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Position-specific oxygenation of benzo[*a*]pyrene by different forms of purified cytochrome *P*-450 from rabbit liver

(chemical carcinogenesis/detoxification/hydroxylases/hepatic microsomes/aryl hydrocarbons)

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ABSTRACT High-pressure liquid chromatography was used to detect oxygenated products of benzo[*a*]pyrene formed in a reconstituted microsomal mixed-function oxidase system containing cytochrome *P*-450 (*P*-450LM), phospholipid, and NADPH-cytochrome *P*-450 reductase (NADPH: ferricytochrome oxidoreductase, EC 1.6.2.4). Three cytochrome fractions purified from a single source, hepatic microsomes from phenobarbital-treated rabbits, were studied; the various forms of the cytochrome are designated by their relative electrophoretic mobilities. The total benzo[*a*]pyrene oxygenation rate was greatest for *P*-450$LM_{1,7}$, intermediate for *P*-450LM_2, and least for *P*-450LM_4. The phenolic products were eluted in two peaks, A and B, that contained primarily 9-hydroxy- and 3-hydroxybenzo[*a*]pyrene, respectively. The ratio of peak A to peak B phenols was 0.11 for *P*-450LM_2 and 0.45 for *P*-450LM_4. Thus, the relative amounts of the various phenols formed by these two cytochrome fractions differ markedly. The positional specificity of the hydroxylation is also indicated by large differences in the fluorescence spectra of the phenolic products formed by the two cytochromes. *P*-450LM_2 and *P*-450LM_4 did not form benzo[*a*]pyrene dihydrodiols, thereby showing that benzo[*a*]pyrene oxide hydratase activity was absent from these purified preparations. Ninety percent of the phenols formed by *P*-450$LM_{1,7}$ were eluted in peak B; the metabolites produced by this preparation also included dihydrodiols, thus indicating the presence of hydratase activity. The positional specificities of different forms of cytochrome *P*-450 may channel polycyclic aromatic hydrocarbon metabolism into the various activation and detoxification pathways and thereby help determine the cytotoxic and carcinogenic activity of these compounds.

Microsomal mixed-function oxidases are involved in the detoxification of polycyclic aromatic hydrocarbons (1–3), but in the process may form metabolites which are toxic (4) and carcinogenic (5). These cytochrome *P*-450-containing enzyme systems oxygenate polycyclic aromatic hydrocarbons at many positions to metabolites which are further converted by enzymatic and nonenzymatic reactions to a variety of products, including phenols, dihydrodiols, quinones, and conjugates (6–11). Separation and characterization of the metabolites have been greatly facilitated by the application of high-pressure liquid chromatography (HPLC) (9–11).

Several studies suggested that the broad substrate specificity of the mixed-function oxidases and the multiplicity of products might be due to the existence of more than one form of the enzyme (12–16). Recently, four individual forms of cytochrome *P*-450 have been purified from solubilized rabbit liver microsomes by column chromatographic procedures carried out in the presence of a nonionic detergent (15). These forms differ in their catalytic activities toward a variety of substrates, including benzphetamine, *p*-nitroanisole, biphenyl, and testosterone. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was used to show that the various forms also differ in their subunit molecular weights (15).

The differences in cytotoxic and carcinogenic potencies of polycyclic hydrocarbon metabolites may be largely a function of the position on the molecule where the primary oxygenation step occurs. Positional specificities of different forms of cytochrome *P*-450 for the substrate may thus be crucial determinants in the biological activities of polycyclic hydrocarbons.

In the present study we used spectrofluorometry and HPLC to examine the specificity of the conversion of benzo[*a*]pyrene (BP) to oxygenated products by purified forms of cytochrome *P*-450 from phenobarbital-treated rabbit liver microsomes.

