Metabolic Activation of Dibenz[a,h]anthracene and Its Dihydrodiols to Bacterial Mutagens

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ABSTRACT

Dibenz[a,h]anthracene and the three metabolically possible trans-dihydrodiols of dibenz[a,h]anthracene were tested for mutagenicity toward Salmonella typhimurium strain TA 100 in the presence of hepatic microsomes from Aroclor 1254-pretreated rats or the cytochrome P-450-dependent monooxygenase system isolated and purified to near homogeneity from these microsomes. With either enzyme system trans-3,4-dihydroxy-3,4-dihydrodibenzo[a,h]anthracene was activated to products that were more mutagenic to the bacteria than were the metabolites of dibenz[a,h]anthracene or the metabolites of the 1,2- and 5,6-dihydrodiols of dibenz[a,h]anthracene. With microsomes but not with the purified monooxygenase system, trans-5,6-dihydroxy-5,6-dihydrodibenzo[a,h]anthracene was also activated to products that were highly mutagenic to the bacteria. However, significant activation was seen only when high concentrations of trans-5,6-dihydroxy-5,6-dihydrodibenzo[a,h]anthracene were incubated with relatively high amounts of enzyme. Saturation of the double bond at position 1,2 of trans-3,4-dihydroxy-3,4-dihydrodibenzo[a,h]anthracene produced a tetracyclic dihydrodiol that was poorly activated in the presence of either microsomes or the purified monooxygenase system. The high activity of trans-3,4-dihydroxy-3,4-dihydrodibenzo[a,h]anthracene in both metabolic activation systems and the importance of the double bond in 1,2-position of the dihydrodiol strongly suggest that a bay-region 3,4-diol-1,2-epoxide is a biologically important active metabolite of dibenz[a,h]anthracene, thereby providing support for the bay region theory.

INTRODUCTION

The first evidence that a chemically pure substance could induce neoplasia was reported in 1930 when Kennaway and Hieger (14) demonstrated that the polycyclic hydrocarbon DBA3 was carcinogenic to mice. In the intervening half-century, a considerable body of evidence has accumulated to indicate that polycyclic hydrocarbons and many other cancer-causing chemicals exert their carcinogenic activity only after metabolism to highly reactive products (ultimate carcinogens) capable of covalent binding to macromolecules (7, 20, 21, 26).

Since carcinogenicity induced by DBA represents an important milestone in the field of chemical carcinogenesis, there has been considerable interest in identifying the ultimately reactive metabolites of this hydrocarbon. Toward that goal this study examines the extent to which DBA and the 3 metabolically possible trans-dihydrodiols of DBA (Chart 1) can be activated by microsomal enzymes to products mutagenic to Salmonella typhimurium. Attention has been focused on the metabolic activation of dihydrodiols because recent studies have shown that benzo ring dihydrodiols of the carcinogens benzo(a)pyrene, benzo(a)anthracene, chrysene, and 7-methylbenzo(a)anthracene are highly mutagenic after metabolism (6, 18, 19, 35-38), and these dihydrodiols are carcinogenic in mice (3, 4, 11, 12, 15-17, 27, 30, 34). Indeed, in some tumor models trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, trans-3,4-dihydroxy-3,4-dihydrobenzo(a)anthracene, and trans-3,4-dihydroxy-3,4-dihydro-7-methylbenzo(a)anthracene are more potent carcinogens (proximate carcinogens) than are the respective parent hydrocarbons (4, 11, 12, 15, 16, 30, 34). The unique structural feature of the active dihydrodiols of these hydrocarbons appears to be the presence of a nonaromatic double bond, which forms part of the bay region of the hydrocarbon and which is adjacent to the dihydrodiol. Epoxidation of this double bond forms a diol-epoxide, which is the proposed ultimate carcinogenic metabolite. This conclusion is based on chemical factors, quantum mechanical calculations, and a reevaluation of existing carcinogenicity data for variously substituted polycyclic aromatic hydrocarbons (8-10). Bay-region diol-epoxides of benzo(a)pyrene and benzo(a)anthracene possess high intrinsic mutagenic activity in bacterial and

