Metabolism of Benzo[a]pyrene and Benzo[a]pyrene Derivatives to Mutagenic Products by Highly Purified Hepatic Microsomal Enzymes

(Received for publication, January 26, 1976)

ALEXANDER W. WOOD,* WAYNE LEVIN,* ANTHONY Y. H. LU,* HARUHIKO YAGI,‡ OSCAR HERNANDEZ,‡ DONALD M. JERINA,‡ and A. H. CONNEY*

From * the Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, New Jersey 07110 and ‡ the Section on Oxidation Mechanisms, Laboratory of Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

A highly purified and reconstituted hepatic microsomal monooxygenase system, completely free of epoxide hydase and consisting of cytochrome P-448 from 3-methylcholanthrene-treated rats, NADPH-cytochrome c reductase, phosphatidylcholine, and NADPH, metabolizes benzo[a]pyrene to products highly mutagenic in strains TA 98 and TA 1538 of Salmonella typhimurium. The formation of mutagenic metabolites is completely dependent on the presence of benzo[a]pyrene, NADPH, NADPH-cytochrome c reductase, and cytochrome P-448 and is partially dependent on phosphatidylcholine. Mutation frequency in both strains is linearly related to amount of cytochrome P-448 and to time of incubation. Highly purified cytochrome P-450 from phenobarbital-treated rats is relatively poor in catalyzing the formation of mutagenic metabolites from benzo[a]pyrene. Addition of 7.5 to 75 units of highly purified epoxide hydase to the cytochrome P-448-dependent monooxygenase system decreases the number of mutations by approximately 50% and 30% in strains TA 1538 and TA 98, respectively. Additional amounts of epoxide hydase (300 units) fail to further suppress mutations, indicating that at least some, but probably not all, of the mutagenic metabolites of benzo[a]pyrene are arene oxides. In the absence of a monooxygenase system, mutations induced by benzo[a]pyrene 4,5-oxide are readily quenched by epoxide hydase, whereas mutations induced by a diol epoxide metabolite of benzo[a]pyrene [(++)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene] are not. Several known and potential phenolic and dihydrodiol metabolites of benzo[a]pyrene are metabolized to products mutagenic in the Salmonella. The number of mutations induced per nmol of hemoprotein is approximately 3- to 4-fold higher when trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene replaces benzo[a]pyrene as a substrate for the cytochrome P-448-dependent monooxygenase system. Little or no mutagenic activity is observed with trans-dihydrodiols at positions 4,5, 9,10, or 11,12 of the hydrocarbon, either in the absence or presence of the active monooxygenase system. Of the 12 possible isomeric monophenols of benzo[a]-pyrene, only 6- and 12-hydroxybenzo[a]pyrene are moderately active bacterial mutagens; 1-, 2-, 3-, 6-, 9-, and 12-hydroxybenzo[a]pyrene are premutagens (i.e. metabolized to mutagenic products); and 4-, 5-, 7-, 8-, 10-, and 11-hydroxybenzo[a]pyrene have little or no mutagenic activity with or without further oxidative metabolism. Benzo[a]pyrene 7,8-oxide, a carcinogen on mouse skin, is weakly mutagenic but can be further metabolized to a highly active bacterial mutagen(s), presumably diol epoxide(s), by a combination of epoxide hydase and the cytochrome P-448 monooxygenase system. This is the first example of a direct role of epoxide hydase in the metabolic activation of a chemical to a toxic product.

Polycyclic hydrocarbons and many other cancer-causing chemicals exert their carcinogenic effects only after metabolic activation to reactive metabolites (ultimate carcinogens) that bind to critical cellular constituents (1-4). The widespread occurrence of benzo[a]pyrene in man's environment, and its potent carcinogenic activity, have prompted numerous studies attempting to delineate the metabolic fate of this polycyclic hydrocarbon and to identify its biologically active metabolite products. Mutagenicity tests utilizing microorganisms or cultured mammalian cells have been used with increasing frequency to identify bioactivated metabolites of carcinogens. The rationale for the use of these tests is derived from the belief that somatic cell mutation may be involved in the chemical initiation of cancer (5), and from the finding that a large percentage of known carcinogens are mutagenic per se or are mutagenic after metabolism (6, 7).