MATERIALS AND METHODS

The separation and purification of the several forms of liver microsomal cytochrome *P*-450 (*P*-450LM) and of NADPH-cytochrome *P*-450 reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4) from phenobarbital-treated rabbits have been described previously (15, 17, 18). The individual forms are designated by their relative electrophoretic mobilities as *P*-450LM_2, *P*-450LM_4, and *P*-450$LM_{1,7}$ (15). Reaction mixtures contained in 1 ml: 0.6 nmol of *P*-450LM; NADPH-cytochrome *P*-450 reductase (48 μg of protein; specific activity, 19 μmol of cytochrome *c* reduced per min/mg of protein); 60 μg of dilauroylglyceryl-3-phosphorylcholine; 50 μg of deoxycholate; 50 μmol of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, pH 7.7; 15 μmol of $MgCl_2$; 1 μmol of NADPH; and 50 μmol of [^{3}H]BP (179 Ci/mol) added in 20 μl of methanol. The specific content of the cytochrome preparations (nmol/mg of protein) was as follows: *P*-450LM_2, 16.8; *P*-450LM_4, 5.1; and *P*-450$LM_{1,7}$, 4.3. In some experiments the cytochrome, reductase, and phospholipid fractions were replaced by rabbit liver microsomes (340 μg of protein) containing 1 nmol of *P*-450LM. Volumes of reaction mixtures were 7–15 ml for metabolite analysis by HPLC. After incubation at 30° for 10 min, aliquots of the reaction mixture were removed for fluorescence determinations and the remainder was extracted with ethyl acetate for HPLC. The samples were prepared and chromatographic analysis was performed as described previously (9, 10). [^{14}C]BP metabolites formed by rat liver, which were charac-

Abbreviations: BP, benzo[*a*]pyrene; HO-BP, monohydroxybenzo[*a*]pyrene; HPLC, high-pressure liquid chromatography; *P*-450LM, liver microsomal cytochrome *P*-450. The various forms of cytochrome *P*-450 are designated by their relative electrophoretic mobilities, e.g., *P*-450LM_2.

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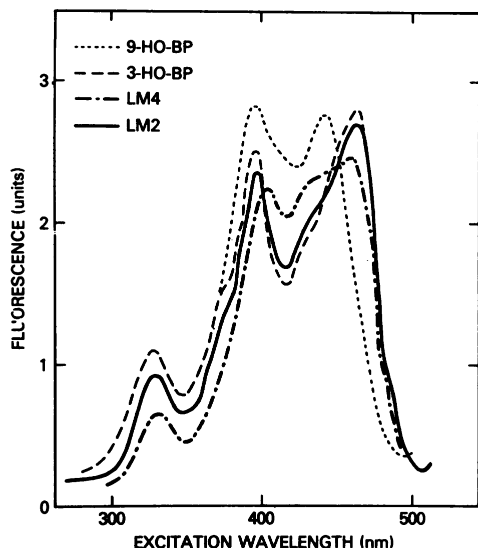

FIG. 1. Fluorescence spectra of alkali-extractable BP products formed by P-450LM$_2$ or P-450LM$_4$. Aliquots of the reaction mixtures were extracted with 4 volumes of acetone-hexane (1:3, v/v), and 1 ml of the organic phase was extracted with 1 ml of 1 N NaOH (20). The excitation spectra were recorded at an emission of 522 nm. The spectra of authentic 3-HO-BP and 9-HO-BP were measured in 1 N NaOH at a final concentration of 1 μM.

terized previously (9, 10), were chromatographed together with the [^{3}H]BP metabolites formed by the reconstituted oxygenase system, and served as internal standards for the retention times of each chromatographic separation.

RESULTS AND DISCUSSION

Phenolic metabolites formed from BP by the purified P-450LM$_2$ and P-450LM$_4$ were extracted into alkaline solution and their fluorescence excitation spectra were measured as shown in Fig. 1. The spectrum of the phenols produced by P-450LM$_2$ closely resembles that of authentic 3-hydroxybenzo[a]pyrene (3-OH-BP) with distinct peaks at 395 nm and 465 nm. The shift of a few nanometers to longer wavelengths and a slightly shallower trough in the extract from the enzyme reaction mixture indicate the presence of some fluorescent material other than 3-OH-BP. Phenols formed by P-450LM$_4$ show a distinctly different excitation spectrum. A small trough separates a narrow peak around 405 nm from a broad peak between 425 and 475 nm. A comparison of the spectrum of the BP phenols formed by P-450LM$_4$ with those of authentic 9-HO-BP and 3-HO-BP suggests that this cytochrome may form a mixture of these isomers and possibly other phenols. This is supported by the results of the chromatographic separation (see below).