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3 The abbreviations used are: DBA, dibenzo(a,h)anthracene; DBA 1,2-dihydrodiol, trans-1,2-dihydroxy-1,2-dihydrodibenzo(a,h)anthracene; DBA 3,4- and 5,6-dihydrodiol, other trans-dihydroxydihydrodibenzo(a,h)anthracene derivatives; DBA 3,4-diol, trans-3,4-dihydroxy-1,2,3,4-tetrahydrodibenzo(a,h)anthracene; DBA 3,4-diol-1,2-epoxide, either or both diastereomers of 3,4-dihydroxy-1,2-epoxy-1,2,3,4-tetrahydrodibenzo(a,h)anthracene derived from DBA 3,4-dihydrodiol in which the epoxide ring is either cis or trans to the benzylic 4-hydroxyl group. All compounds are racemic mixtures where optical enantiomers are possible.

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Hepatic microsomes were obtained from immature male Long-Evans rats pretreated with sodium phenobarbital (5). Reconstitution of the reductase and hemoprotein in the presence of the appropriate amount of phosphatidylcholine and NADPH resulted in a highly active monooxygenase system that had less than 1% of the epoxide hydrase activity found in microsomes.

Antibodies to purified cytochrome P-450 and cytochrome P-448 from rats pretreated with sodium phenobarbital and 3-methylcholanthrene, respectively, were produced in rabbits as previously described (28, 29). Only the IgG fractions from control rabbit sera or immune rabbit sera were used in this study, and these fractions were at least 96% homogeneous as determined by agarose gel electrophoresis. Antibodies and enzymes were stored at -90°.

**Metabolic Activation Assays.** Strain TA 100 of histidine-dependent S. typhimurium was obtained from Dr. B. Ames, University of California, Berkeley, Calif., and cultured as described (32). Mutagenesis experiments with microsomes as the source of monooxygenase activity were conducted as described by Ames et al. (1) with the exception that 1 unit of glucose-6-phosphate dehydrogenase was added to each reaction and the complete incubation mixture was incubated for 5 min at 37° before addition of the top agar. Experiments with the purified monooxygenase system were conducted essentially as previously described (37, 38) with 300 units of NADPH-cytochrome c reductase per incubation. After incubation of the histidine-dependent bacteria with either enzyme system, 2 ml of top agar were added to each reaction, and the entire mixture was poured onto a histidine-deficient agar Petri dish. Mutations to histidine-independent growth were assessed by counting the macroscopic colonies of bacteria after a 2-day incubation of the plates at 37°. All experiments were performed in triplicate, and coefficients of variation of the colony counts rarely exceeded 15%. Comparisons of absolute mutation frequencies among experiments performed during different weeks showed somewhat larger variations, but the same relative activities among the derivatives were always observed, and all the data presented in a single figure or table were obtained from a 1-day experiment.

**MATERIALS AND METHODS**

**Chemicals.** Aroclor 1254 was obtained from Monsanto Chemical Company, St. Louis, Mo.; cyclohexene oxide was from Aldrich Chemical Company, Milwaukee, Wis.; dilaurophosphatidylcholine was from Sedary Research Laboratories, Ontario, Canada; and DBA was from Eastman Organic Chemicals, Rochester, N. Y. Other commercially available biochemicals were obtained from Sigma Chemical Company, St. Louis, Mo.

The 1,2-, 3,4-, and 5,6-dihydrodiols of DBA were synthesized by unequivocal chemical procedures, and the structures of the analytically pure compounds were confirmed by nuclear magnetic resonance spectrometry (13). The hydrocarbons were stored at -90° and dissolved immediately before use in acetone:ammonium hydroxide (1000:1) containing 10% (v/v) anhydrous dimethyl sulfoxide.