Metabolic activation of chemicals to mutagenic metabolites has been most commonly performed by a procedure developed by Ames and his associates (6, 8). Generally, the chemical, bacteria, and an NADPH-generating system are co-incubated...
with a 9000 × g supernatant fraction of a tissue homogenate (generally liver) in a semisolid agar gel for 48 h. The 9000 × g supernatant fraction of liver has been utilized because of the relative ease in obtaining a sterile preparation and because it contains high levels of microsomal NADPH-dependent monoxygenases, which are the principal enzymes involved in the metabolism of many carcinogens. While the procedure has been used successfully to activate a number of carcinogens to bacterial mutagens, it appeared to us to have several limitations for a detailed examination of benzo[a]pyrene activation. The 9000 × g supernatant fraction is relatively crude and contains many enzymatic and structural proteins, nucleic acids, and numerous nucleophile and electrophile groups which could interact with the bioactivated metabolites before they reach the bacterium. Secondly, regardless of the source of monooxygenase activity, reaction rates for metabolism of many carcinogens under usual incubation conditions in a buffer medium remain maximal for only a few minutes. In the case of benzo[a]pyrene, longer incubation times can result in spontaneous or enzymatic breakdown of primary metabolites to well-defined products. Use of the prolonged incubation times routinely employed for metabolic activation studies in agar gel would but all eliminate the possibility of obtaining a profile of the metabolites formed under the conditions which induced the mutations. We therefore sought to develop an enzymatically well-defined model system which would activate benzo[a]pyrene and its derivatives to mutagenic metabolites under conditions which would permit the analysis and identification of the metabolites.

The monomolecular monooxygenase system has been resolved and purified from rat liver into three components consisting of NADPH-cytochrome c reductase, cytochrome P-450 (or cytochrome P-448), and phosphatidylcholine (9-12). Purification of the reductase and the hemoproteins to near-homogeneity has permitted the reconstitution of an active monooxygenase system free of microsomal epoxide hydrase, an enzyme which hydrates a number of arene and alkene oxides to their corresponding diols. In the absence of epoxide hydrase, the monooxygenase system metabolizes benzo[a]pyrene predominantly to phenols and quinones (13). Addition of highly purified microsomal epoxide hydrase to the monooxygenase system results in formation of dihydriodiol fractions corresponding to benzo[a]pyrene 4,5-, 7,8-, and 9,10-dihydriodiol, with an attendant decrease in the phenol fractions. These results demonstrate that a substantial portion of the metabolism of benzo[a]pyrene by the purified monooxygenase system occurs via arene oxide intermediates. The ability (a) to manipulate the amount of various metabolites formed through alteration of the ratio of epoxide hydrase to the monooxygenase system, (b) to identify and quantify metabolites formed from benzo[a]pyrene, and (c) to limit the extent of inactivation of active metabolites by extraneous proteins, nucleic acids, and small molecules suggested to us the use of the purified enzyme system as an activating system for mutagenesis studies with benzo[a]pyrene. In the present report, we describe the metabolism of the hydrocarbon to mutagens by the highly purified cytochrome P-450 or P-448 monooxygenase systems in the presence of purified epoxide hydrase, under short and well-defined incubation conditions.

Previous studies from our laboratories (14-17) have examined the mutagenicity of a large number of chemically synthesized benzo[a]pyrene derivatives, many of which are known metabolites of the hydrocarbon. Since primary oxidative metabolites of benzo[a]pyrene may undergo further oxidative metabolism (18-21), the present report also describes the metabolic activation of several benzo[a]pyrene phenols and dihydriodiol by the purified enzymes. Experimental Procedures

Materials—Dilauroyl phosphatidylcholine was obtained from Serva Research Laboratories, Ontario, Canada, and bacterial media were obtained from the Biological Division of Becton, Dickinson and Co., Cockeyesville, Md. Other commercially available biochemicals were obtained from Sigma Chemical Co., Inc., St. Louis, Mo.

