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HYDROXYLATION OF BENZO[*a*]PYRENE AND BINDING OF (−)*trans*-7,8-DIHYDROXY-7,8-DIHYDROBENZO[*a*]PYRENE METABOLITES TO DEOXYRIBONUCLEIC ACID CATALYZED BY PURIFIED FORMS OF RABBIT LIVER MICROSOMAL CYTOCHROME P-450

EFFECT OF 7,8-BENZOFLAVONE, BUTYLATED HYDROXYTOLUENE AND ASCORBIC ACID*

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Abstract—The catalytic activities of hepatic microsomes from untreated, phenobarbital-treated and 3-methylcholanthrene-treated adult rabbits with respect to benzo[*a*]pyrene hydroxylation and the activation of (−)*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene[(−)*trans*-7,8-diol] to DNA-binding metabolites were determined in the absence and presence of mixed-function oxidase inhibitors and compared to the corresponding activities of the individual enzyme systems. Treatment of rabbits with phenobarbital led to induction of P-450LM_2 and a concomitant 3-fold enhancement in microsomal benzo[*a*]pyrene hydroxylase activity, whereas the conversion of (−)*trans*-7,8-diol to DNA-binding products was unaffected. Homogeneous phenobarbital-inducible P-450LM_2 exhibited the highest activity and specificity toward benzo[*a*]pyrene and the lowest activity toward (−)*trans*-7,8-diol. Conversely, P-450LM_4 was the major form of cytochrome P-450 induced in rabbit liver by 3-methylcholanthrene or β-naphthoflavone, and this was associated in microsomes with an increase in the metabolism of (−)*trans*-7,8-diol but not of benzo[*a*]pyrene. Homogeneous P-450LM_4 preferentially catalyzed the oxygenation of (−)*trans*-7,8-diol, but was largely ineffective with benzo[*a*]pyrene. Partially purified P-450LM_7 lacked substrate specificity, for it metabolized both benzo[*a*]pyrene and (−)*trans*-7,8-diol at comparable rates. Additionally, 7,8-benzoflavone strongly inhibited benzo[*a*]pyrene hydroxylation by P-450LM_4 and phenobarbital-induced microsomes, as well as (−)*trans*-7,8-diol metabolism by P-450LM_4 and 3-methylcholanthrene-induced microsomes; in contrast, the activity of control microsomes with either substrate, and the activities of P-450LM_4 and LM_2 with benzo[*a*]pyrene and (−)*trans*-7,8-diol, respectively, were only partially or slightly decreased by 7,8-benzoflavone. Unlike 7,8-benzoflavone, butylated hydroxytoluene inhibited benzo[*a*]pyrene hydroxylation only. Thus, different forms of rabbit liver microsomal cytochrome P-450 were involved in the metabolism of benzo[*a*]pyrene and its 7,8-dihydrodiol. The results also demonstrate that the changes in substrate specificity and inhibitor sensitivity seen in phenobarbital- and 3-methylcholanthrene-induced microsomes relative to control rabbit liver microsomes can be accounted for by the catalytic properties of a specific form of cytochrome P-450 that prevails in these preparations, P-450LM_2 and LM_4, respectively.

The hepatic microsomal cytochrome P-450-containing mixed-function oxidases and related membrane-bound enzymes catalyze the oxidation of metabolically important substances as well as a host of xenobiotics [1, 2]. Many foreign compounds are detoxified by these versatile catalysts, while in other instances metabolism results in activation to cytotoxic or carcinogenic species [3–5].

Following the resolution of the mixed-function oxidase system of liver microsomal membranes and reconstitution of an active hydroxylation system from the components, namely cytochrome P-450, NADPH-cytochrome P-450 reductase and phosphatidylcholine [6, 7], investigations in this laboratory focused on the separation and characterization of

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multiple forms of rabbit P-450LM* [8, 9]. PB-inducible P-$450LM_2$ and BNF-inducible P-$450LM_4$ were purified to homogeneity and shown to be distinct proteins, as judged by their physichochemical, immunological and kinetic properties [8–12]. Additional forms of rabbit P-450LM possessing different physical properties and catalytic activities, e.g. P-$450LM_1$, LM_{3a}, LM_{3b} and LM_7, have been isolated in a partially purified state [8, 9, 11]. Several other laboratories have recently reported the separation and purification to varying extents of individual forms of the cytochrome from liver microsomes of untreated rats and rabbits, as well as from microsomes of animals treated with different inducers [13–20]. The existence of multiple forms of P-450LM, which exhibit different but often overlapping substrate specificities [8, 11, 14, 17, 18, 20, 21], provides a reasonable explanation for the remarkably broad substrate specificity of the liver microsomal hydroxylase system, while variations in the levels of the individual forms of the cytochrome can readily account for the differences in catalytic activity observed with microsomal suspensions from animals differing in age, sex, strain and type of inducer used for pretreatment.