**Microsomal Enzymes and Antibody to Cytochrome P-450.** Hepatic microsomes were obtained from immature male Long-Evans rats pretreated with the polychlorinated biphenyl mixture Aroclor 1254. Cytochrome P-450 was purified from these microsomes as described (24), and NADPH-cytochrome c reductase was purified from the livers of male Long-Evans rats pretreated with sodium phenobarbital (5).

**RESULTS**

**Activation of DBA Derivatives by Liver Microsomes.** The mutagenic activities of metabolically activated DBA, the 3 DBA dihydrodiols, and DBA H₃-3,4-diol as a function of the amount of microsomal monooxygenase in the reaction mixtures are shown in Chart 2. When 75 nmol of each compound were incubated, mutations increased with increasing amounts of cytochrome P-450, and the metabolic products of DBA 3,4-dihydrodiol were 4 times as mutagenic to the bacteria as were the products formed from DBA. DBA 1,2-dihydrodiol and DBA H₃-3,4-diol were less active in this system than was the parent hydrocarbon. DBA 5,6-dihydrodiol was poorly activated when incubated with low amounts of cytochrome P-450 (0.075 to 0.30 nmol), but with higher amounts of cytochrome P-450 (24). Unless indicated otherwise, the term cytochrome P-450 used in the text and charts designates this mixture of cytochrome P-450 and cytochrome P-448 obtained from Aroclor-pretreated rats.
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Microsomal activation in the standard Ames test proceeds for an undefined period of time and may last for several hr (1). In contrast, metabolic activation with the purified monoxygenase is terminated after the 5- or 10-min incubation (37). An extended 0.5-hr incubation of the 3,4- and 5,6-dihydrodiols of DBA was undertaken to examine the possibility that the low activation of DBA 5,6-dihydrodiol was due to its relatively slow rate of metabolism. However, the data (Chart 6) indicate that, under conditions that caused a marked activation of DBA 3,4-dihydrodiol, DBA 5,6-dihydrodiol was still inactive.

Role of Epoxide Hydrase in the Metabolic Activation of the DBA Derivatives. Cyclohexene oxide decreased the microsomal activation of DBA and DBA 5,6-dihydrodiol in a dose-dependent manner but had no effect on the activation of DBA 3,4-dihydrodiol (Chart 7). Since cyclohexene oxide is a potent inhibitor of epoxide hydrase (23), these results suggest that dihydrodiol formation is an important metabolic step in the activation of both DBA and DBA 5,6-dihydrodiol. The inability of cyclohexene oxide to affect the activation of DBA 3,4-dihydrodiol by liver microsomes is consistent with the hypothesis that DBA 3,4-diol-1,2-epoxides are the activated metabolites of DBA 3,4-dihydrodiol since epoxide hydrase is not needed for the formation of these diol-epoxides from DBA 3,4-dihydrodiol. By extension of the results obtained with diol-epoxides of benzo(a)pyrene

amounts of the monoxygenase system mutation frequency increased rapidly with a slope that paralleled the DBA 3,4-dihydrodiol activation curve.

The effect on mutation induction of varying the substrate concentration over a 16-fold concentration range in the presence of a relatively high amount of microsomes (0.90 nmol cytochrome P-450) is shown in Chart 3. DBA 3,4-dihydrodiol was more active than was DBA at all substrate concentrations and was again over 4 times more active than was the parent hydrocarbon at saturating substrate levels. DBA 5,6-dihydrodiol showed minimal activation at low substrate concentrations but was metabolized to highly mutagenic products at higher substrate concentrations. Additional studies were initiated on the microsomal activation of the 3,4- and 5,6-dihydrodiols of DBA at low amounts of hydrocarbon and enzyme (Chart 4). When the mutation frequency is expressed either as a function of cytochrome P-450 (Chart 4A) or as a function of the dihydrodiol concentration (Chart 4B), the products of DBA 3,4-dihydrodiol metabolism were more mutagenic to the bacteria than were the metabolites of DBA 5,6-dihydrodiol, especially at the lower levels of hydrocarbon and enzyme.