Synthesis of Benzo[a]pyrene Derivatives—All benzo[a]pyrene derivatives were obtained by unequivocal chemical synthesis, with the exception of BP 9,10-diol, which was formed by the action of purified epoxide hydrase on synthetic BP 9,10-oxide (see below). The synthetic procedures used for the preparation of the K-region BP 4,5-oxide (22), non-K-region BP 7,8- and 9,10-oxides (23), the 12 isomeric benzo[a]pyrene phenols (24), BP 4,5- and 11,12-diols, BP 7,8-diol (25), (+)-7,8a-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BP 7,8a-diol-9,10,13-epoxide), and the stereoisomeric (+)-7a,8dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BP 7a,8d-diol-9,10,13-epoxide) (26, 27) have been previously described in detail, as have structural proofs, criteria of purity, and procedures for storage, handling, and stability assessment (14, 19). All compounds used in this study were of analytical purity.

Enzyme Purifications—Complete procedures for the solubilization, purification, and assay of cytochrome P-450 (9), cytochrome P-448 (9), NADPH-cytochrome c reductase (28), and epoxide hydrase (29) from rat liver microsomes have been reported. All enzyme preparations used in the present study were free of contamination by any of the other enzyme activities, as well as cytochrome b5. Cytochrome P-450 and cytochrome P-448 were purified in this study contained 10.4 and 12.8 nmol of hemoprotein/mg of protein, respectively, when protein was assayed by the method of Lowry et al. (30) using bovine serum albumin as standard. However, based on amino acid composition, the specific content of cytochrome P-450 and cytochrome P-448 was 14.1 and 17.4 nmol of hemoprotein/mg of protein, respectively (10). Units of NADPH-cytochrome c reductase are defined as nanomoles of cytochrome c reduced/min, and the method of Phillips and Langdon (31) was used to determine enzymatic activity. Units of epoxide hydrase are defined as nanomoles of styrene glycol formed from styrene oxide/15 min.

Bacterial Strains—Strains TA 1538 and TA 98 of Salmonella typhimurium were developed by Ames and his associates (32, 33) and kindly provided by his laboratory at the University of California, Berkeley. Strain TA 1538, derived from S. typhimurium LT 2, contains the histidine mutation his D3052 which renders the organism completely dependent on histidine for growth. Frameshift mutagens which interact with the appropriate DNA sequences in or near the histidine operon will correct the original mutation and revert the bacteria to histidine independence. As a result of two additional mutations, the strain lacks a functional DNA excision repair system and the poly saccharide side chain of the lipopolysaccharide that coats the bacterial surface. The former mutation increases the sensitivity of the strain to mutagens by eliminating the excision repair of altered DNA, and the latter mutation increases the permeability of the bacteria to chemical mutagens. Strain TA 98 was developed by introducing an error-prone recombinational repair system into strain TA 1538. As a result, TA 98 is much more sensitive to mutagens than many chemical mutagens is the parental strain. Mutations (reversions) are quantified in both strains by counting the number of colonies which grow on a histidine-free medium.

The abbreviations used are: BP 4,5-diol, trans-4,5-dihydroxy-4,5-dihydrobenzo[a]pyrene; BP 7,8- and 9,10-diol, other trans-dihydrodiols of benzo[a]pyrene; I-HOBP, 1-hydroxybenzo[a]pyrene; 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12-HOBP, other benz[a]pyrene phenols; BP 4,5-oxide, benzo[a]pyrene 4,5-oxide; BP 7,8- and 9,10-oxide, other benzo[a]pyrene oxides; BP 7,8,9,10-epoxide, (+)-7,8,9,10-dihydroxy-9,10,13-epoxide, 7,8,9,10-tetrahydrobenzo[a]pyrene; BP 7a,8d-diol-9,10,13-epoxide, (+)-7a,8d-dihydroxy-9,10,13-epoxide, 7,8,9,10-tetrahydrobenzo[a]pyrene; BP 7a,8d-diol-9,10-epoxide, either or both cis epoxide stereoisomers.

Enzyme-mediated Mutagenesis of Benzo[a]pyrene

Bacterial inocula were grown at 37 °C in 5.0 ml of ‘Tryptose (BBL Inc.)’ soy broth, containing 1% yeast extract, and harvested in mid-logarithmic growth phase. After determining cell density by measuring optical density at 540 nm, the bacteria were collected by centrifugation for 15 min at 2000 x g and resuspended in 0.9% NaCl/0.005 M sodium phosphate (final pH, 6.8). The final cell density was 1 x 10^8 bacteria/ml.

