity by about 7-fold. The induced activity was not considerably enhanced by the addition of the glucose-glucose oxidase system. Michaelis-Menten kinetic analysis of NADPH-supported BP 4,5-oxide reductase activity of liver microsomes from 3-MC-treated rats showed a Kₘ of 0.1 mM and a Vₘₐₓ of 3.1 nmol per mg protein per min.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose oxidase activity (nmol/mg protein/min)</th>
<th>BP 4,5-oxide reductase activity (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>0.48 (0.45, 0.50)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.48 (0.52, 0.44)</td>
</tr>
<tr>
<td>3-MC</td>
<td>+</td>
<td>3.06 (3.10, 3.02)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>3.21 (3.30, 3.11)</td>
</tr>
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* Numbers in parentheses, activities of two separate experiments.
markedly stimulated the reduction of BP 4,5-oxide to BP (data not shown). Accordingly, under a nitrogen atmosphere, riboflavin was rapidly reduced by NADPH in the presence of microsomes (Chart 1A). The initial rate of reduction of riboflavin was calculated to be about 35.2 nmol/mg/min. At the steady-state level of reduction, about 88% of added riboflavin was in the reduced form. The reduced riboflavin was reoxidized rapidly by addition of BP 4,5-oxide. Thus, the rate of reduction of BP 4,5-oxide was determined by measuring the initial rate of reoxidation of riboflavin at the steady-state level as reported previously in the reduction of tertiary amine N-oxide (9). The initial rate of reoxidation of reduced riboflavin induced by BP 4,5-oxide was increased by increasing the substrate concentrations. The reduction rates of BP 4,5-oxide were approximately 5, 9, 15, 20, and 24 nmol per mg protein per min on addition of 0.04, 0.08, 0.12, 0.16, and 0.20 mM BP 4,5-oxide, respectively. The amount of BP formed during the incubation (1 min) also increased with the increase in the amount of added BP 4,5-oxide. Under an anaerobic condition, riboflavin could also be reduced by the hypoxanthine-xanthine oxidase system (Chart 1B) (9). At the steady-state reduction level, about 98% of the added riboflavin was calculated to be in the reduced form. The reduced riboflavin was rapidly reoxidized by the addition of BP 4,5-oxide, and the reoxidation rate of riboflavin increased with the increase in the concentration of BP 4,5-oxide added. The rates of reduction of BP 4,5-oxide with 0.04, 0.08, 0.12, and 0.16 mM BP 4,5-oxide were approximately 17, 30, 40, and 49 nmol per mg protein per min, respectively. The reoxidation of riboflavin induced by the addition of BP 4,5-oxide and the formation of BP were almost completely abolished when oxygen-free carbon monoxide was introduced instead of nitrogen gas.

Comparison of Reduction Rates of Various Epoxides by 3-MC-treated Rat Liver Microsomes. The reduction rates of various epoxides were compared by measuring the reoxidation rates of reduced riboflavin (Table 2). For this experiment, the reoxidation of dithionite-reduced riboflavin was measured. Among the substrates used, polycyclic aromatic hydrocarbon epoxides such as BP 4,5-oxide and BP 7,8-oxide were reduced more rapidly than were other epoxide substrates. Phenanthrene 9,10-oxide and trans-stilbene oxide were also reduced but at slower rates, and nonaromatic epoxides such as BP 7,8-diol-9α,10α-epoxide, BP 7β,8α-diol-9β,10β-epoxide, and styrene oxide were not reduced. In addition, the reduction rates were not detectable when cyclohexene oxide, dieldrin, 1,2-epoxy-3,3,3-trichloropropene, and 5α,6α-epoxycholestane-3β-ol were used as substrates.

Effect of Soluble Fraction on the Activity of Microsomal BP 4,5-Oxide Reductase. As mentioned above, the activity of microsomal epoxide reductase was inhibited by oxygen. As shown in Chart 2, aerobic incubation of the mixture containing microsomes from uninduced rats, BP 4,5-oxide, and other necessary components exhibited very low reductase activity. The activity obtained by aerobic incubation was only 7% that obtained by anaerobic incubation. We found that the decrease in the reductase activity by aerobic incubation was less when 10,000 x g supernatant (soluble plus microsomal fractions) was utilized instead of microsomes. Thus, it was assumed that a factor(s) present in the soluble fraction reversed the inhibition by oxygen of the activity of microsomal epoxide reductase. In support of this idea, addition of the soluble fraction back to microsomes resulted in a partial reversal of the inhibition by oxygen. Reversal by the addition of soluble fraction was also seen when microsomes and soluble fraction from 3-MC-treated rats were used (Chart 3). Reversal of the oxygen inhibition by the soluble fraction increased with the amounts of the soluble fraction added (Chart 4). This observation lends support to our view that a factor(s) in the soluble fraction acts as an activator of the epoxide reductase under the physiological oxygen tension.

Table 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reduction rate (^{a}) (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP 4,5-oxide</td>
<td>21.6</td>
</tr>
<tr>
<td>BP 7,8-oxide</td>
<td>28.9</td>
</tr>
<tr>
<td>Phenanthrene 9,10-oxide</td>
<td>1.2</td>
</tr>
<tr>
<td>trans-Stilbene oxide</td>
<td>0.1</td>
</tr>
<tr>
<td>BP 7β,8α-diol-9α,10α-epoxide</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>BP 7β,8α-diol-9β,10β-epoxide</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Styrene oxide</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

The rates of reduction of the epoxides were determined by the reoxidation of reduced riboflavin as described in "Materials and Methods." Concentration of the epoxides was 0.16 mM.