Activation of DBA Derivatives by a Purified Monoxygenase System. The relative mutagenic activities of the metabolic products of DBA 3,4-dihydrodiol, DBA, DBA H2-3,4-diol, and DBA 1,2-dihydrodiol were retained when the highly purified and reconstituted monoxygenase system was used to metabolize the compounds (Chart 5). In contrast to the results obtained with microsomes, DBA 5,6-dihydrodiol could not be activated at any concentration of hemeprotein tested.
(37, 39) and benzo(a)anthracene (32), the diol-epoxides of DBA would be expected to be poor substrates for epoxide hydrase. The inability of cyclohexene oxide to affect the activation of DBA 3,4-dihydrodiol also indicates that non-specific inhibition of the monooxygenase system is not the basis for the action of cyclohexene oxide with DBA or DBA 5,6-dihydrodiol as substrates. While the greater than 50% decrease in mutation induction by cyclohexene oxide suggested that epoxide hydrase activity is necessary for the activation of DBA 5,6-dihydrodiol by liver microsomes, addition of various amounts of highly purified epoxide hydrase to the purified monooxygenase system did not result in activation of the dihydrodiol (data not shown). The persistent 30% activation of DBA in the presence of relatively high amounts of cyclohexene oxide suggests that some of the metabolism of DBA to mutagens is not dependent on epoxide hydrase activity. This observation is consistent with the activation of DBA by the purified monooxygenase system (Chart 5), which is free of epoxide hydrase. Dibenzo(a,h)anthracene 5,6-oxide, which has low but significant mutagenic activity, may be 1 of the metabolites formed by the purified monooxygenase system.

Inhibition of DBA Dihydrodiol Activation by Antibody to Cytochrome P-448. Antibody to highly purified cytochrome P-448 from 3-methylcholanthrene-pretreated rats inhibited the microsomal activation of the DBA 3,4- and 5,6-dihydrodiols as shown in Chart 8. At all concentrations of antibody tested, metabolism of DBA 3,4-dihydrodiol to mutagens was inhibited to a greater extent than was the metabolism of DBA 5,6-dihydrodiol. Approximately 7 times more antibody was needed to inhibit the activation of DBA 5,6-dihydrodiol by 50% as was needed to inhibit DBA 3,4-dihydrodiol activation by 50%. These results demonstrate that the monooxygenase system is the enzyme complex in microsomes that is activating the dihydrodiols and further suggest that more than 1 form of cytochrome P-450 (P-448) metabolizes the 2 dihydrodiols to mutagenic products. Antibody to highly purified cytochrome P-450 from sodium phenobarbital-pretreated rats had no inhibitory effect on the activation of either dihydrodiol (data not shown).

DISCUSSION

Although a quantitative assessment of dihydrodiol formation from DBA has not been described in detail, there is evidence for the in vitro formation of significant amounts of all 3 of the metabolically possible dihydrodiols of this
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hydrocarbon (2, 25). The results of the present study indicate that in the presence of microsomal enzymes 2 of the dihydrodiols, DBA 3,4-dihydrodiol and DBA 5,6-dihydrodiol, are more mutagenic to S. typhimurium than is the parent hydrocarbon. DBA 3,4-dihydrodiol was considerably more active than was DBA 5,6-dihydrodiol when low amounts of the hydrocarbons or low amounts of microsomal enzymes were incubated. Since activation with low substrate concentrations is probably more representative of in vivo exposure to environmental hydrocarbons, the formation and metabolism of DBA 3,4-dihydrodiol are probably more biologically significant than are the formation and metabolism of DBA 5,6-dihydrodiol.