**Metabolic Activation Assay—Oxidative metabolism of benzo[a]pyrene by the reconstituted monoxygenase system was performed essentially as previously described (10), except for the presence of bacteria in the reaction mixture. Generally, 2 x 10^9 bacteria were suspended in a total incubation volume of 0.5 ml containing 2.5 μmol of sodium phosphate, 75 μmol of sodium chloride, 0.08 μmol of phosphatidylcholine, 150 units of NADPH-cytochrome c reductase, 0.025 to 0.19 nmol of cytochrome P-448, 25 nmol of benzo[a]pyrene (added in 12.5 μl of acetone) and 0.1 μmol of NADPH. The final pH was 6.8, and the reaction was initiated by the addition of NADPH. After incubation at 37 °C for 5 min, 9 nmol of menadione in 0.1 ml of aqueous solution was added to stop the reaction. Menadione diverts reducing equivalents from the hemoprotein (34), and studies with the reconstituted monoxygenase system indicated that a 15 μM final menadione concentration effectively blocks benzo[a]pyrene metabolism, as measured by the absence of fluorescent metabolites, without interfering with the expression of mutations induced by BP 4,5-oxide. Immediately after the addition of menadione, 2.0 ml of molten (45 °C) top agar, containing 12 μg each of sodium chloride and agar, and 0.10 μmol each of a-histidine and histidine, was added to the reaction mixture, and the entire contents of the culture tube (13 x 100 mm) were mixed and poured into a Petri dish containing 15 ml of Vogel-Bonner medium with a 2% agar base. After 15 min at room temperature, the Petri dishes were incubated for 48 to 76 h at 37 °C. At the end of this time, the revertant colonies which were capable of growing in the absence of L-histidine were counted. All solutions and components of the reaction, except benzo[a]pyrene and the bacteria, were sterilized by filtration through 0.20 μm membrane filters.

In agreement with published kinetic values for benzo[a]pyrene hydroxylation by the purified monoxygenase system (35), preliminary experiments with TA 1538 showed that mutagenic metabolite formation was optimal at benzo[a]pyrene concentrations of 10 μM and was constant with respect to substrate concentration up to at least 50 μM benzo[a]pyrene. Experiments with excess histidine (1.0 μmol), which permitted all viable bacteria to grow for 120 min, showed that under the assay conditions just described, an insignificant amount of cell death was attributable to toxic metabolic products. Mutation frequency is therefore expressed as histidine revertants per plate. Metabolism of BP 7,8-diol, at some concentrations of cytochrome P-448, did result in the formation of toxic metabolites. It is possible that increased cell survival may have had a minor effect on the number of mutations expressed with some of the other benzo[a]pyrene derivatives. All experiments were performed in triplicate, and in those cases where standard deviations of the mean are not shown, the coefficients of variation were less than 15%.

**RESULTS**

**Requirements for Metabolism of Benzo[a]pyrene to Mutagenic Products by a Monoxygenase System—**The reconstituted monoxygenase system, in the presence of NADPH, metabolizes benzo[a]pyrene to products which are highly mutagenic to strains TA 1538 and TA 98 of Salmonella typhimurium (Table I). A 5-min incubation with a rate-limiting amount (0.1 nmol) of cytochrome P-448 and saturating amounts of NADPH-cytochrome c reductase, phospholipid, NADPH, and benzo[a]pyrene induced a 15-fold increase in histidine-independent colonies in both strains. Induction of mutations was completely dependent upon the presence of benzo[a]pyrene, NADPH, NADPH-cytochrome c reductase, and cytochrome P-448 and partially dependent upon phosphatidylcholine.

**Comparison of Cytochrome P 150 and Cytochrome P 448 in**

**TABLE I**

<table>
<thead>
<tr>
<th>Addition</th>
<th>His+ revertants per plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA 1538</td>
</tr>
<tr>
<td></td>
<td>TA 98</td>
</tr>
<tr>
<td>None</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>Complete hydroxylase system*</td>
<td>145 ± 10</td>
</tr>
<tr>
<td>145 ± 10</td>
<td>440 ± 29</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>30 ± 6</td>
</tr>
<tr>
<td>Cytochrome P-448</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>14 ± 5</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>NADPH</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>34 ± 12</td>
<td></td>
</tr>
</tbody>
</table>

*The complete hydroxylase system consisted of 0.08 μmol phosphatidylcholine, 150 units of purified NADPH-cytochrome c reductase, 0.1 nmol of purified cytochrome P-448, 25 nmol of benzo[a]pyrene, and 0.10 μmol of NADPH incubated in the presence of 2 x 10^9 bacteria as described under “Experimental Procedures.” Each value is the mean ± S.D. from three replicate assays.