The biotransformation of the ubiquitous environmental pollutant BP, which is itself essentially nontoxic, proceeds by a number of pathways, some yielding biologically inactive products and some leading to the formation of highly reactive metabolites which bind to cellular macromolecules and thus exert cytotoxic, mutagenic or carcinogenic effects [3, 22–25]. The liver microsomal mixed-function oxidase systems metabolize BP to phenols, quinones and unstable arene epoxide intermediates [26–29], which are further converted to dihydrodiols by epoxide hydrase [30, 31]. Most of the oxygenated products appear *in vivo* as water-soluble glutathione, glucuronic acid and sulfate conjugates [32–34]. It is presently believed that the most significant pathway of BP activation, at least in 3-MC-induced rat liver microsomal suspensions, involves enzymatic oxygenation of this polycyclic aromatic hydrocarbon in three remarkably stereospecific steps [35], ultimately giving rise to two stereoisomeric (−)*trans*-7,8-diol-9,10-epoxides, namely, a major diol-epoxide I and a minor dio-epoxide II in a ratio of about 10 to 1 [35–37]. The binding of reactive metabolites of BP and of other hydrocarbon carcinogens to DNA is highly correlated with the tumor-initiating ability of these compounds [38]. Although the binding of BP to DNA can occur via the K-region epoxides, e.g. by recycling of phenols such as 9-hydroxybenzo[*a*]pyrene through the mixed-function oxidase system [39–41], diol-epoxides I and II appear to be the predominant BP species bound to nucleic acids *in vitro* and *in vivo* [42–47] and the most mutagenic in cultured mammalian cells (diol-epoxide I) [48–50] and in strains TA 98 and TA 100 of *Salmonella typhimurium* (diol-epoxide II) [51].

In previous studies [52, 53], we examined the metabolism of BP and of the two optical isomers of *trans*-7,8-diol by homogeneous and partially purified forms of rabbit P-450LM in the reconstituted enzyme system, using high pressure liquid chromatography to detect the metabolites formed. It was shown that BP is metabolized predominantly by P-$450LM_2$ [52], whereas the oxidation of (−)*trans*-7,8-diol is catalyzed twenty to thirty times faster by P-$450LM_4$ than LM_2 [52, 53]. Moreover, P-$450LM_4$ exhibits a pronounced preference for the (−) as compared to the (+) enantiomer of *trans*-7,8-diol [53], and stereoselectively converts the former to diol-epoxide I [52, 53]. The striking stereospecificity of P-$450LM_4$ is also evident from the fact that this cytochrome inserts the oxygen atom primarily on one side of the plane of the *trans*-7,8-diol molecule, independent of the configuration of the 7,8 hydroxyl groups [53]. It follows that the relative distribution of the various forms of P-450LM in a tissue, coupled with the substrate specificity and the high degree of regio- and stereoselectivity of these catalysts, will most likely influence the metabolic disposition of a xenobiotic substrate and thus regulate the balance between its detoxification and activation.

In the present studies, we have investigated the effects of 7,8-BF, a strong inhibitor of BP metabolism and binding to DNA [54], and of two other compounds which may modulate chemical carcinogenesis, namely, BHT and ascorbic acid [55, 56], on BP and (−)*trans*-7,8-diol oxidation by control, PB- and 3-MC-induced rabbit liver microsomes, and by homogeneous P-$450LM_2$ and LM_4 and partially purified P-$450LM_7$ in the reconstituted enzyme system. The metabolism of BP was determined by the rapid and sensitive AHH assay, while the binding of (−)-*trans*-7,8-diol metabolites (diol-epoxides I and II) to DNA was used as an index of the catalytic activity of the various preparations toward (−)*trans*-7,8-diol. These studies may help in providing possible approaches to the modulation of chemical carcinogenesis.

MATERIALS AND METHODS

Treatment of animals and preparation of microsomes for metabolism studies. Male New Zealand rabbits (1–1.2 kg in weight), obtained from the NIH animal supply, were treated, i.p., with 80 mg PB (in 0.9% NaCl)/kg body wt once a day for 4 days, or treated one time with 40 mg 3-MC (in corn oil)/kg body wt; control animals received 0.9% NaCl or corn oil, respectively. The animals were killed 24 hr after the last injection, and the livers were homogenized in 5 vol. of 0.05 M Tris-chloride buffer, pH 7.5, containing 0.25 M sucrose. The microsomes were prepared as described previously [29] and were

* The following abbreviations are used: P-450LM, liver microsomal cytochrome P-450; BP, benzo[*a*]pyrene; (+) or (−)*trans*-7,8-diol, (+) or (−)*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; diol-epoxide I, *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; diol-epoxide II, *r*-7,*t*-8-dihydroxy-*c*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; in the latter two abbreviations, *r*-7 indicates that the substituent at the 7-position is the reference, and *t* and *c* indicate that the substituents are *trans* and *cis*, respectively, to the reference substituent; 7,8-BF, 7,8-benzoflavone; BHT, butylated hydroxytoluene; AHH, aryl hydrocarbon hydroxylase; PB, phenobarbital; BNF, β-naphthoflavone; 3-MC, 3-methylcholanthrene; and dilauroyl-GPC, dilauroylglyceryl-3-phosphorylcholine.

stored at −70°. Although there is some loss of activity upon freezing (Table 4), no substantial loss of enzymatic activity occurs during storage for a several-month period. The concentration of cytochrome P-450 in these preparations was determined from the spectrum of the reduced CO complex using an extinction coefficient of 91 $mM^{-1} cm^{-1}$ for the difference between A_{max} (447–451 nm, depending on the preparation) and A_{490} [57].