BP metabolism by the purified cytochromes and by microsomes was further examined by HPLC (Fig. 2). The profile of [^{3}H]BP products formed by P-450LM$_2$ and P-450LM$_4$ showed four major fractions. A portion of the radioactive material chromatographed with and trailed the solvent front (fractions 4–7). This material has not been characterized. Another portion of the radioactivity eluted with authentic BP quinones (1,6-, 3,6-, and 6,12-quinones). Two distinct peaks appeared in the phenol region which we term "phenol A" and "phenol B". The major peak eluting beyond fraction 100 is unmetabolized BP. Extracts of reaction mixtures containing rabbit liver microsomes yielded additional metabolites which cochromatographed with authentic 9,10-, 4,5-,

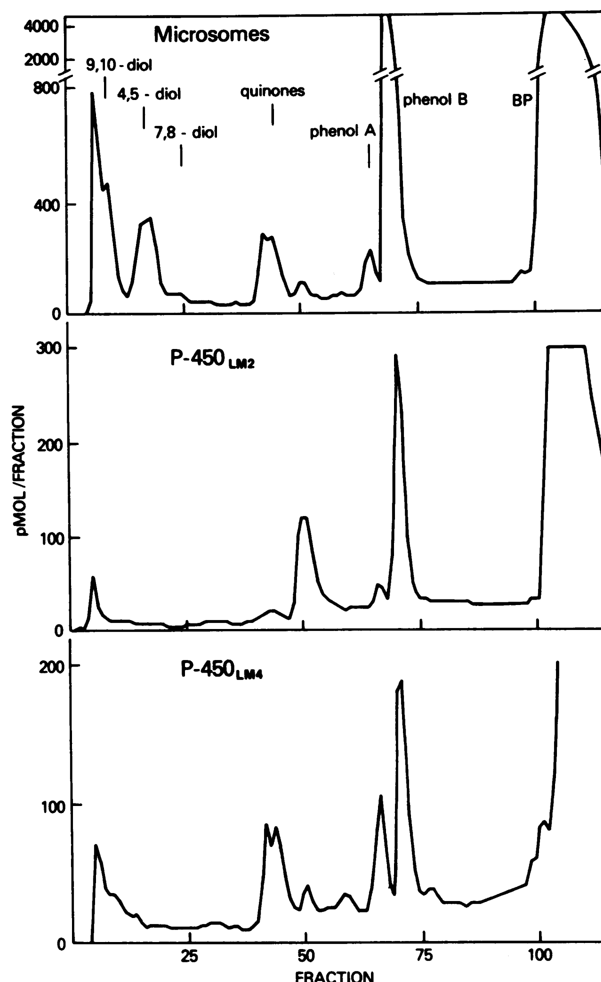

FIG. 2. BP metabolism by P-450LM$_2$, P-450LM$_4$, and liver microsomes. Incubation mixtures were extracted twice with ethyl acetate (3 volumes and 1 volume, respectively). The combined organic phases were evaporated under vacuum.

and 7,8-BP dihydrodiols (Fig. 2). In other chromatograms, the 9,10-dihydrodiol was separated from the initial peak and was clearly not identical with the early eluting products formed by the purified P-450LM$_2$ and P-450LM$_4$.

The relative amounts of BP metabolites formed by various cytochrome preparations and by liver microsomes are shown in Table 1. Total BP metabolism, i.e., the sum of the oxygenated organic-soluble products, differs more than 10-fold among the different forms of cytochromes. BP-oxygenating activity is lowest with P-450LM$_4$, intermediate with P-450LM$_2$, and highest with P-450LM$_{1,7}$. Phenols comprised about 60% of the metabolites from P-450LM$_2$ and P-450LM$_4$. The remaining 40% consisted largely of quinones. Phenols, largely in peak B, are the major metabolites produced by the microsomes.

The relative amounts of phenols in peaks A and B formed by P-450LM$_2$ and P-450LM$_4$ differ markedly. About 11% of the phenols formed by P-450LM$_2$ migrate with peak A, whereas about a third of the phenols formed by P-450LM$_4$ appear as peak A. The rates of formation of peak A phenols are similar for both cytochrome preparations; it is the variation in peak B phenol formation which primarily accounts for the different ratios of phenolic products.