**Activating Benzo[a]pyrene to Mutagenic Metabolites—**Cytochrome P-448 obtained from hepatic microsomes of rats pretreated with 3-methylcholanthrene was up to 8 times more effective than cytochrome P-450 obtained from hepatic microsomes of phenobarbital-pretreated rats in catalyzing the formation of benzo[a]pyrene metabolites which were mutagenic to strains TA 1538 (Fig. 1A) and TA 98 (Fig. 1B). In a 5-min incubation, reversions to histidine independence were proportional to the amount of hemoprotein added from 0.0063 nmol to 0.10 nmol of cytochrome P-448. In contrast, cytochrome P-450 induced a lower number of reversions in both strains which was not proportional to the amount of hemoprotein added.

**Effect of Incubation Time on Mutagenic Activity of Benzo[a]pyrene—**The number of bacteria, of either strain TA 1538 or TA 98, reverted from histidine auxotrophy to histidine independence was directly proportional to the duration of the monoxygenase-dependent reaction (Fig. 2). Using 0.1 nmol of cytochrome P-448, saturating amounts of NADPH-cytochrome c reductase and phosphatidylcholine, and a final benzo[a]pyrene concentration of 50 μM (15 to 20 times K_m), (35), mutations were linear with time for at least 10 min. Generally, to avoid any possibility of nonlinear kinetics, we have restricted incubation times to 5 min.

**Effect of Epoxide Hydrase on Cytochrome P-448-mediated Activation of Benzo[a]pyrene—**Hepatic microsomal epoxide hydrase converts arene oxides to trans-dihydrodiol. Addition of the enzyme to the purified monoxygenase system, which is devoid of epoxide hydrase activity, lowers the concentration of arene oxide intermediates and the levels of those phenols which are spontaneous isomerization products of the corresponding arene oxides (13). Addition of highly purified epoxide hydrase to the monoxygenase incubation mixture during the metabolism of benzo[a]pyrene decreased the mutation frequency in both strains of S. typhimurium, although strain TA 1538 appeared more sensitive to the enzyme’s inhibitory effects (Fig. 3). Approximately 18 units of epoxide hydrase inhibited the metabolic activation of benzo[a]pyrene to mutagens by 50% in strain TA 1538, while 75 units of the enzyme reduced mutations in TA 98 by 30%. Addition of up to 300 units of epoxide
Enzyme-mediated Mutagenesis of Benzo[a]pyrene

Fig. 1. Effect of cytochrome P-450 and cytochrome P-488 concentration on the metabolism of benzo[a]pyrene to products mutagenic to strains TA 1538 (A) and TA 98 (B) of Salmonella typhimurium. Incubation conditions were as described under “Experimental Procedures,” with the exception that hemoprotein concentration was varied as indicated. Reaction mixtures which contained no hemoprotein but cytochrome P-448 present in the complete monooxygenase system. The number of histidine revertants induced per plate without added hemoprotein (ordinate intercept) is a measure of the intrinsic mutagenicity of the derivative, since the monooxygenase system is inactive without the cytochrome.

It can be seen in Fig. 5 that 6- and 12-HOBP were active bacterial mutagens in strain TA 98 without further metabolism. At a final concentration of 25 μM, 6- and 12-HOBP induced a 5.5- and 10-fold increase, respectively, in the mutation frequency. Six phenols (1-, 2-, 3-, 6-, 9-, and 12-HOBP) were metabolized to mutagenic products and can thus be considered pre-mutagens. Studies with strain TA 1538 and phenol concentrations of 10 μM, 25 μM, and 50 μM showed the same pattern of activation as was seen in strain TA 98. The BP 1,6-, 3,6-, and 6,12-quinones which are formed from 3- and 6-HOBP (21, 36), as well as BP 11,12-quinone, a potential metabolite of 12-HOBP, cannot account for the mutagenic activity of metabolically activated 1-, 3-, 6-, or 12-HOBP since these quinones are nonmutagenic in strains TA 98 and TA 1538 (16). Addition of 75 units of epoxide hydrase to the monooxygenase system had a minimal effect (less than 30% decrease) on the number of mutations induced in strain TA 1538 from further metabolism of the phenols. None of the phenols was as effectively converted to mutagenic products as benzo[a]pyrene in either strain. The six remaining phenols (4-, 5-, 7-, 8-, 10-, and 11-HOBP) had little or no mutagenic activity with or without further oxidative metabolism.