Treatment of animals and preparation of microsomes for enzyme purification. Male New Zealand rabbits (2–2.4 kg in weight) were purchased from Langshaw Farms, Kalamazoo, MI, U.S.A. The animals were allowed to drink a 0.1% solution of sodium PB (adjusted with HCl to pH 7) for 5 days and were fasted for 16 hr. Other rabbits were given BNF (80 mg/kg body wt) by an intraperitoneal injection of a 1% suspension in corn oil, and after 24 hr the animals were fasted for 16 hr [9]. The livers were homogenized in 4 vol. of 0.1 M Tris-acetate buffer, pH 7.4, containing 0.1 M KCl, 1.0 mM EDTA and 0.023 mM BHT, in a Waring Blendor for two 40-sec intervals, and the mixture was centrifuged at 10,000 *g* for 30 min [58]. Liver microsomes were prepared, extracted with 0.1 M sodium pyrophosphate buffer containing 1.0 mM EDTA (in the absence of glycerol) and stored at −20° in the presence of glycerol [9]. The microsomes were subsequently thawed, solubilized with sodium cholate, and fractionated with polyethylene glycol as described previously [9, 58].

Purification of microsomal enzymes. The various forms of P-450LM are designated by their relative mobilities when submitted to discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and β-mercaptoethanol [8, 9, 11]. Ouchterlony diffusion analysis and sodium dodecylsulfate–polyacrylamide gel electrophoresis have shown that PB-inducible P-$450LM_2$ is present in only trace amounts in microsomes from untreated and BNF-treated rabbits, whereas BNF-inducible P-$450LM_4$ is present at significant levels in microsomes from untreated and PB-treated animals [8, 11]. As reviewed elsewhere [11, 59, 60], and in agreement with the induction studies of Atlas *et al.* [61], P-$450LM_4$ preparations purified from these three sources [9], as well as from 3-MC-induced (P-448) [15, 62] and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced rabbits (P-450c) [17], are essentially the same protein based on several independent lines of evidence [9, 10, 12, 17, 21, 52, 62, 63].

The purification of P-$450LM_2$ from liver microsomes of PB-treated rabbits and of P-$450LM_4$ from the same source, as well as from microsomes of BNF-treated animals, was carried out according to procedures published previously [9, 64]. These preparations were homogeneous as judged by sodium dodecylsulfate–polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol. Partially purified P-$450LM_7$, which is present in all of the microsomal preparations and is not known to be inducible, was isolated from microsomes of untreated animals [8, 11] and contained traces of epoxide hydrase. The concentrations of P-$450LM_2$ and LM_4 were determined from the absolute spectra of the reduced CO complexes using extinction coefficients of 110 $mM^{-1} cm^{-1}$ for P-$450LM_2$ (A_{max} at 451 nm) and 110 $mM^{-1} cm^{-1}$ for P-$450LM_4$ (A_{max} at 488 nm) [9]. The concentration of P-$450LM_7$ was estimated from the spectrum of the reduced CO complex using an extinction coefficient of 91 $mM^{-1} cm^{-1}$ for the absorbance difference between 450 and 490 nm [57]. The specific content of the purified P-450LM samples used in these studies was as follows (expressed as nmoles of P-450LM per mg of protein): P-$450LM_2$, 17.5; P-$450LM_4$ from PB- and BNF-induced rabbits, 14.4 and 13.1, respectively; and P-$450LM_7$, 1.2.

NADPH-cytochrome P-450 reductase was purified from liver microsomes of PB-treated rabbits by DEAE-cellulose ion exchange chromatography followed by affinity chromatography on 2′,5′-ADP-agarose by procedures [11, 65] similar to those previously described for the rat liver enzyme [11, 66, 67]. The resulting preparation was homogeneous, by sodium dodecylsulfate–polyacrylamide gel electrophoresis, and catalyzed the reduction of 40 μmoles cytochrome *c*/min/mg of protein in 0.3 M potassium phosphate buffer, pH 7.7, at 30°. One unit of the reductase is defined as the amount which catalyzes the reduction of 1.0 μmole cytochrome *c*/min under these conditions. The concentration of the reductase was determined from the absolute spectrum of the oxidized flavoprotein using an extinction coefficient of 21 $mM^{-1} cm^{-1}$ at 456 nm [11, 65, 67].

AHH assay of BP metabolism. The NADPH-dependent oxidation of BP by liver microsomal suspensions from untreated (control), PB- or 3-MC-treated rabbits, and by purified forms of rabbit P-450LM in the reconstituted enzyme system, was measured under aerobic conditions in the absence and presence of mixed-function oxidase inhibitors. The reaction mixtures, in a total volume of 0.5 ml, contained either intact microsomes (100 μg protein) or the following components added in the order indicated: 0.15 nmole P-450LM, 0.25 nmole (0.9 units) NADPH-cytochrome P-450 reductase and 16 μg dilauroyl-GPC which were mixed and equilibrated at room temperature for 5 min. Tris-chloride buffer (pH 8.5) (25 μmoles) and NADPH (0.13 μmole) were then added, and the reactions were initiated with BP (5 nmoles added in 0.005 ml methanol). The final concentrations of the various modifiers, when present, were as follows: 7,8-BF or BHT, 0.1 mM (added in 0.005 ml methanol); and ascorbic acid, 10 mM (added in 0.01 ml water). Incubations were carried out at 37° for 15 min; the reactions were terminated by the addition of 1.0 ml acetone at 4°, and the mixtures were extracted with 3.0 ml hexane (spectrograde). Two-ml aliquots of the organic phase were added to 1.0 ml of 1.0 N NaOH, and the fluorescence of the phenolic products (primarily 3-hydroxybenzo[*a*]pyrene) in the alkaline solution was measured with an Aminco-Bowman model SPF-500 spectrophotofluorometer (AHH assay). A calibration curve was constructed by extracting known amounts of 3-hydroxybenzo[*a*]pyrene from the reaction mixtures prepared in the absence of enzymes.