The bay region theory of polycyclic hydrocarbon carcinogenicity (8–10) predicts that DBA 3,4-diol-1,2-epoxide mediates the high mutagenicity of metabolically activated DBA 3,4-dihydrodiol. DBA 3,4-dihydrodiol was not intrinsically mutagenic to the bacteria, and mutation induction was dependent on the microsomal fraction of liver and an NADPH-generating system. Since epoxidation of hydrocarbons is catalyzed by the cytochrome P-450-dependent monoxygenase system of microsomes, activation of DBA 3,4-dihydrodiol (if via subsequent formation of the 1,2-epoxide) should be catalyzed by a purified monoxygenase system that does not contain epoxide hydrase. The present results confirm this expectation and indicate that the puri-
fied enzyme system induced more mutations/nmol of cytochrome P-450 than did microsomes. The 1,2-double bond is essential for bay-region epoxidation of DBA 3,4-dihydrodiol, and saturation of this double bond to produce DBA H₂ 3,4-diol provided a means of testing whether metabolic activation occurred at the 1,2-position or at other positions of the hydrocarbon. Since DBA H₂ 3,4-diol was considerably less active than was DBA 3,4-dihydrodiol in the presence of either microsomes (Chart 2) or the purified monoxygenase system (Chart 5), most of the mutations induced by DBA 3,4-dihydrodiol appear to be due to its conversion to DBA 3,4-diol-1,2-epoxide. Similar studies with trans-7,8-dihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene (36) and trans-1,2-dihydroxy-1,2,3,4-tetrahydrochrysene (38) have also provided evidence for bay region activation of these 2 hydrocarbons.

Previous studies utilizing the purified hepatic monoxygenase system or liver microsomes have shown that trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene (18, 36, 37), trans-3,4-dihydroxy-3,4-dihydrobenzo(a)anthracene (35), trans-3,4-dihydroxy-3,4-dihydro-7-methylbenzo(a)anthracene (19), and trans-1,2-dihydroxy-1,2-dihydrochrysene (38) are metabolized to potent bacterial mutagens. The bay region epoxides of the benzo(a)pyrene and benzo(a)-anthracene dihydrodiols have high mutagenic activity (6, 22, 31–33, 39), and they are highly carcinogenic in the mouse (11, 12, 15). The diol-epoxides of benzo(a)pyrene and benzo(a)-anthracene dihydrodiols, unlike the tetrahydroepoxides and arene oxides of these hydrocarbons, are poor substrates for epoxide hydrase (32, 37, 39). Accordingly, the inability of the epoxide hydrase inhibitor cyclohexene oxide to affect the metabolic activation of DBA 3,4-dihydrodiol by liver microsomes (Chart 7) is consistent with the formation of a diol-epoxide.

The metabolic activation of DBA 5,6-dihydrodiol was unexpected since K-region dihydrodiols do not have an adjacent olefinic double bond for diol-epoxide formation. The K-region dihydrodiols of benzo(a)pyrene, benzo(a)anthracene, and chrysene have been inactive in vitro activation systems similar to those used here (18, 35, 37, 38), and these dihydrodiols are also weakly or noncarcinogenic (3, 30, 34). The results indicating that the metabolic activation of DBA 5,6-dihydrodiol requires both monoxygenase (Chart 8) and epoxide hydrase (Chart 7) activity suggests that some type of diol-epoxide of the 5,6-dihydrodiol may be the ultimately reactive metabolite. Quantum mechanical calculations used to predict chemical reactivity of diol-epoxides (10) indicate that the 1,2-diol-3,4-epoxide and the 10,11-diol-8,9-epoxide derivatives of DBA 5,6-dihydrodiol would be expected to be the most reactive diol-epoxides (ΔE(2) = 0.814 and 0.658, respectively, compared to 0.738 for DBA 3,4-diol-1,2-epoxide). Regardless of the pathway of DBA 5,6-dihydrodiol metabolism to mutagens, these studies indicate that DBA 3,4-dihydrodiol undergoes metabolic activation to a greater extent than does DBA or the other 2 metabolically possible dihydrodiols of DBA. Studies are currently in progress to determine whether DBA 3,4-dihydrodiol, the immediate metabolic precursor of the bay region 3,4-diol-1,2-epoxide, is also more carcinogenic than are DBA and the other DBA derivatives.
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