When peak B was isolated and analyzed by further HPLC it yielded only a single peak under conditions where 10 of

Table 1. Benzo[*a*]pyrene metabolism by different forms of cytochrome *P*-450 from rabbit liver

BP metabolites formed	Activity of *P*-450LM preparations and microsomes* (pmol/nmol of *P*-450 per min)							
	LM_2		LM_4		$LM_{1,7}$		Liver microsomes	
BP metabolites, total	711	(100)	158	(100)	2127	(100)	1495	(100)
BP 9,10-dihydrodiol	<1	(<0.1)	<1	(<0.1)	657	(31)	200	(13)
BP 4,5-dihydrodiol	<1	(<0.1)	<1	(<0.1)	85	(4)	123	(8)
BP 7,8-dihydrodiol	<1	(<0.1)	<1	(<0.1)	111	(5)	10	(1)
BP quinones	296	(42)	54	(34)	427	(20)	128	(9)
BP phenols, peak A	30	(4)	30	(20)	90	(4)	35	(2)
BP phenols, peak B	283	(53)	67	(45)	757	(36)	999	(67)

Numbers in parentheses give the percent of total BP metabolites.

* Metabolites formed from [^{3}H]BP by *P*-450LM preparations and liver microsomes were separated and quantitated by HPLC as described in Fig. 1 and in *Materials and Methods.*

the 12 possible phenols (except 1- and 3-HO-BP) are separated.§ This peak is primarily 3-HO-BP, since the presence of appreciable amounts of 1-HO-BP (excitation maximum 425 nm at an emission of 530 nm) is excluded by the fluorescence spectrum of the peak B phenol(s) that closely resembled that of 3-HO-BP. The phenol(s) in peak A consisted largely of 9-HO-BP as determined by radiometric and spectrofluorometric methods. Thus both simple differential extraction of the phenolic products (Fig. 1) and the analysis by HPLC (Fig. 2 and Table 1) indicate that the predominant phenolic metabolite of peak B is 3-HO-BP and the predominant phenol of peak A is 9-HO-BP. The data indicate a distinct difference in the positions of hydroxylation by the two cytochromes. Thus the *P*-450LM_2 shows about a 10-fold greater hydroxylation in the 3 position of BP relative to the 9 position whereas the *P*-450LM_4 shows only a 2-fold difference in the ratio of hydroxylation at these two positions.

No dihydrodiols are formed by the purified *P*-450LM_2 and *P*-450LM_4 preparations, indicating the absence of contaminating hydratases. The metabolites formed by *P*-450$LM_{1,7}$ (Table 1), however, include sizable amounts of dihydrodiols, predominantly at the 9,10 position, apparently due to the presence of BP oxide hydratase activity in this preparation.¶ The reason for the apparent differences in the quinone peaks formed by *P*-450LM_2 and *P*-450LM_4 (Fig. 2) is unknown. It may possibly be due to additional positional specificities of the enzymatic oxygenation. The quinones formed from [^{3}H]BP by rabbit liver microsomes, *P*-450LM_2, and *P*-450LM_4 (Fig. 2) cochromatographed with standard [^{14}C]BP quinones (data not shown). Thus the apparent differences in the R_F values of the quinones formed by the various fractions may be due to positional specificities of the different enzymes or may simply reflect technical variations in the chromatographic elution pattern. The more polar radioactive material which migrates close to the origin might be products of further oxidative metabolism of primary BP products. Material with similar chromatographic properties has been found as products of 3-HO-BP metabolism by rat liver microsomes (20).

It has previously been observed that BP-metabolizing enzymes in crude microsomal preparations from animals of different sex, age, or state of induction can be distinguished by their kinetic properties (21, 22), their sensitivity to inhibitors and stimulators (23–25), and the profile of metabolites formed (8, 26). This report shows that different purified forms of hepatic cytochrome *P*-450 isolated from a single source differ in their catalytic properties towards the hydroxylation of BP at different positions. A positional specificity of the purified enzyme preparations has also been observed for the hydroxylation of biphenyl and testosterone (15). Since the mixed-function oxidases are engaged in both the activation and detoxification of polycyclic hydrocarbons (5), differences in the relative distribution of the multiple forms of cytochrome *P*-450 might be a key factor in determining the susceptibility of tissues, individuals, and species to the cytotoxic and carcinogenic action of polycyclic aromatic hydrocarbons.

§ R. G. Croy, J. K. Selkirk, and H. V. Gelboin, in preparation.

¶ Direct measurement of hydratase activity using BP 4,5-oxide as substrate (19) confirmed its presence in *P*-450$LM_{1,7}$ preparations and its absence in *P*-450LM_2 and *P*-450LM_4 (J. C. Leutz and H. V. Gelboin, unpublished).

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