Assay of DNA binding activity. The binding of (−)*trans*-7,8-diol metabolites to DNA was estimated by a modification of procedures published previously [68, 69]. The composition of the reaction mixtures

was as described above for BP metabolism, except that calf thymus DNA (0.25 mg) was also included and BP was replaced by [^{3}H](−)*trans*-7,8-diol (5 nmoles added in 0.005 ml of a 9:1 mixture of methanol–tetrahydrofuran). After incubation at 37° for 15 min, the samples were placed on ice and extracted twice with 3.0 ml ethyl acetate to remove unbound substrate and metabolites. $MgCl_2$ (3 μmoles) was added to the aqueous phase which was subsequently increased to 1.0 ml in volume and extracted with 0.9 ml of phenol reagent (600 g of phenol, 0.5 g of 8-hydroxyquinoline, 75 ml of cresol and 55 ml of water). The resulting mixture was centrifuged at 2000 r.p.m. for 20 min, and the DNA in the aqueous phase was precipitated with 3 vol. of ethanol at 4°. The precipitate was dissolved in 1.0 ml of a 1.0 M Tris–chloride–0.003 M $MgCl_2$ solution, washed three times with 2.0 ml ethyl acetate, and reprecipitated with 3 vol. of ethanol. The DNA was then hydrolyzed in 0.5 M perchloric acid (1.2 ml) at 80° for 15 min. After cooling the samples at room temperature, a 0.5-ml aliquot of the hydrolysate was diluted with 5.0 ml Aquasol and counted in a Beckman model LS 8100 scintillation counter having a greater than 30 per cent efficiency for ^{3}H. Another 0.5-ml aliquot of the hydrolysate was analyzed for DNA content by the absorption at 260 nm, using a Gilford model 2400 spectrophotometer. The DNA content was estimated by a modification of the method of Shack [70] in which 40 μg of native calf thymus DNA/ml has an A_{260} of 0.66 after a 22 per cent correction for the hyperchromic effect due to the perchloric acid hydrolysis.

Other methods. Protein concentrations were determined according to Lowry *et al.* [71] with correction for the effect of other components of the solutions [9]; bovine serum albumin served as the standard.

The data represent the mean ± S.D. of values obtained in at least two separate experiments with duplicate determinations in each experiment. Student's *t*-test was used to establish the significance of differences between means. Unless indicated otherwise, $P < 0.01$ was chosen as the level of significance.

Chemicals. Nonlabeled racemic *trans*-7,8-diol was synthesized through National Cancer Institute Contract Nol-CP-33387, and the pure (+) and (−) optical isomers were prepared by high pressure liquid chromatography as already described [35, 72]. [^{3}H](−)*trans*-7,8-Diol was obtained by incubating liver microsomes from 3-MC-treated rats with [^{3}H]BP (sp. act. 20–50 Ci/nmole) in the presence of NADPH and oxygen [36, 37]. The biosynthetic [^{3}H]-(−)*trans*-7,8-diol thus formed was isolated by high pressure liquid chromatography [73] and diluted to an appropriate specific activity (300 nCi/nmole) with nonradioactive (−)*trans*-7,8-diol. BP was obtained from Eastman Organic Chemicals (Rochester, NY) and was recrystallized from ethanol. 7,8-BF and BHT were purchased from the Aldrich Chemical Company (Milwaukee, WI) and were recrystallized from methanol. Calf thymus DNA and ascorbic acid were from the Sigma Chemical Co. (St. Louis, MO).

Table 1. Effects of modifiers on BP metabolism by purified forms of rabbit P-450LM and by intact liver microsomes*

	Activity (pmoles phenolic products formed/min/nmole P-450LM)			
		With modifier		
P-450LM preparation	Without modifier	7,8-BF	BHT	Ascorbic acid
Purified P-450LM				
LM_2	60.3 ± 3.4	13.2 ± 2.2 (78)†	16.4 ± 1.9 (73)	35.8 ± 2.4 (41)
$LM_{4(BNF)}$‡	6.8 ± 0.4	5.1 ± 0.5 (25)	5.2 ± 0.4 (24)	3.7 ± 0.5 (46)
$LM_{4(PB)}$‡	2.7 ± 0.1	2.1 ± 0.5§ (22)	2.2 ± 0.4§ (19)	1.7 ± 0.1 (37)
LM_7	40.2 ± 3.6	30.1 ± 7.1§ (25)	29.0 ± 6.1 (28)	23.9 ± 5.2 (41)
Liver microsomes				
Control	28.5 ± 3.6‖	19.1 ± 1.6 (33)	23.0 ± 4.2 (19)	18.3 ± 3.1 (36)
PB-induced	54.5 ± 5.5‖	27.1 ± 2.2 (50)	13.4 ± 2.2 (75)	25.0 ± 4.6 (54)

* AHH activities were determined by incubating BP with rabbit liver microsomal suspensions or with purified forms of rabbit P-405LM (homogeneous P-450LM_2 and LM_4 or partially purified P-450LM_7) in the reconstituted enzyme system. Reaction mixtures contained Tris-chloride buffer, pH 8.5 (25 μmoles), BP (5 nmoles), NADPH (0.13 μmole), and either intact microsomes from the indicated source (100 μg protein) or purified P-450LM (0.15 nmole) homogeneous NADPH-cytochrome P-450 reductase (0.25 nmole), and dilauroyl-GPC (16 μg) in a total volume of 0.5 ml. Incubations were carried out for 15 min at 37°, and the phenolic metabolites formed were extracted into NaOH and estimated by spectrophotofluorimetry as described in Materials and Methods. When present, 7,8-BF, BHT and ascorbic acid were added at a final concentration of 0.1, 0.1 and 10 mM, respectively. The microsomes used in these metabolic studies had been stored at −70° following their isolation. All the values are significant at a value of $P < 0.01$ except where indicated otherwise.