Metabolic Activation of Benzo[a]pyrene Dihydrodiols by a Cytochrome P-448-dependent Monooxygenase System—Of four trans dihydrodiols tested BP 7,8 diol was most actively
metabolized to mutagenic products (Fig. 6). As little as 6 pmol of cytochrome P-448 mediated a 10-fold increase in mutatios from BP 7,8-diol in strain TA 98, whereas 20 to 25 pmol of cytochrome P-448 were necessary to induce this mutation rate when benzo[a]pyrene was the substrate. Addition of increasing amounts of cytochrome P-448 to the incubation mixture resulted in a decrease in the mutation frequency with BP 7,8-diol probably as a result of the formation of increased amounts of the highly cytotoxic diol epoxide4 (15). Relatively few or no mutagenic products were formed when BP 4,5-, 9,10-, and 11,12-diols were incubated with the active monooxygenase system. Qualitatively similar results were observed in strain TA 1538 at final diol concentrations of 25 μM (Fig. 6) and at 10 μM and 50 μM concentrations (data not shown).

Metabolic Activation of Benzo[a]pyrene 7,8-Oxide—Since BP 7,8-diol was readily metabolized to a mutagenic product(s) by the cytochrome P-448 monooxygenase system, we examined the effect of the monooxygenase system on the mutagenic activity of its arene oxide precursor, BP 7,8-oxide (Fig. 7). In the absence of added epoxide hydrase, the low intrinsic mutagenic activity of the non-K-region arene oxide remained unchanged up to 0.1 nmol of cytochrome P-448. However, addition of epoxide hydrase to the monooxygenase system resulted in a marked increase in the induction of histidine prototrophs in strain TA 98. These results demonstrate that the BP 7,8-oxide is hydrated to the dihydrodiol, which in turn is oxidatively metabolized to the active mutagen. The low and constant mutagenic activity of BP 7,8-oxide without added epoxide hydrase clearly demonstrates the absence of epoxide hydrase from the monooxygenase system.

**DISCUSSION**

The addition of a mammalian metabolizing system in bacterial mutation assays has been essential for the detection of mutagenic activity of those carcinogens and mutagens that must be bioactivated to their reactive forms (6-8, 37, 38). Although prolonged co-incubation of bacteria, liver homogent, cofactors, and test chemicals in agar is effective as a screening system, this procedure all but precludes identification of the bioactivated mutagenic metabolite(s). We therefore chose to develop an enzymologically well defined mutation assay system in which benzo[a]pyrene activation would be proportional to incubation time and enzyme concentration, and in which metabolism could be readily terminated. Only the studies on the activation of dialkylaminoalkanes (37, 38) appear to have been performed under such conditions. Major advances have recently been made in the identification of the
products of benzo[a]pyrene metabolism and in the unequivocal chemical synthesis of these and other potential metabolites. We therefore designed our mutation assay system to make maximal use of the information already obtained from our metabolite analysis studies (13, 19) and our systematic investigations of the intrinsic mutagenicity (14-17) of the chemically synthesized derivatives. As discussed in further detail below, the results of high pressure liquid chromatographic analysis of benzo[a]pyrene metabolism by the reconstituted monooxygenase system and the results on the mutagenicity of authentic metabolites after the enzyme is added to the reconstituted system to the corresponding dihydrodiols, which have little or no intrinsic mutagenic activity, represent the mean number of histidine revertants/plate of three replicate incubations. O--O, BP 4,5-oxide, strain TA 98; O—O, BP 7,8a-diol-9β,10β-epoxide, strain TA 1538; ■—■, BP 7,8a-diol-9β,10β-epoxide, strain TA 98.