![Chart 1](image1.png)

**Chart 1.** NADPH-dependent (A) and xanthine oxidase-supported (B) reductions of riboflavin and its reoxidation by BP 4,5-oxide with liver microsomes from 3-MC-pretreated rats. The content of cytochrome P-450 was 1.64 nmol/mg protein. The other experimental conditions were as described in "Materials and Methods." The change in the absorbance at 450 nm was recorded under an atmosphere of nitrogen.

![Chart 2](image2.png)

**Chart 2.** Effect of soluble fraction on BP 4,5-oxide reductase activity in liver microsomes from 3-MC-induced rats. Incubations were carried out under an atmosphere of air as described in "Materials and Methods." 1.69 mg of microsomal protein (0.1 g of liver equivalent), 1.27 mg of protein of soluble fraction (0.05 g of liver equivalent), and 10,000 x g supernatant fraction equivalent to 0.1 g of liver were used as enzyme source. Ms, microsomes; S, soluble fractions; 7,000×gS, 10,000×g supernatant fraction.
BP 4,5-oxide formation even in an aerobic environment (7). The nature of the soluble factor has not yet been clarified but is under investigation in our laboratory.

As shown in Table 2, BP 7,8-oxide as well as BP 4,5-oxide were reduced by liver microsomes when measured by the reoxidation of dithionite-reduced riboflavin. Thus, it seems reasonable to assume that the BP 7,8-oxide formed by liver microsomes is reduced back to the parent compound, BP, in liver cells.

As shown in Table 2, BP 7,8-oxide was reduced by liver microsomes at a rate comparable to or even faster than BP 4,5-oxide, while phenanthrene 9,10-oxide and trans-stilbene oxide were reduced at much slower rates. No reductase activities were detected when BP 7β,8βα-diol-9α,10α-epoxide, BP 7β,8α-diol-9β,10β-epoxide, styrene oxide, cyclohexene oxide, dieldrin, 1,2-epoxy-3,3,3-trichloropropene, and 5α,6α-epoxycholestan-3β-ol were used as substrates. These results are in accordance with the results reported by Booth et al. (1), who reported that 7,8-dihydrobenzo(a)pyrene 9,10-epoxide was not reduced to 7,8-dihydrobenzo(a)pyrene by rat liver microsomes. These results indicate that arene oxides rather than alkene oxides are good substrates for the microsomal epoxide reductase.

The carcinogenicity of BP appears to be closely correlated with the amounts of bay-region and K-region epoxides covalently bound to DNA in the target tissues. Thus, the overall assessment of the carcinogenicity of BP should be considered as the results of relative rates of activation and detoxification reactions. BP is known to be activated to BP 4,5-oxide, BP 7,8-oxide, etc., by microsomal cytochrome P-450. These epoxides are reduced to BP and then hydrated to dihydrodiol (13) or conjugated with glutathione (5). Some of these reactions lead to the irreversible loss of carcinogenicity. Assuming the possible role of the reductase as the detoxification reaction, the reduction of BP 4,5-oxide and BP 7,8-oxide to BP may increase the formation of hydroxy derivatives followed by conjugation with glucuronic acid (16) or sulfuric acid. The activity of epoxide hydrase is assumed to be much higher than that of epoxide reductase when the epoxide substrates are supplied exogenously (13). However, it seems reasonable that the epoxide metabolites formed endogenously by cytochrome P-450 are reduced more efficiently by the same enzyme, cytochrome P-450, prior to the attack by the epoxide hydrase. It is noteworthy that, once BP 7,8-oxide is hydrated to BP 7,8-diol (13) or conjugated with glutathione (5), these diol-epoxides cannot be reduced. However, the metabolic pathways of BP in vivo in relation to tumorigenesis are so complicated (19) that the role of epoxide reductase as a detoxification mechanism in the tumorigenesis of BP remains to be clarified.

The addition of riboflavin to the incubation mixture resulted in a marked increase in the reduction rate of BP 4,5-oxide (Tables 1 and 2; Chart 1). This activity was markedly inhibited by carbon monoxide. The reduced form of riboflavin scarcely reduced BP 4,5-oxide in the absence of liver microsomes. These results are similar to those observed in the reduction of tertiary amine N-oxides, suggesting that reduced riboflavin accelerates the reduction of cytochrome P-450 and that the reduced cytochrome P-450 in turn reduces the substrate. In addition, the hypoxanthine-xanthine oxidase system reduces cytochrome P-450, as reported previously (9), and this system...
markedly stimulates the activity of BP 4,5-oxide reductase without NADPH. These results probably suggest that the rate-limiting step in the reduction of BP 4,5-oxide is the reduction of cytochrome P-450. The mechanism by which cytochrome P-450 reduces epoxide substrates is not clear as yet; however, it may be similar to that previously proposed for the reduction of tertiary amine N-oxides (20). In the case of the reduction of arene oxides, it seems possible to speculate that the oxygen atom of an arene oxide coordinates with the sixth ligand of the heme of the reduced cytochrome P-450 to form the ferrous cytochrome P-450-epoxide complex; then the complex accepts a second electron from an electron donor such as NADPH via NADPH-cytochrome P-450 reductase or cytochrome b, to produce water and the reduced metabolite. The mechanism of the reduction proposed here is only tentative and must be confirmed in further studies.

REFERENCES


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