† The numbers in parentheses denote per cent inhibition of AHH activity.

‡ The P-450LM_4 used was isolated from either BNF- or PB-treated animals as indicated.

§ $P < 0.05$.

‖ The specific activities, expressed as pmoles products formed/min/mg of protein, were 32.8 and 93.8, respectively, for control and PB-induced microsomes incubated in the absence of modifier.

Aquasol was purchased from New England Nuclear (Boston, MA). Synthetic dilauroyl-GPC was obtained in chloroform solution from Serdary Research Laboratories, London, Ontario, Canada; prior to use, the solvent was removed under nitrogen and a 1 mg/ml aqueous suspension was prepared by sonication. The source and preparation of other materials are given elsewhere [8, 9].

RESULTS

The metabolism of BP in liver microsomal suspensions and in the reconstituted enzyme system containing the different forms of P-450LM was determined by spectrofluorimetric estimation of the phenolic metabolites formed (AHH assay). The differential effects of selected mixed-function oxidase inhibitors on the catalytic activity of the purified P-450LM preparations and of microsomes from untreated and PB-treated rabbits toward BP are compared in Table 1. PB-inducible P-450LM_2 catalyzed the conversion of BP to phenols about ten to twenty times faster than P-450LM_4, while rates of BP hydroxylation by partially purified P-450LM_7 were somewhat intermediate to those obtained with P-450LM_2 and LM_4. Although P-450LM_4 from BNF-induced microsomes appears to have been more active than the corresponding preparation from PB-induced microsomes, the opposite was found in a previous study in which phenols and other metabolites formed from BP were analyzed by high pressure liquid chromatography [52]. Small differences in the relative activities of the P-450LM_4 proteins isolated from different sources are invariably seen from one preparation to another regardless of the type of microsomes used as source, and may be due to the presence of varying amounts of apoenzyme or to differences in the composition of reaction mixtures and in the incubation conditions employed [53]. However, there is presently no strong evidence suggesting that these preparations are significantly dissimilar, catalytically or otherwise [11, 59, 60]. In fact, the oxidation of BP was catalyzed at comparable rates by the various purified preparations of the polycyclic hydrocarbon-inducible form of rabbit P-450LM isolated in different laboratories, i.e. P-450LM_4 from BNF- or PB-induced microsomes (Table 1) [52, 59], P-448 from 3-MC-induced microsomes [62] and P-450*c* from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced microsomes [17, 60]. The three P-450LM_4 enzymes, purified in this laboratory from different sources, were indistinguishable based on electrophoretic and spectral properties, identity of the COOH-terminal amino acids [9], immunochemical methods [10] and catalytic activity toward a variety of substrates besides BP [11, 21, 59].

Table 1 also shows that treatment of rabbits with PB led to an increase in microsomal BP hydroxylase activity, as reported previously by others [61]. Moreover, the P-450LM_2-mediated hydroxylation of BP was markedly inhibited by 7,8-BF and BHT, whereas only a 25 per cent loss in the activity of P-450LM_4 or LM_7 was observed in the presence of either of these compounds. The pronounced inhibitory effect of BHT and, to a smaller extent, of 7,8-BF on BP hydroxylation in PB-induced microsomes most likely reflects inhibition of P-450LM_2; since this cytochrome is induced by PB, it possesses the highest activity and degree of specificity towards BP, and its BP hydroxylase activity is the most sensitive to 7,8-BF and BHT. On the other hand, the effect of 7,8-BF and BHT on BP metabolism in microsomes from untreated rabbits was considerably smaller, and similar in magnitude to that seen with purified P-450LM_4 and LM_7. This finding is consistent with the observation that microsomes from untreated rabbits contain significant amounts of all P-450LM forms, except for P-450LM_2 which is present in only trace amounts [8, 11]. In contrast to the differential effects of 7,8-BF and BHT, ascorbic acid displayed no selectivity, for it inhibited all preparations uniformly by about 40 per cent. This indicates that ascorbic acid is not a specific inhibitor of a particular form of P-450LM in the BP hydroxylation reaction, but may affect overall activity by inhibiting electron transport or some step in the redox cycle of P-450LM, or possibly phenol formation from arene oxide intermediates.

The activity of the various preparations from rabbit liver toward (−)*trans*-7,8-diol, as well as the effects of inhibitors on this activity, are depicted in Table 2. The conversion of (−)*trans*-7,8-diol to isomeric 9,10-epoxides which bind to DNA was catalyzed by P-450LM_7 at rates about 2-fold and 10-fold greater than those obtained with P-450LM_4 and LM_2, respectively. However, P-450LM_7 was devoid of substrate specificity since it was equally as active with the (−)*trans*-7,8-diol as with BP. Inspection of the activities shown in Tables 1 and 2 reveals that the specificity for (−)*trans*-7,8-diol in the reconstituted enzyme system resides essentially in P-450LM_4. This conclusion is also supported by the results of a previous study in which BP and (−)*trans*-7,8-diol metabolites were measured directly by high pressure liquid chromatography [52].