The reconstituted NADPH dependent monooxygenase system, solubilized and purified from rat liver microsomes, effectively activates benzo[a]pyrene and several of its non-mutagenic derivatives to metabolites which are highly mutagenic to S. typhimurium strains TA 98 and TA 1538. The requirements of cytochrome P-450, NADPH-cytochrome c reductase, phosphatidylcholine, and NADPH for mutagen formation are identical to those necessary for benzo[a]pyrene metabolism with the reconstituted system (39). The superior-
Enzyme-mediated Mutagenesis of Benzo[a]pyrene

Fig. 5. Metabolism of the 12 isomeric phenols of benzo[a]pyrene to mutagenic products by a cytochrome P-448-dependent monooxygenase system. Incubation conditions were exactly as described in the legend to Fig. 1 and under "Experimental Procedures." The final concentration of benzo[a]pyrene or the phenols was 25 µM, and the hemoprotein concentration was as indicated. The values shown represent the means of three replicate determinations after subtraction of the revertants induced by reaction mixtures which lacked benzo[a]pyrene or phenol but were otherwise complete.

Fig. 6. Effect of cytochrome P-448 concentration on the metabolism of benzo[a]pyrene dihydrodiols to mutagenic products. The final concentration of benzo[a]pyrene or dihydrodiol was 25 µM. All other experimental details were identical to those described in Fig. 5.
lism of BP 7,8-oxide to mutagenic product(s) (Fig. 7) is the first
direct demonstration of a bioactivating role for this enzyme.
Thus, like the microsomal monoxygenase system, epoxide
hydrate activates as well as detoxifies polycyclic hydrocarbons.
The 30 to 50% decrease in mutation frequency observed upon
the addition of epoxide hydrase to the monoxygenase system
demonstrates that some of the mutagenic metabolites are
arene oxides. BP 4,5-oxide is a product of benzo[a]pyrene
metabolism by the purified monoxygenase system (13), and it
is likely that the K-region arene oxide accounts for a significant
number of the mutations in the absence of epoxide hydrase.
The inability of epoxide hydrase to completely block the
cytochrome P-448-mediated mutagenesis of benzo[a]pyrene
suggests (a) that non-arene oxide metabolites of benzo[a]pyrene
may also have mutagenic activity in S. typhimurium, (b) that
some mutagenic arene oxides are poorly metabolized by the
purified epoxide hydrase, and/or (c) that the effects of epoxide
hydrate shown in Fig. 3 represent a composite of inactivation of
arene oxide metabolites and formation of the highly mutagenic
BP 7,8-diol-9,10-epoxide from BP 7,8-diol. Support for all three
possible explanations can be found in the present results, but
without additional experiments it is impossible to make a
definitive judgment on the relative importance of the several
possible mechanisms underlying the incomplete inactivation of
metabolites by epoxide hydrase. With respect to the first
hypothesis, it is worth noting that 6-HOBP and 12-HOBP are
mutagenic to strain TA 98 (Fig. 5) and are the most mutagenic
of approximately 20 nonepoxide derivatives of benzo[a]pyrene
we have examined to date (16). However, the amount of phenol
and quinone metabolites produced by the reconstituted mono-
oxygenase system as determined by high pressure liquid
chromatography (13) under conditions comparable to those
described here, make it unlikely that these phenols contribute
significantly to the mutagenicity observed from benzo[a]pyrene
metabolism.
Several studies have indicated that primary oxidative me-
tabolites of benzo[a]pyrene can be further metabolized by the
hepatic monoxygenase system. To ascertain whether sec-
tory oxidative metabolites of benzo[a]pyrene were mutagenic,
we used several derivatives of the hydrocarbon as substrates in
the monoxygenase mutagenesis system. Of the 4 trans-dihy-
drodiols and 12 monohydroxyl derivatives of benzo[a]pyrene
examinined in this study (Figs. 5 and 6), only BP 7,8-diol was
more active than benzo[a]pyrene as a substrate for further
oxidation to a mutagenic metabolite(s). These results are
consistent with the study of Borgen et al. (18) which
demonstrated that metabolites of BP 7,8-diol bind more extensivly
to DNA than do metabolites of benzo[a]pyrene. A recent study
by Malaveille et al. (41), published during the preparation of
this manuscript, demonstrates that on further metabolism by
a 9000 x g supernatant fraction of rat liver BP 7,8-diol is more
active than benzo[a]pyrene or BP 4,5- and 9,10-diols in inducing
his revertants in strain TA 100 of S. typhimurium. Sims et al.
(20) have provided evidence that BP 7,8-diol-9,10-epoxide is the
product of BP 7,8-diol metabolism which binds to DNA.
Benzo[a]pyrene 7,8-diol-9,10-epoxide is highly mutagenic to
bacteria (15, 16, 41) and mammalian cells (15, 16, 42), and it
is likely that this compound is responsible for the mutations
observed from the metabolism of BP 7,8-diol. However, it is
certain that the diol-epoxide is not one of the mutagenic
metabolites formed during metabolism of benzo[a]pyrene by the
reconstituted monoxygenase system which is free of
epoxide hydrase. We do not know whether the diol-epoxide is
being formed from the metabolism of benzo[a]pyrene by the
reconstituted monoxygenase system in the presence of
epoxide hydrase. High pressure liquid chromatographic analysis of
metabolites formed from benzo[a]pyrene by the monoxygen-
ase system in the presence of epoxide hydrase indicates that a
significant amount of BP 7,8-diol (approximately 10% of the
metabolites) is formed (13). While additional studies are
necessary to demonstrate further metabolism of the diol to
diol-epoxide under these conditions, we have shown (4) that BP
7,8-diol is a good substrate for the reconstituted monoxygen-
ase system and is metabolized to both stereoisomers of BP
7,8-diol-9,10-epoxide.
Although the mutagenicity of 1-, 2-, 3-, 6-, 9-, and 12-HOBP
towards S. typhimurium TA 98 can be increased by co-incuba-
tion of the bacteria and phenols with the reconstituted
monoxygenase system (Fig. 5), benzo[a]pyrene was activated
to mutagenic metabolites to a much greater extent than any of
the phenols. Wieland (91) has recently reported that 3-, 6-, and
9-HOBP are metabolized by rat liver microsomes, and Cape-
devila et al. (43) have demonstrated that rat lung microsomes
can metabolize 3-HOBP to a product(s) which covalently binds
to DNA.
The carcinogenicity of a number of known and possible
benzo[a]pyrene metabolites is currently being evaluated in our
laboratory. We have recently reported that BP 7,8-oxide, un-
like BP 4,5-oxide and BP 9,10-oxide, was a potent carcino-
gen on mouse skin (44). In contrast to its carcinogenic activity,
BP 7,8-oxide is a weak bacterial and mammalian cell mutagen

1 D. R. Thakker, H. Yagi, W. Levin, A. Y. H. Lu, A. H. Conney, and
Enzyme-mediated Mutagenesis of Benzo[a]pyrene

(14). Since mouse skin epidermis contains both epoxide hydase (45, 46) and monoxygenase activity (47, 48), the carcinogenicity and mutagenicity data may be reconciled if the BP 7,8-oxide is not carcinogenic per se but must be metabolized to BP 7,8-diol-9,10-epoxide. The demonstrated mutagenicity of BP 7,8-diol-9,10-epoxide*(4, 15, 16, 41, 42) and our data indicating that BP 7,8-oxide can be metabolized to a highly active mutagen by the reconstituted monoxygenase system only in the presence of epoxide hydase supports the metabolic activation of benzo[a]pyrene according to the scheme: BP → BP 7,8-oxide → BP 7,8-diol → BP 7,8-diol-9,10-epoxide. We are currently evaluating the carcinogenicity of the latter two metabolites of this pathway. While data are not yet available on the carcinogenicity of the two BP 7,8-diol-9,10-epoxides, BP 7,8-diol is a very potent carcinogen on mouse skin*. The complete inactivation of BP 7,8-oxide (K-region oxide) as a bacterial mutagen by low amounts of epoxide hydrase (Fig. 4) and the lack of mutagenic activity of BP 4,5-diol, both in the absence and presence of the active monoxygenase system (Fig. 6) may explain, in part, why BP 4,5-oxide was a weak carcinogen on mouse skin.

Acknowledgments—We wish to thank Dr. R. L. Goode for many helpful suggestions and discussion in the initial phases of this study, and Mrs. M. A. Augustin, Mrs. C. Chvoasta, and Mrs. A. Szilagyi for their assistance in the preparation of this manuscript.

REFERENCES


Metabolism of benzo(a)pyrene and benzo (a)pyrene derivatives to mutagenic products by highly purified hepatic microsomal enzymes.


Access the most updated version of this article at http://www.jbc.org/content/251/16/4882

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/251/16/4882.full.html#ref-list-1