As in the case of BP hydroxylation, 7,8-BF markedly inhibited those forms of P-450LM which were the most active toward (−)*trans*-7,8-diol, i.e. P-450LM_4 and LM_7, but did not have an appreciable effect on P-450LM_2 which was largely incapable of catalyzing the oxygenation of this substrate (Table 2). BHT, unlike 7,8-BF, did not act in this dual fashion, for the metabolism of (−)*trans*-7,8-diol as substrate. Ascorbic acid caused a striking decrease in the activity of P-450LM_4 but inhibited the activities of P-450LM_7 and LM_2 to a lesser extent.

It may also be seen in Table 2 that the metabolism of (−)*trans*-7,8-diol in microsomes remained unaltered following treatment of the animals with PB; this is contrary to the effect of this compound on microsomal BP hydroxylase activity. 7,8-BF inhibited both microsomal preparations by about 45 per cent, probably by inhibiting the activities of P-450LM_4 and LM_7. In agreement with its lack of effect on the purified proteins, BHT did not inhibit (−)*trans*-7,8-diol metabolism in microsomes from untreated or PB-treated rabbits.

BP hydroxylation and the binding of (−)*trans*-7,8-diol metabolites to DNA were also investigated in 3-MC-induced microsomes, as illustrated in Table 3. Whereas 3-MC treatment led to a decrease in the specific activity of microsomal BP hydroxylase, the binding of (−)*trans*-7,8-diol metabolites to DNA was

Table 2. Effects of modifiers on (−)*trans*-7,8-diol oxidation to DNA binding metabolites by purified forms of rabbit P-450LM and by intact liver microsomes*

P-450LM preparation	Activity (pmoles metabolite bound/min/mg DNA/nmole P-450LM)			
	Without modifier	With modifier†		
		7,8-BF	BHT	Ascorbic acid
Purified P-450LM				
LM_2	4.5 ± 0.5	3.7 ± 1.4 (18)‡	4.7 ± 1.1	2.9 ± 0.4 (36)
$LM_{4(BNF)}$	19.5 ± 5.6	5.9 ± 1.8 (70)	21.3 ± 4.8	3.5 ± 1.0 (82)
$LM_{4(PB)}$	24.9 ± 0.2	3.2 ± 0.2 (87)	28.6 ± 6.2	6.1 ± 0.4 (76)
LM_7	41.9 ± 1.6	6.2 ± 0.7 (85)	36.1 ± 1.5 (14)	18.7 ± 1.3 (55)
Liver microsomes				
Control	13.4 ± 2.8§	7.9 ± 2.2 (41)	12.8 ± 1.9	8.5 ± 0.9‖ (37)
PB-induced	11.0 ± 1.1§	6.0 ± 1.0 (45)	9.9 ± 0.5	8.1 ± 1.8‖ (26)

* The catalytic activity of the various preparations toward (−)*trans*-7,8-diol was ascertained by measuring the binding to DNA of (−)*trans*-7,8-diol metabolites formed upon incubation of the diol with microsomes (100 μg protein) or with purified P-450LM (0.3 μM) in the reconstituted enzyme system. Conditions were identical to those described in Table 1, except that [^{3}H]-(−)*trans*-7,8-diol (10 μM) served as the substrate and the reaction mixtures contained calf thymus DNA (0.25 mg) in addition to the other components. Incubations were carried out for 15 min at 37° and the amount of (−)*trans*-7,8-diol metabolites bound to DNA was determined as described in Materials and Methods. The microsomes used in these experiments had been stored at −70° following their isolation. All values are significant at a value of $P < 0.01$ except where indicated otherwise.

† The final concentration of the various modifiers was as in Table 1.

‡ Numbers in parentheses indicate per cent inhibition of the binding of (−)*trans*-7,8-diol metabolites to DNA.

§ The specific activities, expressed as pmoles metabolite bound/min/mg DNA/mg of protein, were 15.4 and 18.9, respectively, for control and PB-induced microsomes incubated in the absence of modifier.

‖ $P < 0.05$.

Table 3. Effects of modifiers on BP metabolism and (−)*trans*-7,8-diol oxidation to DNA binding metabolites catalyzed by liver microsomes from untreated and 3-MC-treated rabbits*

Reaction assayed and source of microsomes	Activity†			
	Without modifier	With modifier‡		
		7,8-BF	BHT	Ascorbic acid
BP hydroxylation				
Control	36.7 ± 0.1	26.4 ± 4.7 (28)§	26.6 ± 1.9 (28)	24.4 ± 3.3 (34)
3-MC-induced	27.3 ± 0.9	17.0 ± 1.3 (38)	26.5 ± 4.9	26.3 ± 4.2
Binding of (−)*trans*-7,8-diol metabolites to DNA				
Control	43.7 ± 6.0	17.4 ± 1.3 (60)§	39.9 ± 0.7	29.5 ± 5.8 (32)
3-MC-induced	87.4 ± 6.4	18.3 ± 1.6 (79)	83.3 ± 1.8	57.5 ± 6.4 (34)

* AHH and DNA binding activities were determined by incubating microsomes from the indicated source (100 μg protein) with BP (10 μM) or with [^{3}H]-(−)*trans*-7,8-diol (10 μM) in the presence of calf thymus DNA (0.25 mg) under incubation conditions identical to those described in Tables 1 and 2, respectively. The phenolic products formed from BP and the binding of (−)*trans*-diol metabolites to DNA, which reflects the catalytic activity of these preparations toward (−)*trans*-7,8-diol, were measured as described in Materials and Methods. The microsomes used in these experiments had been stored at −70° following their isolation.

† Activities are expressed as pmoles products formed/min/mg of protein (BP hydroxylation) or pmoles metabolite bound/min/mg DNA/mg of protein [(−)*trans*-7,8-diol oxygenation].

‡ The final concentration of the various modifiers was the same as in previous experiments and is given in Materials and Methods and Table 1.

§ Numbers in parentheses indicate per cent inhibition of AHH activity or of the binding of (−)*trans*-7,8-diol metabolites to DNA.

Table 4. Effects of 7,8-BF on BP metabolism and (−)*trans*-7,8-diol oxidation to DNA binding metabolites by fresh and previously frozen liver microsomes from untreated rabbits*

	Activity†	
Reaction assayed	Without 7,8-BF	With 7,8-BF‡
BP hydroxylation		
Fresh microsomes	49.2	87.4 (177)§
Microsomes stored at −70°	28.5	19.1 (67)
Binding of (−)*trans*-7,8-diol metabolites to DNA		
Fresh microsomes	22.6	16.5 (73)§
Microsomes stored at −70°	13.4	7.9 (59)

* Experimental conditions were as in Table 3. Product formation (BP) and the binding of (−)*trans*-7,8-diol metabolites to DNA were assayed as described in Materials and Methods. Microsomes were used within 1–2 days of their isolation (fresh) or after they had been stored at −70° for several weeks.

† Activities are expressed as pmoles products formed/min/nmole P-450LM (BP hydroxylation) or pmoles metabolite bound/min/mg DNA/nmole P-450LM [(−)*trans*-7,8-diol oxygenation].

‡ 0.1 mM.

§ Numbers in parentheses are the per cent of control activities [AHH and binding to DNA of (−)*trans*-7,8-diol metabolites, respectively] obtained in the absence of 7,8-BF.

twice as great in 3-MC-induced microsomes as in control microsomes. These effects are the opposite of those obtained with PB treatment. The extent of inhibition of BP and (−)*trans*-7,8-diol oxygenation by 7,8-BF or BHT in 3-MC-induced microsomes was as expected from the effects of these compounds on the corresponding activities of P-450LM$_4$ and LM$_7$.

Freezing and storage of microsomes from untreated rabbits at −70° resulted in a 40 per cent loss of both BP hydroxylase activity and the binding of (−)*trans*-7,8-diol metabolites to DNA (Table 4), indicating that labile forms of P-450LM may have been present in these preparations. 7,8-BP stimulated BP hydroxylation about 2-fold in fresh microsomes, but inhibited this activity by 30 per cent in microsomes stored frozen for several weeks. On the other hand, the binding of (−)*trans*-7,8-diol metabolites to DNA was decreased by 7,8-BF in both preparations. The differential effect of 7,8-BF on BP hydroxylase activity suggests that one or more P-450LM forms were lost upon freezing and storage of control microsomes at −70°.

DISCUSSION

In the present studies we have examined the relationship between individual purified forms of rabbit P-450LM and intact microsomes of different P-450LM composition in the metabolism of BP and (−)*trans*-7,8-diol, and investigated the degree of selectivity of mixed-function oxidase inhibitors toward the various preparations catalyzing these reactions. It is well known that 7,8-BF produces a profound decrease in BP hydroxylase activity of 3-MC-induced rat liver microsomes [74], but only partially inhibits this activity in microsomes from adult 3-MC- [75] (Table 3) or TCDD-treated rabbits [76]. On the other hand, stimulation of BP hydroxylation by 7,8-BF is seen in control microsomes from rats [74], adult rabbits [75, 76] and humans [77]. BHT and similar phenolic antioxidants are capable of blocking the carcinogenic effects of a substantial number of polycyclic aromatic hydrocarbons *in vivo*, presumably by acting as scavengers of reactive metabolic species of the chemical carcinogens [78]. The inhibition of BP hydroxylation by BHT *in vitro* is believed to result from the direct interaction of this compound with cytochrome P-450 in liver microsomes [55]. Ascorbic acid has been found to inhibit BP metabolism in rat liver microsomes [79] and to reduce dimethylbenzanthracene-initiated tumor formation when applied to mouse skin [56]. However, limited information is currently available concerning the role of these modifiers in the oxygenation of (−)*trans*-7,8-diol by microsomes and by the reconstituted enzyme system containing the different forms of P-450LM.

Atlas *et al.* [61] have shown that certain compounds of the polycyclic aromatic hydrocarbon class exert essentially identical effects when administered to adult rabbits *in vivo*. Thus, 3-MC, BNF and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induce microsomal cytochrome P-448 (P-450LM$_4$) to the same extent (about 2.5-fold), and either do not alter cytochrome P-448-associated mono-oxygenase activities or, when induction is seen, give rise to comparable increases in substrate hydroxylation rates [61]. Accordingly, the P-450LM$_4$ proteins purified from microsomes of 3-MC- [15, 62], BNF- [9] and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated adult rabbits [17] are identical, as ascertained from electrophoretic, immunochemical and substrate specificity studies [11, 59, 60]. This is also true of P-450LM$_4$ preparations isolated from PB-induced and control rabbit liver microsomes. These observations support the conclusion that it is both appropriate

and valid to explain the relative catalytic effectiveness of 3-MC-induced microsomes (Table 3) on the basis of the substrate specificity inherent in P-450LM$_4$, regardless of the type of microsomes used as source for purification of this cytochrome (Tables 1 and 2).

It has been pointed out recently that the catalytic properties of microsomes should correspond to those of a specific form of P-450LM when that particular cytochrome predominates as a result of induction [59, 76]. The findings in this paper clearly demonstrate that the changes in substrate specificity and inhibitor sensitivity seen with microsomes from PB- or 3-MC-treated rabbits do indeed reflect the prevalence of a specific form of cytochrome P-450 in these preparations. In this regard, a single form of rabbit P-450LM, P-450LM$_2$, is induced to a predominant level by PB [8, 11], and this is accompanied by more than a 2-fold increase in microsomal BP hydroxylase activity; the oxygenation of (−)*trans*-7,8-diol remains unchanged in PB-induced, as compared to control, microsomes. In accordance with these results, P-450LM$_2$ is fifteen to twenty times more active with BP than with (−)*trans*-7,8-diol and, of the several forms of P-450LM, has the highest specificity toward BP. Furthermore, BHT and 7,8-BF inhibited BP hydroxylase activities of P-450LM$_2$ and of PB-induced microsomes by 75 per cent, whereas no significant change in the metabolism of this substrate by control or 3-MC-induced microsomes and by purified P-450LM$_4$ or LM$_7$ was seen in the presence of either of these compounds.

Conversely, treatment of rabbits with 3-MC leads to an increase in the specific content of P-450LM$_4$ [15, 59, 62] and a concomitant 2-fold enhancement in the binding of (−)*trans*-7,8-diol metabolites to DNA, but has no effect on BP metabolism in microsomes; induction of P-450LM$_4$ in adult rabbits by BNF [8–11, 61] or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [17, 60, 61] is likewise not associated with an increase in microsomal BP hydroxylase activity [61, 75]. Homogeneous P-450LM$_4$ possessed the highest specificity for (−)*trans*-7,8-diol and catalyzed the oxygenation of this substrate five times faster than P-450LM$_2$, but was largely ineffective with BP. In fact, P-450LM$_4$ from PB- or BNF-induced microsomes is by far the least active of the various forms of rabbit P-450LM in BP hydroxylation, whether activity is expressed in terms of total metabolites [52], or total phenols estimated fluorometrically (Table 1), or by high pressure liquid chromatography [52]. Similar results have been obtained with P-450LM$_4$ preparations from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated adult rabbits [17, 60, 76]. Moreover, 7,8-BF strongly inhibited (−)*trans*-7,8-diol oxygenation by P-450LM$_4$ and 3-MC-induced microsomes and caused a considerable diminution of this activity in control microsomes, which are known to contain P-450LM$_4$ at significant levels [8, 11]. However, in contrast to 7,8-BF, inhibitor selectivity appears to be the sole property of BHT since this compound did not alter (−)*trans*-7,8-diol oxygenation in any of the microsomal preparations or in the reconstituted enzyme system containing the different forms of P-450LM. In other experiments (not shown) treatment of rabbits with 3-MC ostensibly did not alter the overall activity of microsomes toward (−)*trans*-7,8-diol, as judged by the total metabolites formed, but a substantially greater amount of diol-epoxide I than diol-epoxide II was obtained with 3-MC-induced as compared to control microsomes. It is noteworthy in this connection that P-450LM$_4$ exhibited a striking preference for the pure (−) relative to the (+) enantiomer of *trans*-7,8-diol, and stereoselectively oxygenated the former to yield 10-fold more diol-epoxide I than diol-epoxide II. These results provide strong evidence in favor of the view that the catalytic properties of each form of P-450LM, as determined in reconstituted enzyme systems, are characteristic of and intrinsic to that form and by no means due to artifacts of the purification or reconstitution techniques.

In conclusion, our results demonstrate that the oxygenation of BP and BP(-)*trans*-7,8-diol is catalyzed by different forms of rabbit P-450LM, and that each of these processes is affected differently by mixed-function oxidase inhibitors. The data also illustrate that substrate metabolism in PB- and 3-MC-induced rabbit liver microsomes can be accounted for by the catalytic properties of the predominant forms of P-450LM in these preparations, P-450LM$_2$ and LM$_4$, respectively. In sharp contrast to the results obtained with rabbit P-450LM, it is well established that BP and *trans*-7,8-diol oxygenase activities are both induced several-fold in microsomes from rats treated with polycyclic aromatic hydrocarbons [80]. Accordingly, rat 3-MC-inducible cytochrome P-448 is seven times more active than PB-inducible cytochrome P-450 with BP [80] and approximately thirty times more active with racemic *trans*-7,8-diol [81]. The stereospecificity of individual forms of P-450LM, whose relative proportion in a given tissue is invariably altered by drugs and environmental pollutants and by hormonal and genetic factors, may play a key role in determining the balance between detoxification and activation; this, in turn, may govern the susceptibility of specific tissues and animals to the carcinogenic action of polycyclic hydrocarbons.

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